Molecular mechanisms of the human pathogen *Candida glabrata* involved in the interaction with the host

PhD Thesis

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Faculty of Biology

submitted by
Pia Schmidt

born in
Halle/Saale, Germany

Göttingen, September 2007

Affidavit

Herewith I declare that my PhD thesis "Molecular mechanisms of the human pathogen *Candida glabrata* involved in the interaction with the host" has been written independently and with no other sources and aids than quoted.

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Pia Schmidt, Göttingen, September 30th 2007

Meiner Familie

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ABSTRACT

Besides superficial skin and vaginal infections *Candida glabrata* causes life threatening disseminated candidiasis. *C. glabrata* is now the second most common cause of systemic Candidiasis in humans and isolated strains show a high rate of Fluconazole resistance, which aggravates successful treatment. *C. glabrata* occupies a variety of niches in its human host (skin, vaginal mucosa, blood) and must therefore respond and adapt effectively to different environmental conditions. The cell wall is of utmost importance as it is the front line in respect to host pathogen interaction as well as the recognition of environmental clues.

Despite the immense medical importance of *C. glabrata*, data on molecular mechanisms of pathogenicity are still rare. In the present study we have investigated the function of one of the most abundant GPI-anchored cell wall proteins of *C. glabrata* (Cwp1.1p) and its protein family as well as the proteomic changes which occur upon exposition to different environmental pH values.

We found Cwp1.1p to be a major structural component of the *C. glabrata* cell wall, which mediates protection of the fungus from cell wall degrading enzymes such as β-1,3-glucanase. Deletion of all three *CWP1* genes resulted in reduced growth at alkaline pH and remarkable structural changes in the cell wall, as indicated by enhanced sensitivity towards calcofluor white. These changes also reduced the fungus' ability to adhere to epithelial cells. The exposure of *C. glabrata* to changing ambient pH induced copious alterations in protein expression. We found enzymes of glucose catabolism and the TCA cycle to be expressed at much higher levels at acidic than at alkaline pH, indicating that higher metabolic activity might be needed for intracellular pH homeostasis in acidic environments. At alkaline conditions protein catabolism was induced while protein synthesis was significantly downregulated, thereby shifting protein metabolism to the usage of reserves. Thus, *C. glabrata* seems to perceive acidic pH as less stressful. This suggestion was further supported by the observed induction of stress response proteins at alkaline pH and contrasts with the human pathogen *C. albicans*.

In this study we performed the first systematic proteomic analysis of the global pH response of *C. glabrata* and characterized the important function of structural cell wall proteins, namely Cwp1p, in the cell wall of this pathogenic yeast.

1 INTRODUCTION

1.1 Fungi

Until the end of the 19th century fungi have been considered to be plants, though they are heterotrophic eukaryotic organisms. Today, fungi are grouped in their own taxonomic kingdom, which is estimated to consist of more than one million species. Only a very small fraction of approximately 400 species have been identified as human pathogens, but the numbers are rising (DE HOOG, 2000; FRIDKIN and JARVIS, 1996; GUARRO *et al.*, 1999). As fungi are extremely diverse in regard to morphology, occurrence and biological function, the taxonomical classification is difficult and has changed a lot during the last decades. The classical doctrine divides the Kingdom of fungi into five phyla: the ascomycota, basidiomycota, zygomycota, chytridiomycota and glomeromycota. The first four of the five alluded groups propagate by the production of sexual spores named ascospores, basidiospores, zygospores and oospores, respectively. In general, fungi can propagate in a sexual or asexual way. The sexual form is known as the teleomorph and the asexual form is the anamorph. They are often physically quite distinct forms and many of the asexual forms were discovered and named without evidence that they could reproduce sexually. Therefore, the teleomorph and the anamorph of the same fungus may have received different names. The sexual form of *Candida kefyr* for example is named *Kluyveromyces marxianus* and another prominent example is *Cryptococcus neoformans* (sexual form *Filobasidiella neoformans*) (GUARRO *et al.*, 1999). The classification according to the sexual form of the fungus might be difficult or even impossible as for some species a sexual form has not been identified yet or is non existent. These asexual fungi are classified as the deuteromycetes or *fungi imperfecti*. This group is no true phylum but an artificial taxonomic group. The deuteromycetes do not form sexual spores but asexual conidia for propagation.

Recently, a comprehensive phylogenetic classification has been published based on molecular phylogenetic analyses (HIBBETT *et al.*, 2007). The authors proposed the kingdom of fungi to contain one subkingdom and seven phyla. A number of profound changes in the taxonomical classification were suggested: The two phyla Ascomycota and Basidiomycota are now found in the subkingdom Dikarya. The other five phyla are the Chytridiomycota, Neocallimastigomycota, Blastocladiomycota, Glomeromycota and Microsporidia. Some of the biggest changes in this new classification are that the former phylum Zygomycota is now included in the Glomeromycota, the Blastocladiomycota are now an own phylum whereas they were previously a member of the Chytridiomycota and the intracellular parasitic fungi Microsporidia

are a phylum of their own. However, this study is not complete yet and a number of classifications remain open for discussion.

In the work presented here, we want to focus on the characterisation of the ascomycetous, pathogenic yeast *C. glabrata*.

1.2 Medical relevance of *Candida* infections

Human pathogenic fungi are found in four of the six groups described above – the ascomycetes, basidiomycetes, zygomycetes and deuteromycetes. The glomeromycetes live in mycorrhizal symbiosis with a variety of plants and the chytridiomycetes are the cause of diseases in agriculture and in lower cold-blooded animals. In comparison to bacterial pathogens, fungi were less frequently the cause of infectious diseases in humans. However, with the increased number of immunosuppressed patients fungal infections have gained enormous medical importance. Much progress has been made in successful organ transplantation and cancer chemotherapy. Thus, the number of patients with immunosupression but also with chronic, debilitating illnesses and hematological malignancies has increased. Many of these patients, who formerly often died from bacterial infections, are now surviving due to the availability of more potent antimicrobial agents, but are at high risk for fungal infections.

Examples of medical important fungi are the *Aspergillus species*, *Cryptococcus neoformans* and members of the genus *Candida*. Also the increasing numbers of preterm infants challenge the clinicians, as the neonates often suffer from *Candida* infections (FRIDKIN *et al.*, 2006). Today, *Candida spp.* have become common nosocomial pathogens and serious systemic *Candida* infections frequently lead to death. Often, these nosocomial infections are caused by indwelling devices such as catheters, when they are covered with fungal biofilms (CHANDRA *et al.*, 2001; IRAQUI *et al.*, 2005). At the end of the twentieth century, *Candida* infections have emerged as a significant medical concern (KOMSHIAN *et al.*, 1989; PFALLER, 1996), whereas they had been a rarity at the beginning (HALEY, 1961; STENDERUP, 1962). Today, they are ranked as the fourth most common cause of blood stream infections in the United States (PFALLER *et al.*, 1998b).

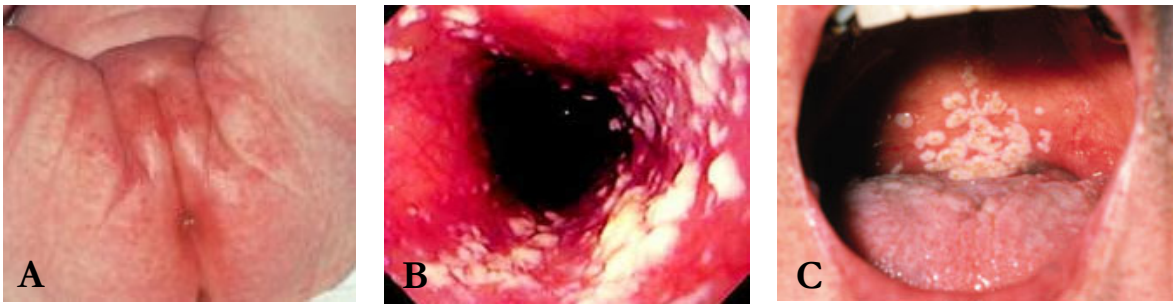

Figure 1-1 Skin and mucosal infections with *Candida*
(A) Diaper dermatitis – skin infection of a neonate with *Candida* (taken from www.emedicine.com), (B) Esophageal infection of an AIDS patient with *Candida* (taken from WILCOX *et al.*2002), (C) Oral thrush – oropharyngeal candidiasis of an AIDS patient (taken from MERCANTE *et al.*2006).

Candida spp. are opportunistic fungal pathogens that can frequently be found in the gastrointestinal tract of healthy humans in balance with different bacteria (e.g. *Lactobacillus acidophilus, Streptococcus faecalis, Escherichia coli*). In Germany for example, about 16% of healthy humans have *C. glabrata* in their stool samples (PD Dr. Dagmar Rimek, Erfurt, Germany, personal communication). In immunocompetent hosts, especially in warm climates, *Candida spp.* can cause superficial infections on skin and mucosa (ODDS, 1994). Furthermore, about 75% of the female population encounters at least one episode of vaginal candidiasis during the life time and about 5-10% of otherwise healthy women suffer from recurrent vulvovaginal candidiasis (BAROUSSE *et al.*, 2005). The reasons for these infections are still not fully understood. Also skin infection of neonates with *Candida*, the so called diaper dermatitis, is quite often seen and mucosal *Candida* infections of the oropharynx or esophagus frequently constitute the first manifestation of AIDS (BODEY, 1993)(Figure 1-1). When *Candida* succeeds to enter the blood stream, it disseminates throughout the body and causes severe and life threatening invasive illnesses in immunosuppressed hosts. Today, *C. albicans* is still the most frequently isolated yeast species from blood cultures and tissue samples. However, throughout the 1990s, an epidemiological change to non-albicans *Candida spp.*, such as *C. glabrata, C. parapsilosis, C. dubliniensis, C. tropicalis* and *C. krusei* has been observed (HOBSON, 2003; KRCMERY and BARNES, 2002; ZEPELIN *et al.*, 2007) and was linked to the frequent use of tri-azole antimycotics (GIRMENIA and MARTINO, 1998). Among these non-albicans *Candida spp.* *C. glabrata* has emerged as the most important pathogen. Today 19% of the invasive yeast infections in the adult population in Germany are caused by this species (ZEPELIN *et al.*, 2007). *C. glabrata* infections are difficult to treat, as this organism shows a high intrinsic resistance against antifungal agents. For *C. albicans* a number of genes which are responsible for antifungal resistance were identified and examined during the last ten years (AKINS, 2005). However, despite being the second most common cause of disseminated candidiasis in

humans, the underlying molecular bases of resistance mechanisms in *C. glabrata* are far less understood. The encountered problems in the treatment of *C. glabrata* infections urge the scientists to put effort into the molecular dissection of the pathogen. The identification of antifungal targets and resistance mechanisms as well as a better understanding of its molecular organization (e.g. in respect to signal transduction pathways, transporter proteins, receptors and cell wall organization) will be the basis to successfully treat *C. glabrata* infections.

1.3 Candida glabrata

1.3.1 Candida glabrata - a pathogenic yeast

C. glabrata is an asexual haploid fungus, which reproduces exclusively by budding. It belongs to the ascomycetes and not to the deuteromycetes, in which the asexual fungi are usually assembled. This is, because it has the genetic prerequisites to mate. A mating type locus has been found (*MTL1*) and studied (SRIKANTHA *et al.*, 2003; WONG *et al.*, 2003). It is the orthologue of the *Saccharomyces cerevisiae MAT* locus (HABER, 1998) and it was shown that *C. glabrata* switches between the *MTL1a* and *MTL1α* genotypes *in vivo*. Brockert and co-workers found that mating type switching occurs also in the patient and that pathogenesis of vaginal infections is not limited to a single mating type (BROCKERT *et al.*, 2003). This homothallic (HO) endonuclease mediated switching occurs in the *Saccharomyces sensu stricto* group as well as its relatives *C. glabrata, K. delphensis and S. castellii* (BUTLER *et al.*, 2004).

The subphylum, in which *C. glabrata* and the closely related baker's yeast *Saccharomyces cerevisiae* are grouped, is called saccharomycotina. The members of this group are mostly single celled and reproduce vegetatively by budding. Therefore, they were at first described as hemiascomycetes. In fact, the relationship of *C. glabrata* to *Saccharomyces cerevisiae* is closer than to the human pathogenic yeast *Candida albicans*, which belongs to the same genus (KAUR *et al.*, 2005) (Figure 1-2). Although the three yeasts, *S. cerevisiae*, *C. glabrata* and *C. albicans*, have a lot in common (e.g. the basic cell wall structure, certain components of signal transduction pathways) they display very distinct individual features on the other hand ranging from the presence or absence of certain surface proteins to reverse phenotypes of orthologous gene deletions.

The earlier taxonomy based on morphological criteria was sometimes rather imprecise, but modern molecular biology techniques have helped to identify close relationships between species, which had at first been classified differentially. The species *Torulopsis glabrata* is a prominent example. After discovery and classification, it has later been integrated into the

genus *Candida* and was thus named *Candida glabrata* (ODDS, 1988). Today, species can be identified and taxonomically classified by DNA sequence analysis and calculating sequence similarity (e.g. the variable D1/D2 domain of 26S rDNA) (BUTLER *et al.*, 2004; SUGITA and NISHIKAWA, 2004), which is by far more precise than phenotypic classification.

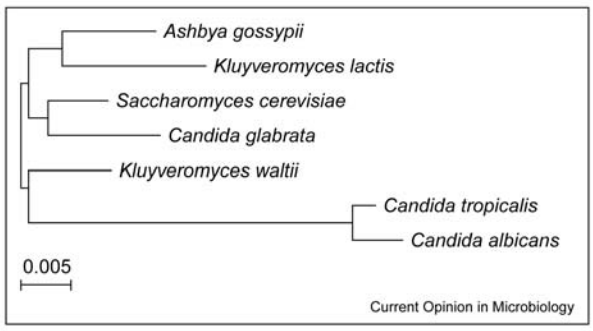

Figure 1-2 The 18S phylogeny of *Candida* species and other hemiascomycetes
C. glabrata and *S. cerevisiae* are phylogenetically closely related and are quite distinct from the other pathogenic *Candida* species (from KAUR *et al.*2005)

In the environment *C. glabrata* can be found on rotten fruits or as a contaminant of fruit juices, but it can also grow on artificial surfaces like plastic catheters where it tends to form biofilms (Figure 1-3) (IRAQUI *et al.*, 2005). On the other hand it is also an opportunistic human pathogen of increasing importance and the second most common cause of systemic candidiasis in immuno compromised patients after *C. albicans* (PFALLER *et al.*, 1998a; VAZQUEZ *et al.*, 1998). Infections caused by *C. glabrata*, often arise from the host's endogenous microflora and meanwhile the fungus accounts for approximately 18 - 21% of yeast blood culture isolates in the USA (PFALLER *et al.*, 2001; PFALLER *et al.*, 2003a; PFALLER *et al.*, 1998b; PFALLER *et al.*, 2003b). In Germany, epidemiological data on yeast cultures obtained from primarily sterile sites of patients displayed a very similar picture: *C. albicans* was the most frequently isolated species (58.5%), followed by *C. glabrata* (19.1%), *C. parapsilosis* (8.0%) and *C. tropicalis* (7.5%) (ZEPELIN *et al.*, 2007).

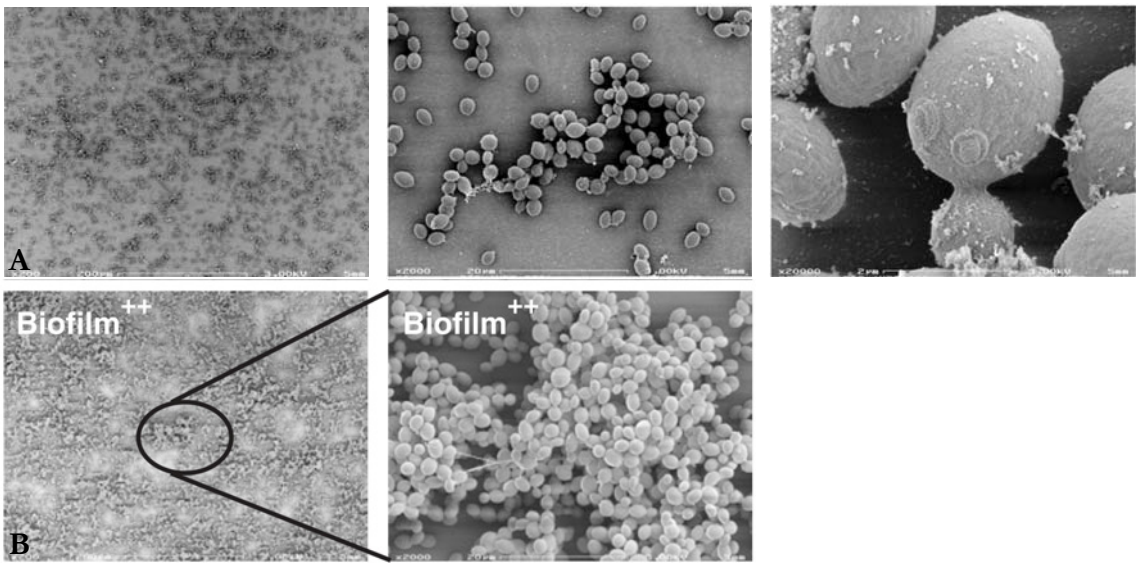

Figure 1-3 Biofilm formation by *Candida glabrata*
(A) *C. glabrata* wild type strains (here BG2) form biofilms on the surface of plastic slides. At high magnification (top right), residual extracellular matrix is visible. **(B)** Using a genetic screen for *C. glabrata* biofilm mutants, biofilm++ mutants were identified. One of these mutants formed extensive biofilms due to a mutation in the *YAK1* gene. The results strongly suggest that Yak1p regulates the transcription of *C. glabrata* adhesins via a subtelomeric silencing-dependent mechanism (from IRAQUI *et al.*2005).

In contrast to *C. albicans*, for which hyphae formation followed by tissue penetration and invasion is discussed as a very important factor of virulence (LO *et al.*, 1997), pathogenicity of *C. glabrata* is solely mediated by the yeast form (FIDEL *et al.*, 1999). Only under *in vitro* conditions of nitrogen starvation pseudomycel, but no true hyphae formation, can be observed (CALCAGNO *et al.*, 2003; CSANK and HAYNES, 2000). Although closely related to the apathogenic yeast *Saccharomyces cerevisiae, C. glabrata* infections show high mortality. This might also be due to the naturally low susceptibility and acquired resistance to treatment with Fluconazole, an established antifungal agent (HITCHCOCK *et al.*, 1993; VANDEN BOSSCHE *et al.*, 1998; VANDEN BOSSCHE *et al.*, 1992). Recent studies in Germany have shown that about 93% of *C. albicans* but only 23% of *C. glabrata* isolates are fully susceptible to treatment with Fluconazole. 50% of the *C. glabrata* isolates are classified as SDD (susceptible dose dependent), which necessitates a higher dosage in the treatment of patients (ZEPELIN, National Reference Centre for Systemic. Mycoses, Göttingen, personal communication).

	C. albicans	*S. cerevisiae*	*C. glabrata*
Pathogenicity	opportunistic	non pathogenic	opportunistic
Infection sites	skin, mucosa, disseminated	non infectious	skin, mucosa, disseminated
Ploidy	diploid	haploid and diploid	haploid
Mating type loci	present	present	present
Phenotypic switching	yes	no	yes
Growth forms	yeast, pseudohyphae, hyphae	yeast, pseudohyphae	yeast, pseudohyphae
Biofilm formation	yes	yes	yes
Adhesion	lectins, Hwp1, Als family	lectins, sexual agglutinins	lectins (EPA)
Azole resistance	Not frequent	no	often

Table 1 - Comparison of *Candida glabrata*, *Candida albicans* and *Saccharomyces cerevisiae*
In contrast to *C. albicans* and *S. cerevisiae*, *C. glabrata* possesses only a haploid set of chromosomes. Although it is unable to form true hyphae, it is a pathogenic fungus like *C. albicans* and frequently displays Fluconazole resistance.

1.3.2 Treatment of Candida glabrata infections

Many intensive-care patients acquire nosocomial infections during their stay at the hospital. Here, the bacterial infections (*Pseudomonas aeruginosa*, *Staphylococcus aureus*) still outnumber those caused by fungi (*Aspergillus fumigatus*, *Candida spp.*). Nevertheless these fungal infections have become a major clinical concern. Due to the metabolic similarities between fungi and their eukaryotic hosts, development of efficient and well tolerated antifungal agents is difficult. The "Gold-standard" for antimycotic treatment of systemic mycoses used to be Amphotericin B (ODDS *et al.*, 2003). It is a very lipohpilic agent and binds to ergosterol, an important component of the fungal cell membrane, forms an irreversible complex and thus changes the membrane's permeability. It is highly effective against most clinically relevant yeasts as well as other fungi like *Aspergillus spp.* Unfortunately it displays very low solubility and strong kidney toxicity. These problems have been partially overcome by the production of Amphotericin B in lipid formulations (e.g. AmBisome), where the Amphotericin B molecules are packed e.g. into a liposome for better availability. Nevertheless its side effects cause certain reluctance towards Amphotericin B medication, especially for the treatment of patients with fever of unknown origin (FUO) or as an antifungal prophylaxis medication in immuno compromised patients.

Fluconazole is now frequently used for treatment of candidiasis patients. This drug, an agent which inhibits the formation of ergosterol, can be used for the treatment of different yeasts (e.g. *C. albicans*, *C. parapsilosis* or *C. tropicalis*) and dermatophytes (e.g. *Microsporum*), but is not effective against various moulds (e.g. *Aspergillus spp.*) (TIETZ, 2004). Although it causes damage to the liver, it is far from being as toxic as Amphotericin B. Unlike most other *Candida spp. C. glabrata* is less sensitive or completely resistant to Fluconazole, (PFALLER *et al.*, 1999). This

usually necessitates an increase in duration and dose of Fluconazole or an alternative therapeutic strategy. Recently, it has been shown that Fluconazole susceptibility of tested *C. glabrata* isolates varies a lot, ranging from 82% to 44%, depending on factors such as the age of patients as well as their geographic location (PFALLER *et al.*, 2003a). An improved medication is now possible with the introduction of new broad-spectrum triazoles (e.g. Voriconazole). These drugs are more effective than their predecessors (PFALLER *et al.*, 2003a; PFALLER *et al.*, 2003b) and can be an option for the treatment of Fluconazole resistant *Candida spp*. However azole cross resistance has been observed (MULLER *et al.*, 2000).

The echinocandins represent a new class of drugs, which attack the fungal cell wall. These polypeptides exhibit a very effective mechanism to kill fungal organisms based on the inhibition of β-1,3-glucan synthesis. Thus, the echinocandins destroy or heavily weaken the outer most protective layer of the fungus' exterior. Interestingly, the antifungal activity of echinocandins has already been known and examined in the early 70s (BENZ *et al.*, 1974; KELLER-JUSLEN *et al.*, 1976). But it took almost 30 years until Caspofungin (in 2002) was the first echinocandin (Cancidas®) being approved by a drug regulatory authority to be used in human healthcare. It is effective against many medically important *Candida spp*. (e.g. *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. dubliniensis*) (PFALLER *et al.*, 2003a; PFALLER *et al.*, 2003b) as well as *Aspergillus spp*. However, there are important pathogenic fungi, which are not effectively eliminated by Caspofungin (e.g. *Cryptococcus neoformans*). This might be brought about by an alternative cell wall composition (e.g. a lack of β-1,3-glucan) or by compensatory mechanisms, like the upregulation of chitin, in order to counterbalance cell wall weakening caused by β-1,3-glucan depletion. Such compensatory mechanisms were observed in mutants, which are impaired in β-1,3-glucan synthesis or have an otherwise weakened cell wall structure (KAPTEYN *et al.*, 1997; POPOLO *et al.*, 1997). Caspofungin also displays very little side effects, which is a major improvement compared to other antimycotics. Nevertheless, the rather tight fungicidal spectrum and the necessity of intravenous application (CORNELY *et al.*, 2002) are a major setback.

To summarize: there are antimycotic drugs available; but they are either markedly toxic (as described for Amphotericin B), can be used for a limited set of susceptible species only (as seen for Fluconazole) or the occurrence of secondary resistance during therapy complicates an effective treatment. With Caspofungin the cell wall has proven to be an excellent target for antimycotic agents. This prove of principle gives reason to put effort in the identification of other cell wall targets eventually leading to improved strategies of antifungal treatment. In analogy to the successful treatment of serious bacterial infections this would also give the possibility for combination therapy. Thus, understanding the molecular organization of the

cell wall as well as the molecular bases of pathogenicity may lead to new therapeutic strategies. This is especially needed for the successful treatment of *C. glabrata* infections.

1.3.3 Adaptation mechanisms

C. glabrata has evolved as a pathogen with a perfect ability to adapt to changes in the environment and therefore is able to infect diverse body sites and organs (e.g. gastrointestinal tract, vaginal mucosa, skin and blood). Due to differences in temperature and ambient pH at the particular infection sites, the fungus must cope with altered protein activities, reduced nutrient availability, maintenance of the proton gradient and the necessity to remodel the cell wall.

It was found that *C. albicans* regulates central metabolic pathways in response to the surrounding environment. Barelle and co-workers found that glyoxylate genes are repressed at glucose concentrations present in the blood (though glyoxylate cycle genes are essential for pathogenicity) (BARELLE *et al.*, 2006). Other studies showed that amino acid biosynthesis genes are upregulated when *C. albicans* grows in biofilms (GARCIA-SANCHEZ *et al.*, 2004). Similar results were observed when the fungus was exposed to human neutrophils or cultured macrophages (FRADIN *et al.*, 2005; LORENZ *et al.*, 2004; RUBIN-BEJERANO *et al.*, 2003). Thus, environmental clues induce profound changes in protein expression. For more than 30 years it has been known that environmental pH strongly influences the morphological differentiation of *C. albicans*. While growth in the yeast form is favoured under acidic conditions, filamentous growth is induced at alkaline pH (Figure 1-4) (BRAUN and JOHNSON, 2000; BUFFO *et al.*, 1984; DAVIS *et al.*, 2000b; EVANS *et al.*, 1974; KONNO *et al.*, 2006; LEE *et al.*, 1975). Hypha formation has been suggested to enable this fungus to penetrate human tissue (Figure 1-5) and cause invasive disseminated disease (GOW *et al.*, 2003; LO *et al.*, 1997).

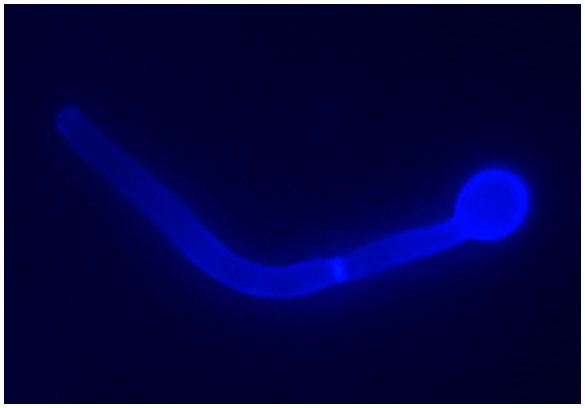

Figure 1-4 The morphology of *Candida albicans* is pH dependent
C. albicans wild type cells form hyphae when grown at alkaline pH. Cell wall chitin was stained with calcofluor white.

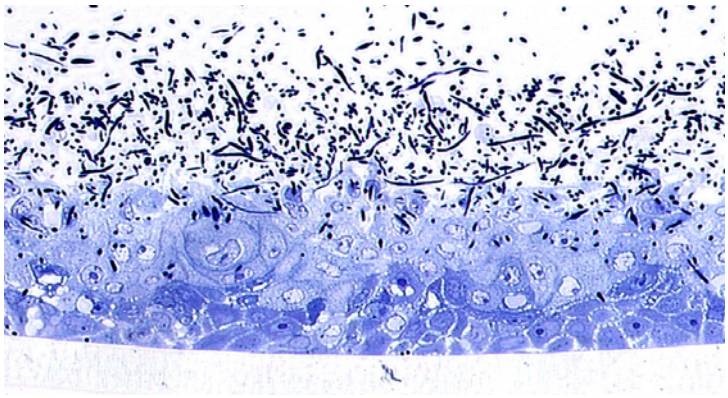

Figure 1-5 *Candida albicans* forms hyphae to penetrate human tissue
The reconstituted human epithelium (RHE) has been successfully used as a model for the pathogenic mechanism of tissue penetration in *C. albicans*.

Thus, the ability to respond to changing pH seems an important virulence factor in *C. albicans*. This change in morphology is a rather outward manifestation of the pH response. However, further investigation of this phenomenon, revealed an important molecular background: the regulated expression of *PHR1*. Expression of this gene was not detected at pH below 5.5 but was enhanced as the pH of the medium was increased. This parallels the pH dependent filamentation pattern even though *PHR1* expression is not linked to cell morphology (GHANNOUM *et al.*, 1995). Later, the inversely expressed *PHR2* gene and other pH regulated genes (e.g. *PRA1*, *RIM101/PRR2 and PRR1*) were identified and characterized (DAVIS, 2003; DAVIS *et al.*, 2000a; MUHLSCHLEGEL and FONZI, 1997; PORTA *et al.*, 1999; RAMON *et al.*, 1999). It was demonstrated that this response to pH occurs not only *in vitro* but also at the infected host site and that *PHR1* and *PHR2* are critical to virulence as knock out of these genes renders *C. albicans* avirulent (DE BERNARDIS *et al.*, 1998; GHANNOUM *et al.*, 1995). Today it is known that *RIM101* is a central regulator of response to alkaline pH in *C. albicans* (DAVIS, 2003). It induces *PRA1* and *PHR1* expression at alkaline pH, and represses *PHR2* at the same condition (DAVIS *et al.*, 2000b).

In contrast to *C. albicans*, *C. glabrata* does not form hyphae and thus the responses to pH can not be seen by similar drastic morphological changes. Nevertheless unravelling the profound transcriptional and translational changes of *C. glabrata* in response to pH should improve our understanding of the fundamental pathogenetical clues of this organism.

1.3.4 Candida glabrata genetics

C. glabrata belongs to the *Ascomycota* and the class of *Endomycetes* (DE HOOG, 2000; ODDS, 1988). As mentioned before, it is a single celled, haploid fungus, which carries 11

chromosomes (MAGEE and MAGEE, 1987). Under standard lab conditions (e.g. on YPD, YNB or Sabouraud plates, incubated at 30°C or 37°C), *C. glabrata* grows in small, glistening colonies with a white to creamy colour. However, when grown on SLAD plates under nitrogen starvation conditions the fungus is able to form pseudohyphae (CSANK and HAYNES, 2000) (Figure 1-6).

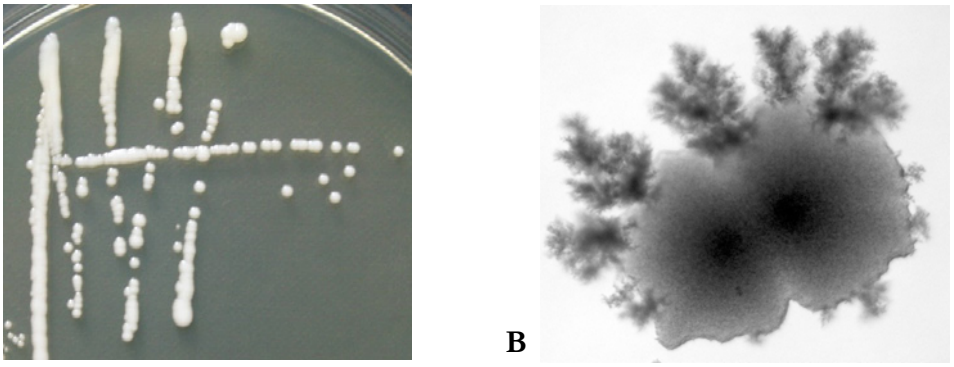

Figure 1-6 Growth forms of *Candida glabrata*
(A) *C. glabrata* ATCC2001 wild type strain was grown on YPD agar plates at 37 °C over night forming small, white, glistening colonies consisting of single celled yeasts. **(B)** After growth on synthetic low ammonia dextrose (SLAD) media plates at 37 °C for five days, *C. glabrata* ATCC2001 wild type strain produced pseudohyphae at the rim of the colony.

The baker's yeast is very well studied and for many years useful genetic tools have been available to analyse gene functions in this organism. However, in the case of *C. glabrata* they have been available only for the last few years. Auxotrophic mutant strains (KITADA *et al.*, 1995; WEIG *et al.*, 2001), plasmid vectors (KITADA *et al.*, 1996; KITADA *et al.*, 1997) and a reporter system using *lacZ* (EL BARKANI *et al.*, 2000) have been generated and it was also shown that in *C. glabrata* homologous recombination events occur (CORMACK and FALKOW, 1999). However, illegitimate non-homologous recombination was observed in the absence of homologous regions on the transformed DNA fragment, indicating the importance of these sequences in a transformation construct (CORMACK and FALKOW, 1999). The auxotrophic mutant strains 2001U (ATCC2001 ⊿*ura3*), 2001TU (ATCC2001 ⊿*trp1* ⊿*ura3*) and 2001HTU (ATCC2001 ⊿*his3* ⊿*trp1* ⊿*ura3*) were generated by gene disruption (KITADA *et al.*, 1995). Working with these auxotrophic *C. glabrata* strains, it was observed that the partial deletion of the marker locus holds disadvantages. Knock out cassettes, carrying for example the *HIS3* gene for selection of transformants, integrated not only at the site of targeted gene knock out but also at the original *HIS3* locus, as the sequence remnants allowed homologous recombination. Therefore, the improved auxotrophic mutants ΔH1 (ATCC2001 ⊿*his3*) and

ΔHT6 (ATCC2001 ⊿*his3* ⊿*trp1*) were constructed, which lacked the auxotrophic markers completely (WEIG *et al.*, 2004). These mutants can now be employed for gene knock out experiments by site specific homologous recombination using knock out cassettes carrying the respective auxotrophic marker. Afterwards, generated knock out strains can be complemented by reintroduction of the deleted gene to prove that an observed phenotype was indeed caused by the gene deletion but not by unexpected und uncontrolled genetic events. For this purpose the gene of interest can be cloned into a plasmid, which must replicate episomally and must be stably maintained in the cells. Plasmids containing such an autonomously replicating sequence (ARS) element generally exist in high copy numbers and are highly unstable. However, it was shown that the introduction of a centromere into an ARS plasmid confers stability and reduces the copy number to one or two per cell (CLARKE and CARBON, 1980). For *C. glabrata*, the plasmid based vectors pCgACH3 and pCgACT14 have been generated (KITADA *et al.*, 1996). Both plasmids carry ARS and centromere sequences from *C. glabrata*, as well as a selection marker (*CgHIS3* or *CgTRP1* gene respectively). They show stabilities of 91.5% (pCgACH3) and 90.3% (pCgACT14) after ten generation-growth in YPD with a loss rate per generation of 0.6% and 0.7% respectively. They have been successfully used for the generation of *C. glabrata* revertants (BADER *et al.*, 2001).

Additionally, a controllable gene-expression system has been developed to elucidate the physiological role of genes e.g. in a mouse model and to examine the function of essential genes. The system uses a tetracycline responsive element (tetO) to control target gene expression and has been successfully employed for the investigation of the two essential genes *TEF3* (transcription elongation factor 3) and *TOP2* (DNA topoisomerase 2) (NAKAYAMA *et al.*, 1998). To analyse pathogen-host interaction a signature-tagged mutagenesis approach was applied (CORMACK *et al.*, 1999). This strategy permits parallel screening of multiple mutants in a pool of mutants, as each mutant carries a unique sequence tag flanked by constant PCR priming sites, which permit the amplification of all tags in a single PCR amplification step. The fate of individual mutants can be mirrored by their cognate oligonucleotide tags.

All these novel tools, available now for *C. glabrata*, enable us to begin studying the molecular bases underlying pathogenic mechanisms as well as general biological processes of this organism.

1.4 The fungal cell wall

1.4.1 Cell wall structure

The formation of an outer protective cell wall layer is crucial for the survival of fungi in the harsh environments they are facing. For some decades the only information about the cell wall was the biochemical nature of its major structural components: polysaccharides and proteins (Figure 1-7). With the development of more sophisticated biochemical and genetic methods, specific cell wall genes and the function of their encoded proteins could be analyzed. As the outer most structure, the cell wall of fungi mediates morphogenesis and plays a key role in host-pathogen interaction. It is synthesized by enzymes, which are specific to fungi, is composed of structures absent in the mammalian cell and therefore is a promising target for antimycotic drugs.

The majority of studies about the composition and structure of the fungal cell wall were done in the model organism *Saccharomyces cerevisiae*. In this ascomycetous yeast the molecular organization of the cell wall is well documented (DE NOBEL *et al.*, 2000b; ECKER *et al.*, 2006; KAPTEYN *et al.*, 1999; KLIS *et al.*, 2006; KLIS *et al.*, 2002; KOLLAR *et al.*, 1995; KOLLAR *et al.*, 1997). Also the cell wall of *C. albicans* is now more and more investigated (CHAFFIN *et al.*, 1998; KLIS *et al.*, 2001; OSUMI, 1998; RUIZ-HERRERA *et al.*, 2006). For *C. glabrata* however, molecular studies of the cell wall are rare (FRIEMAN *et al.*, 2002; WEIG *et al.*, 2004) to the present.

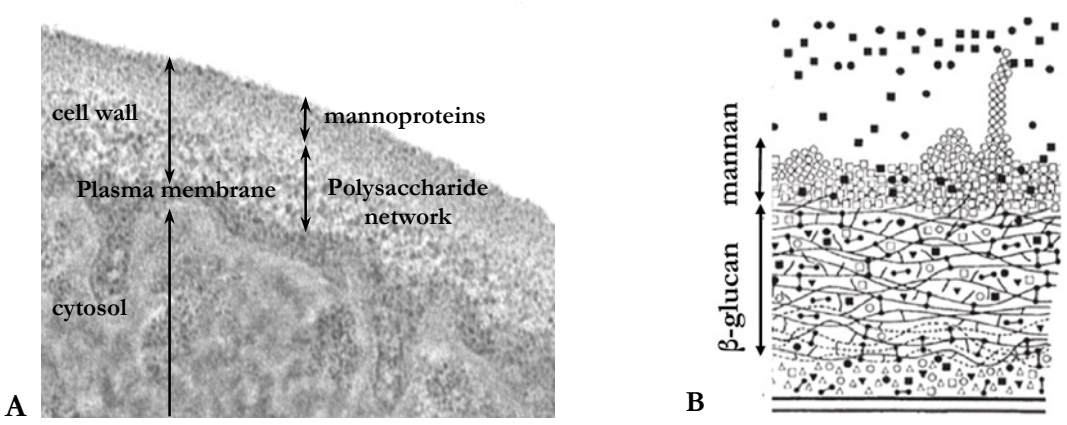

Figure 1-7 The fungal cell wall
In the electron microscopic picture **(A)** two layers of the cell wall (the electron dense mannoprotein layer and the lighter glucan network) can be distinguished. **(B)** Model of the cell wall showing the interconnected β-glucan network, anchored mannoproteins and their glycosylations (mannan) (taken from (WHEELER and FINK, 2006).

The cell wall of the baker's yeast consists of β-1,3-glucan, β-1,6-glucan, chitin and mannoproteins (Figure 1-8). Chitin is an N-acetylglucosamine polymer, which covers the plasma membrane. The levels of chitin in the cell wall vary from 1%-3% in *S. cerevisiae* and 1%-2% in *C. albicans* (BROWN and CATLEY, 1992; FLEET, 1985). Chitin levels in *C. glabrata* are around 1% of cell wall dry weight (see 3.3.3 of this study). In *S. cerevisiae* the β-1,3-glucan forms a three dimensional resilient framework, being responsible for mechanical strength of the cell wall, in which chitin and β-1,6-glucans are interwoven (CHAFFIN *et al.*, 1998; KLIS, 1994). To accomplish this three dimensional network, reducing ends of chitin and β-1,6-glucan polymers are connected to the non reducing ends of β-1,3-glucan side chains (KAPTEYN *et al.*, 1997; KOLLAR *et al.*, 1995; KOLLAR *et al.*, 1997). The cell wall of *S. cerevisiae* as well as *C. albicans* contains approximately 25-30% alkali resistant β-1,3-glucan, whereas the amount of alkali resistant β-1,6-glucan varies between these species (around 9-11% in *C. albicans* and 6-8% in *S. cerevisiae*) (DE GROOT *et al.*, manuscript in preparation). Recent analyses of *C. glabrata* cell walls revealed differences in this polysaccharide composition. De Groot and co-workers found that only about 4% of *C. glabrata* cell wall dry weight was made of alkali resistant β-1,6-glucan and 16-18% of β-1,3-glucan (DE GROOT *et al.*, manuscript in preparation).

The outer layer of the cell wall mainly consists of highly glycosylated proteins, so called mannoproteins (KLIS, 1994; KLIS *et al.*, 2006), which account for certain surface properties of the cell wall such as hydrophobicity, electrical charge, flocculence, sexual agglutinability and pathogenicity, as in the case of *C. albicans* (HOYER, 2001; HOYER and HECHT, 2001; RUIZ-HERRERA *et al.*, 2006; SUNDSTROM, 2002; SUNDSTROM *et al.*, 2002a). The glycosylation of these mannoproteins also determines the permeability of the cell wall for macromolecules (DE NOBEL *et al.*, 1990; ZLOTNIK *et al.*, 1984). Depending on the environmental conditions (e.g. pH, temperature, oxidative or high osmolarity stress, aerobic or anaerobic growth conditions), growth stage (e.g. logarithmic phase vs. stationary phase), growth form (e.g. yeast vs. hypha) and phase of the cell cycle, the composition of the cell wall protein population may vary significantly (ABRAMOVA *et al.*, 2001; CARO *et al.*, 1998; DE NOBEL *et al.*, 1991; KAPTEYN *et al.*, 2001; KLIS *et al.*, 2001; RODRIGUEZ-PENA *et al.*, 2000). Even though not all encoded GPI-proteins might eventually arrive at the cell wall, the number of 106 *in silico* predicted GPI-dependent cell wall proteins in *C. glabrata* indicates the high potential for regulated, specifically adapted cell wall protein expression (DE GROOT *et al.*, 2003; WEIG *et al.*, 2004) in this fungus. Additionally, it has been shown, that regulation of cell wall protein expression and maintenance of cell wall integrity is not only crucial for stress resistance but also for virulence of *C. glabrata* (CALCAGNO *et al.*, 2004; CALCAGNO *et al.*, 2005; CALCAGNO *et al.*, 2003).

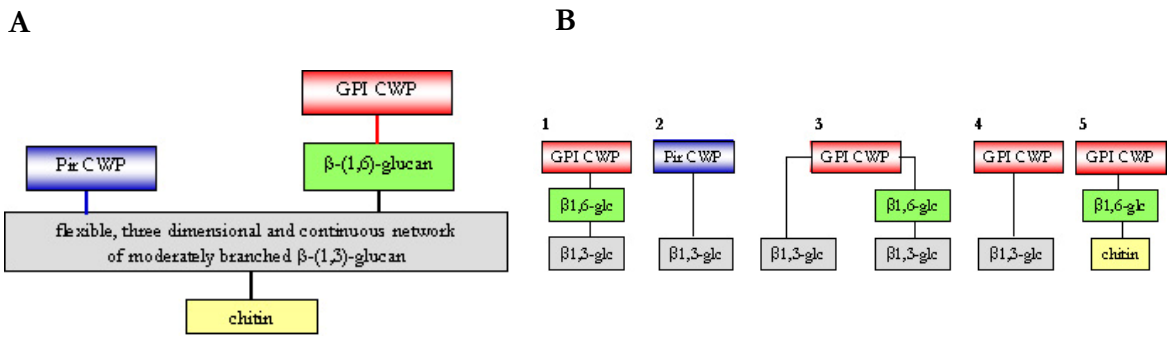

Figure 1-8 Anchoring of proteins in the cell wall of *S. cerevisiae*
(A) A three dimensional polysaccharide network is formed by β-1,3-glucan, in which chitin and β-1,6-glucan are interwoven. Pir-proteins are directly bound to β-1,3-glucan via an alkaline sensitive linkage (blue), whereas GPI-proteins are connected with β-1,3-glucan via the crosslinker β-1,6-glucan through a hydrofluoric acid (HF) sensitive linkage (red). **(B)** So far five possible CWP-polysaccharide complexes have been identified in yeast. Shaded boxes indicate proteins, solid boxes indicate polysaccharides (adapted after KLIS *et al.*2002).

In the baker's yeast there are three major groups of covalently bound cell wall proteins: glycosyl-phosphatidylinositol (GPI) anchored proteins, proteins with internal repeats (Pir) and proteins linked to other proteins by disulfide bridges. The largest class of covalently bound mannoproteins, the GPI-anchored proteins, are attached to the β-1,3-glucan framework via β-1,6-glucan molecules (KAPTEYN *et al.*, 1995; KAPTEYN *et al.*, 1996; KAPTEYN *et al.*, 1997). They can be selectively liberated from purified cell walls by treatment with β-1,6-glucanase or hydrofluoric acid-pyridin complex (HF) (KAPTEYN *et al.*, 1995).

The less abundant group of covalently linked mannoproteins, the Pir-proteins are directly bound to β-1,3-glucan (KAPTEYN *et al.*, 1999) by an alkali sensitive linkage (MRSA *et al.*, 1997). Recently it was suggested that this linkage is an ester linkage between the γ-carboxyl group of glutamic acid residues, which arise from glutamines, and hydroxyl groups of glucose molecules from the β-1,3-glucan (ECKER *et al.*, 2006). This reaction is very similar to the transglutaminase reaction, which the pathogenic yeast *C. albicans* uses to attach itself to buccal epithelial cells during the infection process via the cell wall protein Hwp1 (SUNDSTROM *et al.*, 2002a). There, the γ-carboxamide group of a glutamine residue reacts with primary amines (e.g. the ε-amino group of protein bound lysines) to cross link or polymerize proteins.

As a third possibility, the mannoproteins can also be bound to other proteins via disulfide bridges or can be non-covalently associated to complete the cell wall structure.

Based on the knowledge in *S. cerevisiae*, the cell wall structure of *C. albicans* has been analysed successfully (DE GROOT *et al.*, 2004; KAPTEYN *et al.*, 2000; KAPTEYN *et al.*, 1995) and some features, such as the constitutive assembly of β-1,3-glucan, β-1,6-glucan, chitin and mannoproteins are highly similar (KAPTEYN *et al.*, 2000; KAPTEYN *et al.*, 1995). On the other hand, a number of *C. albicans* specific and pathogenicity determining proteins have been

identified (DE GROOT *et al.*, 2004) and studied as for example Hwp1 (NAGLIK *et al.*, 2006; STAAB *et al.*, 1999; SUNDSTROM *et al.*, 2002b) or the essential Pir1 protein (MARTINEZ *et al.*, 2004). Nowadays, the first step for the characterization of the *C. glabrata* cell wall has been made by the *in silico* identification of putative cell wall proteins (WEIG *et al.*, 2004). The high number of 116 cell wall associated proteins and their suggested functions highlight the potential role of the *C. glabrata* cell wall in pathogenicity.

Thus, the cell wall accounts for cell shape, is responsible for resisting mechanical stress and plays important roles in adhesion and pathogenicity (also see 2.4.2. GPI-anchored cell wall proteins). Characterizing the features of the *C. glabrata* cell wall will give a better understanding of its pathogenic mechanisms and might eventually lead to novel successful treatment strategies.

1.4.2 GPI-anchored cell wall proteins

Genome wide *in silico* analyses identified 66, 104 and 106 putative GPI-proteins in the gnomes of *S. cerevisiae, C. albicans* and *C. glabrata* respectively (DE GROOT *et al.*, 2003; WEIG *et al.*, 2004). These GPI-proteins can either be bound to the plasma membrane or are incorporated into the cell wall, depending on the carboxy-terminal protein sequence (FRIEMAN and CORMACK, 2003). GPI-anchored proteins displaying various functions: some GPI-proteins are important for physical strength, permeability of the cell wall or adhesion properties, whereas others have been shown to exhibit enzymatic functions.

In *S. cerevisiae* for example, the regulated expression of certain mannoproteins accounts for cell wall porosity (ZLOTNIK *et al.*, 1984). On the other hand, the sexual adhesion protein α-agglutinin of *S. cerevisiae* MATα cells can also be found among the group of GPI-proteins. In *C. albicans* several GPI-anchored proteins have been identified to be related to pathogenicity, like HWP1 (STAAB *et al.*, 1999) and different ALS proteins (HOYER, 2001; KLIS *et al.*, 2001), which can not be found in *S. cerevisiae*. The group of adhesins account for a large class of GPI-anchored cell wall proteins found in diverse fungal species including *S. cerevisiae, C. albicans, C. glabrata* and *Aspergillus fumigatus* (BRUL *et al.*, 1997; KAPTEYN *et al.*, 2000; KLIS *et al.*, 2001; LATGE, 1999). Although all adhesins enable fungal cells to bind to other cells or surfaces, their modes of action differ: Adhesion can be sugar-sensitive (lectin-like) or sugar-insensitive. The sugar-sensitive adhesion depends on the lectin-like binding of the proteins to sugar residues on the surface of other cells. Adhesins of this group (e.g. *S. cerevisiae FLO* gene products or *C. glabrata EPA* gene products, also see Figure 1-9) contain an amino-terminal lectin-like carbohydrate binding domain (CORMACK *et al.*, 1999; SEKI *et al.*, 1998; VERSTREPEN and KLIS,

2006), which they use either to bind to glycosides on the surface of mammalian cells (*C. glabrata*) or mannose oligomers on their own surface (aggregation of *S. cerevisiae* cells).

One interesting member of this adhesin group is Epa1p, which mediates about 95% of the adhesion properties of *C. glabrata* to human epithelial cells and thus is essential for the establishment of an infection. It has been identified in *C. glabrata* (CORMACK *et al.*, 1999; DOMERGUE *et al.*, 2005), but cannot be found in *S. cerevisiae* or in *C. albicans* (DE GROOT *et al.*, 2003). Thus, the cell wall of these three different yeasts, although similar in its basic architecture, does show significant differences, especially in regard to proteins relevant for pathogenicity. In *C. glabrata* only a few GPI-proteins have been characterized so far: the family of Epa adhesins (CORMACK *et al.*, 1999; DE LAS PENAS *et al.*, 2003; FRIEMAN *et al.*, 2002), a family of Gas/Phr orthologues Gas1-3 (WEIG *et al.*, 2001) and a family of yapsins (KAUR *et al.*, 2007), extracellular aspartyl proteases which play an important role in cell wall remodelling.

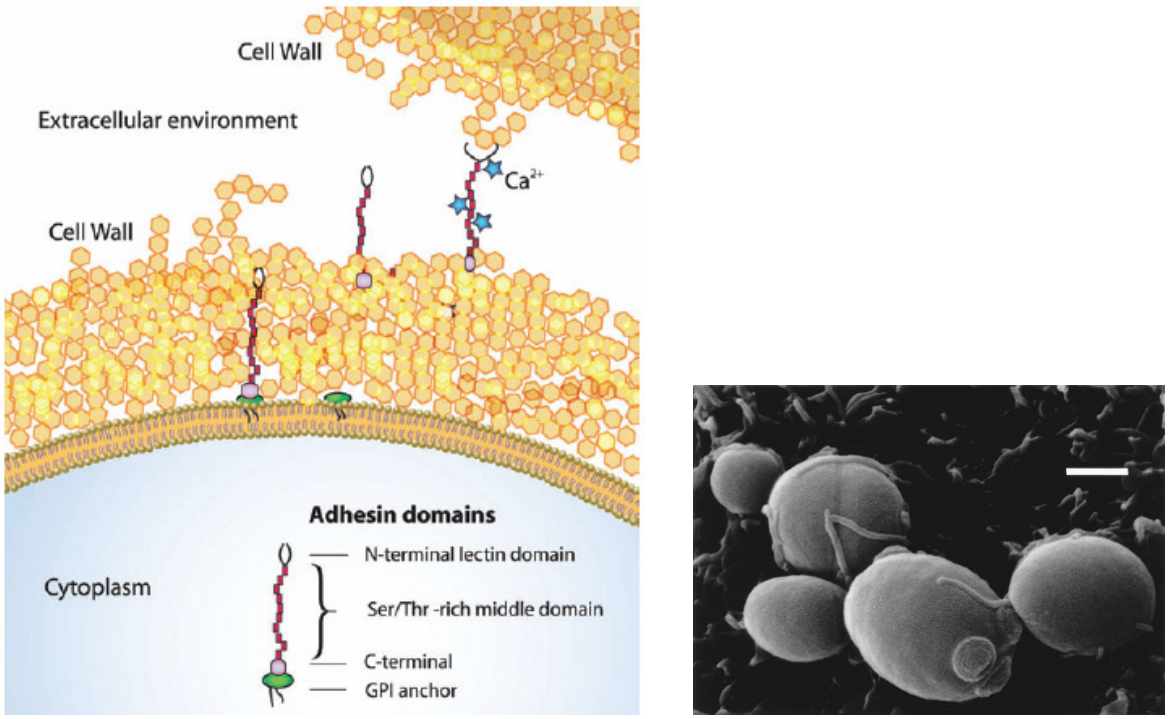

Figure 1-9 Anchoring and function of fungal adhesins
(A) Adhesins display the characteristics of GPI-anchored cell wall proteins. The amino-terminal signal peptide for secretion is removed and a GPI-anchor is attached at the carboxy-terminus. First, the proteins are transported to the plasma membrane, where the GPI-anchor is cleaved off and the adhesins are covalently linked to the β-1,6-glucan via the GPI-remnant. It is believed that the short O-linked oligosaccharide side-chains enable the adhesions to obtain a long, semi-rigid rod-like structure that is stabilized by Ca^{2+} ions. (taken from VERSTREPEN *et al.*2006) **(B)** Scanning electron micrograph of *C. glabrata* adhering to cultured HEp2 cells. Scale bar 1 µm (taken from CORMACK *et al.*1999)

For *C. albicans* as well as for *C. glabrata*, adherence to host cells is hypothesized to be an important factor of virulence, a process in which GPI-anchored proteins have been shown to be involved (CALDERONE and FONZI, 2001; CORMACK *et al.*, 1999). The 106 *in silico* identified GPI-proteins include 51 adhesive proteins, 11 glycoside hydrolases, 17 other enzymatic proteins (proteases, lipases and proteins for cell wall biogenesis) as well as structural proteins (14) and proteins with other or unknown function (WEIG *et al.*, 2004). Thus, GPI-proteins fulfil an enormous variety of functions.

The described GPI-associated proteins have to meet three sequence requirements: an amino-terminal signal sequence for secretion, a hydrophobic, fungal specific GPI-attachment site at the carboxy-terminus and the absence of internal transmembrane domains. Three consecutive parts of the GPI-attachment signal can be distinguished: first the GPI-attachment site itself (the ω site) plus two additional amino acids downstream of the ω site (ω+1 and ω+2 site); second a spacer of 4-19 amino acids and third a hydrophobic tail domain of 11-15 amino acids (FRIEMAN and CORMACK, 2003; MAO *et al.*, 2003; WEIG *et al.*, 2004) (also see Figure 1-10). After synthesis and translocation into the endoplasmic reticulum, the signal sequence for secretion is removed and the precursor protein is cleaved between the ω and ω+1 site. Afterwards a preassembled GPI-anchor, present in the ER membrane, is attached to the carboxy-terminus and the protein is transported to the cell surface (Figure 1-11). Depending on the amino acids present immediately upstream of the ω-site, the protein will end up either in the plasma membrane or the cell wall. Proteins with basic amino acids immediately upstream of the GPI-attachment site are preferably anchored in the plasma membrane (COYNE *et al.*, 1993; DE GROOT *et al.*, 2003; VOSSEN *et al.*, 1997).

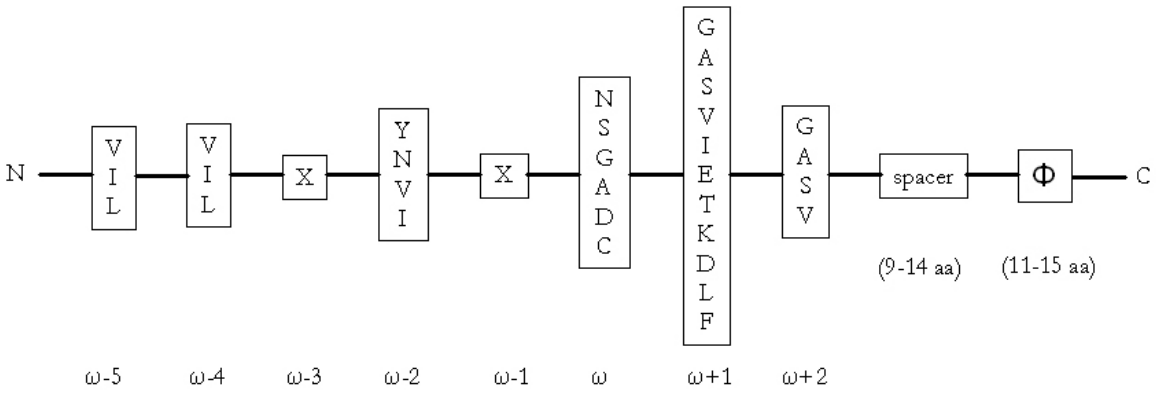

Figure 1-10 Sequence requirements for cell wall localization of a GPI-anchored protein
N (amino-terminus), C (carboxy-terminus), ω (GPI-attachment site), Φ (hydrophobic stretch), X (any amino acid) (after (WEIG *et al.*, 2004)

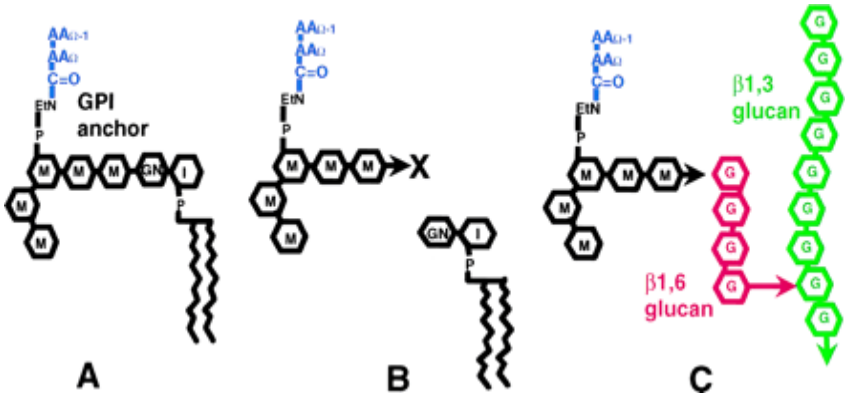

Figure 1-11 Model for the anchoring of GPI-proteins into the fungal cell wall
(A) GPI-anchor: AA, amino acid residue; EtN, ethanolamine; M, mannose; GN, glucosamine; I, myoinositol. **(B)** Proposed cleavage of the glycan of the GPI-anchor. Arrow, glycan reducing end; "X", a hypothetical complex or other "activator." **(C)** Formation of a glycosidic linkage between the GPI remnant and glucan. G, glucose. (LIPKE and OVALLE, 1998)

1.4.3 The GPI-anchored protein Cwp1p

The *in silico* analysis of the *C. glabrata* genome identified a group of GPI-proteins with putative structural function in the cell wall. *S. cerevisiae* homologues of these proteins have been shown to be induced under stress conditions (e.g. Spi1, Tip1, Srp1) or to be important for agglutination and mating (Ccw12). Part of this structural protein group in *C. glabrata* is a family of three homologous proteins named Cwp1.1p, Cwp1.2p and Cwp1.3p. Interestingly, these proteins do not only have the sequence prerequisites for GPI-attachment but also show characteristic Pir protein repeat-like sequences in the carboxy-terminal half of the proteins. Furthermore, no basic residues can be found in the sequence immediately upstream of the GPI-attachment site, which suggests a cell wall associated localization. Thus, the sequence of the Cwp1 proteins suggests that they can be bound to the cell wall either via the GPI-anchor or via a Pir like linkage.

Subsequently, Cwp1.1p was identified as one of the most abundant cell wall proteins in *C. glabrata* (WEIG *et al.*, 2004). A homologue of this protein was previously identified in *S. cerevisiae* (VAN DER VAART *et al.*, 1995) but cannot be found in *C. albicans*. The Cwp1 protein in *S. cerevisiae* was identified as a major cell wall glycoprotein, which is not essential for growth (SHIMOI *et al.*, 1995). A transcriptional analysis of cell cycle regulated genes showed that the protein's expression peaks in late S/early G2 phase (CARO *et al.*, 1998). Furthermore, transcription of the gene is induced by low environmental pH (KAPTEYN *et al.*, 2001) but downregulated during anaerobic growth (ABRAMOVA *et al.*, 2001). Gene deletion of *CWP1* in *S. cerevisiae* resulted in an increased calcofluor white and congo red sensitivity of the generated mutants (VAN DER VAART *et al.*, 1995) underlining the structural role within the cell wall

architecture. Three highly similar *CWP1* genes exist in *C. glabrata*, indicating its importance in the cell wall of monomorphic yeasts.

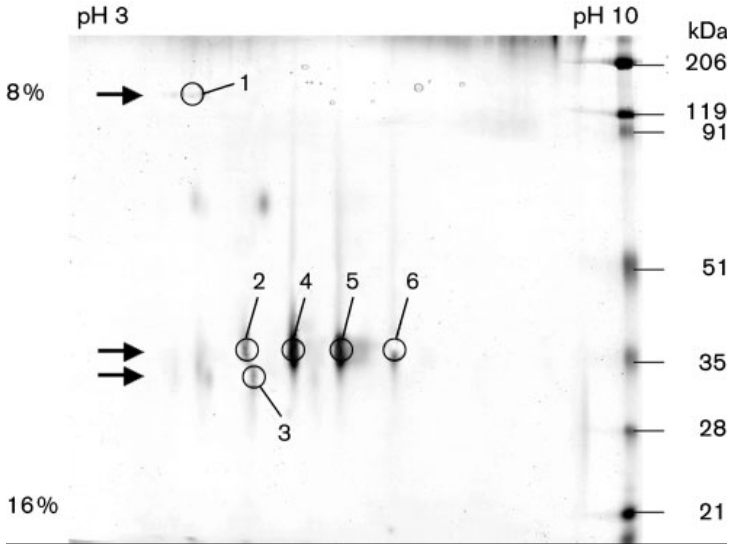

Figure 1-12 HF-pyridine-extracted cell wall proteins on a gradient 2D SDS gel
GPI-proteins were extracted from cell wall material, separated by gradient 2D gel electrophoresis and stained with colloidal Coomassie. Protein spots 2, 4, 5 and 6 were identified as Cwp1.1p and spot number 3 as Cwp1.2p in mass spectrometric analysis. The occurrence of Cwp1.1p at four different pIs is likely to be due to differently phosphorylated O-glycosylations of the protein (taken from WEIG *et al.*2004).

The three members of the *C. glabrata* Cwp1p family are very similar to each other. Cwp1.1p and Cwp1.2p show 95% identity and 97% similarity to each other, whereas Cwp1.3p is less similar to the others. It shows only 40% identity, 55% similarity to Cwp1.1p and 42% identity, 56% similarity to Cwp1.2p. As mentioned above, Cwp1.1p and Cwp1.2p were identified in the cell wall of *C. glabrata* by mass spectrometry analysis (Q-TOF) of GPI-cell wall proteins (WEIG *et al.*, 2004). However, Cwp1.3p was not detectable in MS analysis so far. The two proteins show 50% identity and 64/66% similarity, respectively, to Cwp1 of *Saccharomyces cerevisiae* (Figure 1-13). In the genome, these two genes, possibly originating from gene duplication, can be found directly adjacent to each other with a spacing of about 3kb between the open reading frames.

The molecular masses of the Cwp1 proteins range from 21.3 kDa and 20.7 kDa for Cwp1.1p and Cwp1.2p respectively, to 26 kDa in the case of Cwp1.3p. When separated on SDS gels, Cwp1.1p and Cwp1.2p run at higher molecular masses (about 34–37 kDa) than theoretically assumed. This observation may be due to the post-translational O-glycosylation of Cwp1.1p and Cwp1.2p. Both proteins possess serine/threonine rich regions, which are potential O-glycosylation sites. However, N-glycosylation acceptor sites (Asn-X-Ser/Thr), are not present

in their sequences. The O-glycosylations can be phosphorylated to different extend (NAKAYAMA *et al.*, 1998). This could explain explains why Cwp1.1p is found at various pIs on 2D gels.

Figure 1-13 Alignment of the Cwp1p protein sequences of *Candida glabrata* and *Saccharomyces cerevisiae*.
The degree of conservation in each position is indicated by (*) for identical residues, (:) for conserved substitutions, and (,) for semi-conserved substitutions of the alignment. Peptide sequences of CgCwp1.1p and CgCwp1.2p derived from the mass spectrometric analysis are depicted in bold. Predicted amino-terminal ER-import signals are underlined. Putative GPI-attachment sites are boxed. Pir protein repeat-like sequences (in the carboxy-terminal half of the proteins) are also underlined. (taken from WEIG *et al.*2004).

1.4.4 Tools for the identification of cell wall proteins

2D-gelelectrophoresis is an excellent tool for the large scale separation of proteins. For many years it has been successfully used in combination with mass spectrometric (MS) analysis for proteome examinations in various organisms. It has also been tried to use the same experimental procedure for the identification of fungal cell wall proteins (WEIG *et al.*, 2004). GPI-anchored cell wall proteins were released from *C. glabrata* cell wall preparations, separated on gradient 2D SDS polyacrylamide gels, stained with colloidal coomassie, excised from the gel, trypsin digested and subjected to mass spectrometry. Since mannoproteins are highly glycosylated, presumably only a few small proteins were actually detected on the gel and could

therefore be identified. To overcome these obstacles, de Groot and co-workers (YIN *et al.*, 2005) developed a new experimental approach: The trypsin digestion of proteins prior to mass spectrometry was done with the purified cell wall material directly. Afterwards, the produced tryptic peptides were subjected to mass spectrometry analysis. Using this approach more proteins could be identified than after the conventional gel separation. However, one drawback of this method is that protein expression cannot be quantified as it is possible with gel based methods, but technical and experimental improvements start to overcome this problem. Using the iTRAQ methodology quantification is now possible. (ROSS *et al.*, 2004; YIN *et al.*, 2007). Cell wall peptides, which were previously released from the cell wall via a tryptic digest, were labelled with one or more of the four available iTRAQ reagents. The added mass of the molecule is kept constant so that derivatized peptides from different samples have the same mass, but yield distinct reporter ions at m/z 114, 115, 116 or 117 in a mass spectrometer. The relative concentrations of the peptides are deduced from the relative intensities of the corresponding reporter ions.

Having the required tools available it is now possible to thoroughly examine the cell wall architecture of *C. glabrata*. The knowledge about the immense pathogenetic potential of the cell wall and its incorporated proteins, indicates that an in depth investigation might reveal virulence associated protein functions or unravel molecular evidence for novel putative antifungal targets.

1.5 Aims of the study

C. glabrata has advanced as the second most common cause of systemic Candidiasis in humans and has attracted medical attention as it frequently displays resistance to the commonly used antifungal drug Fluconazole. Mechanisms underlying this resistance are not fully understood to this day and studies analysing pathogenic mechanisms are rare, which demands to study the molecular structure, organization and regulatory mechanisms of the fungus. Here, the cell wall deserves special attention: It is involved in the recognition of environmental signals and mediates the first interaction with the host.

In a proteomic analysis of GPI-anchored cell wall proteins, Cwp1.1p has been identified as one of the most abundant cell wall proteins in *C. glabrata* (WEIG *et al.*, 2004). No homologues of this protein exist in its dimorphic relative *C. albicans*. Therefore, we sought to investigate the function of Cwp1.1p, as well as its highly similar homologues Cwp1.2p and Cwp1.3p, by targeted gene deletion and functional analysis of the generated mutants. We hypothesized that

the absence of a major structural cell wall component might induce severe cell wall weaknesses and could render the fungus less potent to respond to environmental stress conditions. Trying to counterbalance the induced impairment of cell wall architecture, the fungus might disclose the nature of compensatory strategies. These may in turn uncover possible mechanisms involved in antifungal resistance.

As the fungus occupies a variety of infection sites (e.g. the oral cavity, the vaginal mucosa and the blood) the recognition and processing of various environmental signals are the fundamental prerequisites of successful survival and virulence. In the cell wall for example we observed pH-dependent compensatory mechanisms. Despite their enormous importance, these fundamental processes are still widely unexplored in *C. glabrata*. Therefore, we analyzed the protein expression pattern of the fungus upon exposure to different environmental pH values using an overall proteomics approach. In *C. albicans*, an ambient alkaline pH induces filamentation, which is a prerequisite for invasive growth and disseminated infection. Even though *C. glabrata* is not able to form hyphae, it can cause systemic infections. We therefore expected remarkable differences in protein expression patterns when the fungus was exposed to acidic and alkaline pH values.

Thus, the aims of the present study were (i) to elucidate the function of the *C. glabrata* cell wall protein 1 family in the architecture of the fungal cell wall and (ii) to analyse the alterations in protein expression pattern of *C. glabrata* in response to changes in the ambient pH conditions.

2 MATERIALS AND METHODS

2.1 Chemicals and Disposables

Chemicals were purchased from Merck (Darmstadt), Sigma-Aldrich (Munich), GibcoBRL (Eggenstein), Merck (Darmstadt), Pierce (Perbio, Bonn), Roth (Karlsruhe) or Becton Dickinson (Oxford, England) if not stated otherwise. Disposables were purchased from Roth (Karlsruhe), VWR (Darmstadt), Rettberg (Göttingen), Schleicher und Schüll (Dassel) and Whatman (Biometra, Göttingen).

2.2 Enzymes and Reaction kits

Enzymes were purchased from the following companies:

DNA quickligase	New England Biolabs, Frankfurt
Restriction enzymes	New England Biolabs, Frankfurt
Taq DNA polymerase	Roche, Mannheim
Shrimp alkaline phosphatase	Roche, Mannheim
KOD hot start DNA polymerase	Novagen (via VWR), Darmstadt
T4- DNA ligase	Novagen (via VWR), Darmstadt
Quantazym	Q-Biogene, Illkirch
Zymolyase-20T	Medac, Wedel

Plasmid preparations and DNA purification steps (gel extraction, PCR or restriction digest purification) were performed with plasmid preparation and DNA purification kits from Qiagen (Hilden) respectively. For subcloning of PCR products we used either the TOPO-TA cloning kit from Invitrogen (Karlsruhe) or the Perfectly Blunt cloning kit from Novagen (via VWR, Darmstadt). Protein quantification was performed using the BCA protein assay kit from Pierce (Perbio, Bonn).

2.3 Synthetic oligonucleotides and molecular weight standards

Synthetic oligonucleotides used for polymerase chain reactions and sequencing were purchased from Sigma-Aldrich (Munich). A list of the primers can be found in the Appendix. We received DNA molecular weight standards from Eurogentec (Cologne), Dig-labelled DNA standard from Roche (Mannheim) and protein molecular weight standards from Roth (Karlsruhe).

2.4 Plasmids

pBSK+/KS- These standard vectors were used for the subcloning of PCR products to facilitate their specific excision with restriction enzymes e.g. for the generation of knock out cassettes. They carry an ampicillin resistance and a multiple cloning site which is located in the LacZ gene, to allow for blue/white selection of positive transformants on plates with X-Gal and IPTG.

pTW23 We took this plasmid for the construction of knock out cassettes using the *HIS3* gene as the selective marker to be used in *his*⁻ auxotrophic strains. The plasmid has been constructed by integrating *HIS3* into pBSK+ (WEIG *et al.*, 2001).

pCgACT14 With this vector gene knock outs can be complemented in *C. glabrata* by reintroducing the coding sequence of the deleted gene cloned into the plasmid. The plasmid is self replicating, harbours the TRP1 open reading frame as the prototrophic selection marker and an ampicillin resistance for propagation in *E. coli* (KITADA *et al.*, 1996).

pACH3 With this vector gene knock outs can be complemented in *C. glabrata* by reintroducing the coding sequence of the deleted gene cloned into the plasmid. The plasmid is self replicating, harbours the *HIS3* open reading frame as the prototrophic selection marker and an Amp^R for propagation in *E. coli* (KITADA *et al.*, 1996).

pSFS1A The vector uses a cassette, which contains a dominant nourseothricin resistance marker (caSAT1) for the selection of integrative transformants and an inducible recombinase (caFLP under the control of the SAP2 promoter) that allows the subsequent excision of the cassette from the genome. Thus the vector can be used for gene knock out as well as gene complementation. It also carries a chloramphenicol resistance for propagation in *E. coli*.

pQE30 This vector of the pQE series (Qiagen, Hilden) allows the bacterial expression of 6x His-tag fusion proteins. The vector carries an ampicillin resistance.

pGEX-KT This vector of the pGEX series (GE Healthcare, Munich), allows the bacterial expression of glutathione-S-transferase (GST) fusion proteins. The vector carries an ampicillin resistance.

2.4.1 *Constructs and strategies for gene knock out*

We generated a number of knock out strains with single, double or triple gene deletions of the *CWP1* gene family: Single gene deletions of *CWP1.1*, *CWP1.2* and *CWP1.3* as well as the double gene knock out of *CWP1.1/1.2* were done in different wild type parental strains (ATTC 2001, ATTC 90876, ΔHT6 and ΔH1). Triple gene knock out (*⊿cwp1.1/1.2/1.3*) was done in the following wild type strains: ATTC 2001, ATTC 90876 and ΔH1. Different wild type strains and thus knock out strategies were used because the auxotrophic strain ΔHT6 for instance is limited to only one gene knock out and complementation. This will be further explained later on. For a complete list of the generated knock out strains please see Table 2.
For the targeted knock out of genes, we amplified flanking regions upstream (F1, F4, F5) and downstream (F2, F3, F6) of the *CWP1* genes (Figure 2-1) by PCR using primers with restriction enzyme recognition sites at their ends. Thus, the generated PCR fragments could be digested with the respective restriction enzymes and were cloned into the appropriate vectors. The primer list in the appendix contains the primer names, which indicate the fragment they were used for and the corresponding restriction sites, as well as the primer sequences. The size of the flanking regions were:

CWP1.1: 674 bp of the 5' flanking region (F1: -700 to -26 5' of ORF) and
718 bp of the 3' flanking region (F3: +36 to +754 3' of ORF)
CWP1.2: 386 bp of the 5' flanking region (F4: -601 to -215 5' of ORF) and
626 bp of the 3' flanking region (F2: +132 to +758 3' of ORF)
CWP1.3: 483 bp of the 5' flanking region (F5: -500 to -17 5' of ORF) and
184 bp of the 3' flanking region (F6: +1 to +185 3' of ORF)

Two different approaches were used for the gene knock out:

1. We used the auxotrophic strains ΔHT6 and ΔH1 for gene knock out with the selection marker *HIS3* for positive transformants. The *TRP1* gene in ΔHT6 can afterwards be used as a marker for the generation of revertants. However, the use of auxotrophic strains has its limitations as there is a maximum of three auxotrophic markers available in *C. glabrata* so far.

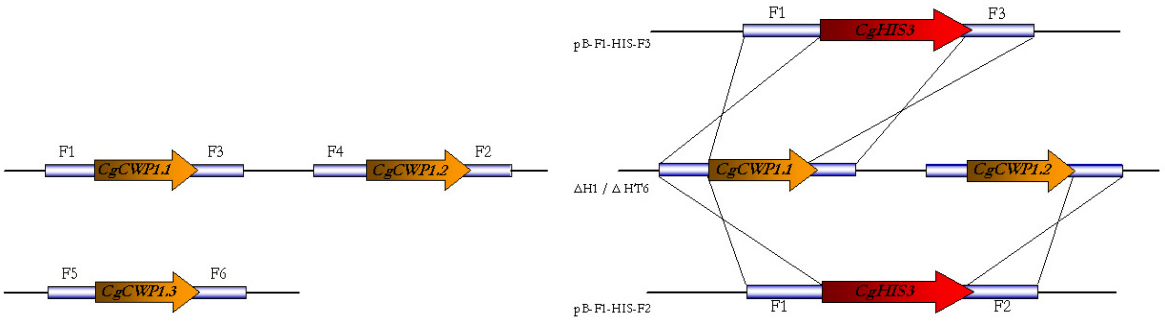

Figure 2-1 Knock out constructs
The PCR amplified flanking regions upstream (F1, F4, F5) and downstream (F2, F3, F6) of the different *CWP1* genes are used for gene knock out using *HIS3* as the selection marker. The knock out strategy for *CWP1.1* and *CWP1.1/1.2* are chosen as an example to demonstrate the homologous recombination at the target gene locus. Since *CWP1.1* and *CWP1.2* are directly adjacent to each other, they can both be deleted in a single transformation step using the homologous region upstream of *CWP1.1* (F1) and downstream of *CWP1.2* (F2).

2. In order to generate triple-mutants we made use of an alternative knock out strategy, which uses a dominant, recyclable selection marker. The flipper construct (REUSS *et al.*, 2004) includes the *SAT1* gene, which confers resistance to nurseothricin in combination with an inducible recombinase (*FLP* under the control of the *SAP2* promoter) for resolution of the knock out cassette after successful gene deletion (Figure 2-2). Thus, the same selection marker can be recycled and reused for several rounds of gene deletion and complementation (Figure 2-3). This system has originally been developed for *C. albicans* but could be successfully adapted in our hands for gene knock out in *C. glabrata* after some modifications in the experimental protocols. When generating revertants the target gene was cloned 5' of the flipper construct. To ensure correct gene transcription of the gene, we cloned the open reading frame plus 1000 bp of 5' and 3' sequences thus including promoter and terminator sequences.

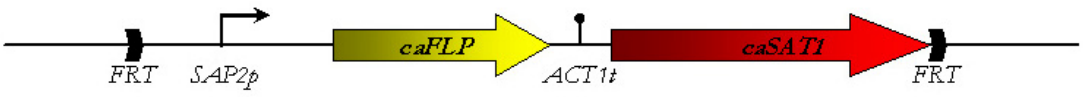

Figure 2-2 The flipper construct (pSFS1A)
Nurseothricin resistance of positive transformants is mediated by the *caSAT1* gene product. The recombinase flippase allows homologous recombination at the FRT (FLP recombination target) sites, which are only 34 nucleotides in length (GAAGTTCCTATACTTTCTAGAAATAGGAACTTC). Flippase transcription is under the control of the tightly regulated *SAP2* promoter, which can be induced in yeast carbon base medium supplemented with BSA as the sole nitrogen source and acidic pH (pH 4.0) (according to (REUSS *et al.*, 2004).

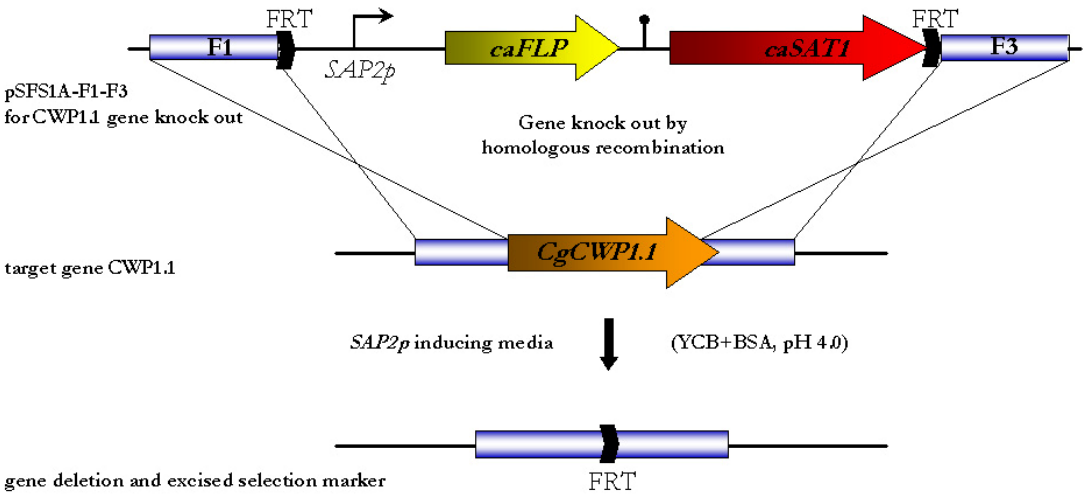

Figure 2-3 Targeted gene knock out using the flipper construct
Homologous regions 5' and 3' of the target gene (here *CWP1.1*) are cloned adjacent of the flipper construct in the pSFS1A plasmid. After targeted gene knock out by homologous recombination the whole construct is excised by recombination at the FRT sites leaving nothing behind but the 34nt FRT sequence. Thus, the same marker can be used for a second gene deletion as well as gene complementation.

2.4.2 Constructs and strategies for gene complementation

To reintegrate the deleted gene in the knock out strains, we amplified the open reading frame (ORF) of the gene of interest and several hundred base pairs of the 5' and 3' sequence in order to include the promoter and terminator sequences that ensure the correct transcription of the gene. (Please see primer list in the appendix for primer names, which indicate the fragment they were used for and the corresponding restriction sites, as well as the primer sequences.) The sizes of the amplified fragments were:

CWP1.1: 2626 bp amplified from -1000 5' of ORF to +969 3' of ORF

CWP1.2: 2697 bp amplified from -1053 5' of ORF to +1005 3' of ORF

CWP1.3: 2284 bp amplified from -959 5' of ORF to +570 3' of ORF

Two different strategies were used for gene complementation.

1. When the auxotrophic strain ΔHT6 was used for gene knock out with the selection marker *HIS3*, the reintegration of the gene occurred with help of the plasmid pCgACT14 (KITADA *et al.*, 1996) (Figure 2-4). This plasmid contains the Cg*TRP1* gene as a selection marker, an autonomously replicating sequence (ARS) and a centromere. These elements are required for the episomal replication and stable maintenance of the plasmid. For the propagation in *E. coli* it also carries an ampicillin

resistance. For gene complementation we cloned the gene into the multiple cloning site of the plasmid, propagated it in *E. coli* and used the purified plasmid for yeast transformation.

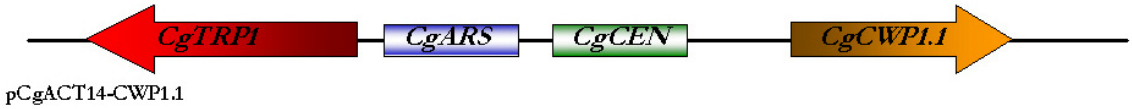

Figure 2-4 Generation of revertants using pCgACT14
To reintegrate the deleted gene and express it from an autonomously replicating plasmid, we cloned the open reading frame of the target gene (here *CWP1.1*) and about 1000bp of promoter and terminator sequence into the multiple cloning site of pCgACT14. The plasmid was then used for the transformation of *⊥cwp1.1* mutants.

2. The flipper construct allows reintegration of the gene at the original gene locus (Figure 2-5). Thus, the present regulatory sequences (e.g. promoter and terminator) assure gene transcription at its original levels. For gene complementation we transformed knock out strains with the ORF of interest including about 1000 bp of 3' and 5' flanking regions.

Figure 2-5 Generation of revertants using the flipper construct
To reintegrate the deleted gene at its original locus, we cloned the open reading frame of the target gene (here *CWP1.1*) and about 1000 bp of promoter and terminator sequence in front of the flipper cassette and the 3' homologous region (F3) behind the cassette. The whole cassette was excised from the plasmid (pSFS1A-CWP1.1-F3) with restriction enzymes and used for the transformation of *⊥cwp1.1* mutants.

2.5 Strains

2.5.1 Escherichia coli strains

DH5α — We used this *E. coli* strain for plasmid transformations during the cloning process. Genotype: F^-,Φ 80Δ*lac*ZΔM15, Δ(lacZYA-argF),U169, *deo*R, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17(r_K^-,m_K^+), *sup*E44, *rel*A1

TOP10 This *E. coli* strain (Invitrogen) was used for high efficient transformations of large plasmids.
Genotype: F⁻, *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), Φ 80Δ*lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139 Δ(*ara-leu*)7697, *gal*K, *rps*L(StrR), *end*A1, *nup*G

BL21 For effective protein expression we used this *E. coli* strain.
Genotype: *F, ompT, gal, dcm, lon, hsdS$_B$ ($r_B^-m_B^-$), rne131*

2.5.2 Candida glabrata strains

Table 2 - Strains used in this study

Strain	Genotype	Parental strain	Reference
Cg2001	wild type	--	Kitada 1995
Cg90876	wild type	--	
CgΔH1	*his3*	2001U	Weig 2001
CgΔHT6	*his3, trp1*	2001TU	Weig 2001
HΔcwp1.1	*his3, cwp1.1*::HIS	CgΔH1	this work
HTΔcwp1.1	*his3, trp1, cwp1.1*::HIS	CgΔHT6	this work
2001 Δcwp1.1(FLP)	*cwp1.1*::FLP	Cg2001	this work
2001 Δcwp1.1 ΔFLP	*cwp1.1*	2001 Δcwp1.1 (FLP)	this work
HΔcwp1.2	*his3, cwp1.2*::HIS	CgΔH1	this work
HTΔcwp1.2	*his3, trp1, cwp1.2*::HIS	CgΔHT6	this work
HΔcwp1.1/1.2	*his3, cwp1.1/cwp1.2*::HIS	CgΔH1	Weig, unpub.
HTΔcwp1.1/1.2	*his3, trp1, cwp1.1/cwp1.2*::HIS	CgΔHT6	Weig, unpub.
2001 Δcwp1.1/1.2 (FLP)	*cwp1.1/cwp1.2*::FLP	Cg2001	this work
2001 Δcwp1.1/1.2 ΔFLP	*cwp1.1, cwp1.2*	2001 Δcwp1.1/1.2 (FLP)	this work
90876 Δcwp1.1/1.2 (FLP)	*cwp1.1/cwp1.2*::FLP	Cg90876	this work
90876 Δcwp1.1/1.2 ΔFLP	*cwp1.1, cwp1.2*	90876 Δcwp1.1/1.2 (FLP)	this work
2001 Δcwp1.3 (FLP)	*cwp1.3*::FLP	Cg2001	this work
2001 Δcwp1.3 ΔFLP	*cwp1.3*	2001 Δcwp1.3 (FLP)	this work
2001 Δcwp1.1/1.2 /1.3 (FLP)	*cwp1.1, cwp1.2, cwp1.3*::FLP	2001 Δcwp1.1/1.2 ΔFLP	this work
2001 Δcwp1.1/1.2/1.3 ΔFLP	*cwp1.1, cwp1.2, cwp1.3*	2001 Δcwp1.1/1.2/1.3 (FLP)	this work
HΔcwp1.1/1.2/1.3 (FLP)	*his3, cwp1.1/cwp1.2*::HIS, *cwp1.3*::FLP	HΔcwp1.1/1.2	this work
HΔcwp1.1/1.2/1.3 ΔFLP	*his3, cwp1.1/cwp1.2*::HIS, *cwp1.3*	HΔcwp1.1/1.2/1.3 (FLP)	this work
90876 Δcwp1.1/1.2/1.3 (FLP)	*cwp1.1, cwp1.2, cwp1.3::FLP*	90876 Δcwp1.1/1.2 ΔFLP	this work
90876 Δcwp1.1/1.2/1.3 ΔFLP	*cwp1.1, cwp1.2, cwp1.3*	90876 Δcwp1.1/1.2/1.3(FLP)	this work
HΔcwp1.1/1.2/1.3 – CWP1.3 (FLP)	*cwp1.1, cwp1.2, cwp1.3*::CWP1.3/FLP	HΔcwp1.1/1.2/1.3 ΔFLP	this work
HΔcwp1.1/1.2/1.3 – CWP1.3 ΔFLP	*cwp1.1, cwp1.2, cwp1.3*::CWP1.3	HΔcwp1.1/1.2/1.3 – CWP1.3 (FLP)	this work
HΔcwp1.1 – CWP1.1	*cwp1.1*; pCgACT14-CWP1.1	HΔcwp1.1	this work

* FLP → construct which consists of nurseothricin resistance marker and the inducible recombinase flippase

2.6 Media

Media components were purchased from Q-Biogene (Illkirch, Germany).

Liquid media were prepared according to the following protocols:

LB 0.5% (w/v) Tryptone, 1% (w/v) Yeast extract, 0.5% (w/v) NaCl in ddH_2O
For selection different antibiotics were added to the media after autoclaving and cooling: ampicillin (100 µg/ml), chloramphenicol (20 µg/ml)

2xYT 1.6% (w/v) Tryptone, 1% (w/v) Yeast extract, 0.5% (w/v) NaCl in ddH_2O

SOC 2% (w/v) Tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 20 mM Glucose, 10 mM $MgCl_2$, 10 mM $MgSO_4$ in ddH_2O

YPD 1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose in ddH_2O

YNB 0.67% (w/v) yeast nitrogen base (without amino acids, with $(NH_4)_2SO_4$), 2% (w/v) glucose in ddH_2O; For the growth of auxotrophic mutants, YNB minimal media was supplemented with tryptophan (40 µg/ml) and/or histidin (20 µg/ml) if necessary.

SC 0.67% (w/v) yeast nitrogen base (without amino acids, with $(NH_4)_2SO_4$), 0.79 g/L CSM amino acid mix, 2% (w/v) glucose in ddH_2O

5% SC 5% SC medium, 2% (w/v) glucose in ddH_2O

YCB 2.34% yeast carbon base (YCB), 0.4% BSA, pH 4.0 in ddH_2O

SLAD The nitrogen starvation medium induces pseudohyphal growth in *C. glabrata*. For the preparation, the following concentrates were prepared and autoclaved:

50% (w/v) glucose in ddH_2O, 4 x YNB (6.8 g/L ddH_2O), 1000 x ammonium sulphate (0.5 mM $(NH_4)_2SO_4$), 4% (w/v) agar

For SLAD media preparation 10 ml 50% glucose, 62.5 ml 4 x YNB, 250 µl 1000 x AS, 62.5 ml, 125 ml 4% 8w/v) agar and 52.5 ml ddH_2O were mixed and plates were poured.

PFM This minimal medium contains essential minerals, trace elements, vitamins, amino acids and 2% (w/v) glucose (Ken Haynes, personal communication).

A) Trace elements - 40 μg/L $Na_2B_4O_7.10H_2O$, 400 μg/L $CuSO_4.5H_2O$, 1.32 mg/L $FeSO_4.7H_2O$, 800 μg/L $MnSO_4.4H_2O$, 696.4 μg/L Na_2MoO_4, 8 mg/L $ZnSO_4.7H_2O$

B) Salts - 0.52 mg/L KCl, 0.52 mg/L $MgSO_4.7H_2O$, 1.52 mg/L KH_2PO_4

C) Vitamins - 500 μg/L thiamine, 1 mg/L nicotinic acid, 2 mg/L DL-pantothenic acid, 2.5 mg/L pyridoxine, 10 μg/L biotin

D) Essential amino acids - 30 mg/L L-leucine, 30 mg/L L-lysine, 20 mg/L L-methionine, 20 mg/L L-histidine, 20 mg/L L-uracil

E) Nitrogen source – 5 mM ammonium tartrate

F) Carbon source - 2% (w/v) glucose

PFM medium was buffered to pH 7.4 or pH 8.0 with 100 mM Tris-HCl, or to pH 4.0 with 100 mM glycolic acid.

For the preparation of media plates, the described media broths were supplemented with 1.5% (w/v) agar in case of LB medium and 2% (w/v) agar for yeast media.

For low pH agar plates, the agar was autoclaved separately, to prevent agar breakdown during autoclaving. After sterilization of the agar and the other media components, they were mixed and plates were poured as usual. For the preparation of the nitrogen starvation medium (SLAD) for pseudohyphae induction, the agar wash washed four times with ddH_2O to remove any traces of nitrogen compounds before it was used.

Thermolabile additives (e.g. antibiotics) were filter sterilized and added to the autoclaved media after it had cooled down to 50 °C.

2.7 Software/web interfaces

Adobe 6.0 Professional	Adobe Systems, San Jose, CA, USA
Adobe Photoshop 7.0.1	Adobe Systems, San Jose, CA, USA
Microsoft Office Applications	Microsoft, Unterschleißheim
NCBI Homepage (National Centre for Biotechnology) database for DNA, proteins, literature etc.	Web site: www.ncbi.nlm.nih.gov
PubMed service of the US National Library of Medicine	Web site: www.ncbi.nlm.nih.gov/entrez
Genolevures database for *C. glabrata* genome	Web site: cbi.labri.fr/Genolevures/elt/CAGL
Laser Gene DNA Star package DNA and protein analysis software	GATC biotech, Munich
Phoretix 2D software	Nonlinear Dynamics, Newcastle upon Tyne, UK
MASCOT Searches for peptide mass fingerprints	Web site: www.matrixscience.com

2.8 Molecular Biology Methods

2.8.1 Polymerase chain reaction (PCR)

PCR was performed for the specific amplification of DNA used to generate knock out or complementation constructs as well as for the screening of generated plasmid construct after ligation and transformation. A standard PCR was performed in a final volume of 50 µl containing template DNA (10-100 ng), 1 x reaction buffer, 200 µM of each dNTP, 1 µM of

each primer and 1 U Taq-polymerase. The reaction was run in a T3 Thermocycler (Biometra, Göttingen): After an initial denaturation step (95 °C, 3 min) 35 cycles of denaturation (95 °C, 30 sec), primer annealing (50-60 °C, 30 sec) and fragment extension (72 °C, ~1 min/kb) were followed by a final extension (72 °C, 5 min) before the end of the PCR.
For proof reading PCR (e.g. for the generation of constructs for gene knock out complementation or protein expression) we used KOD proof reading DNA polymerase (Novagen) to assure exact amplification.

2.8.2 Plasmid DNA isolation from Escherichia coli cells

Plasmid DNA from *E. coli* was extracted from over night cultures using the DNA extraction mini- or midi-prep kit (Qiagen) depending on the amount of desired DNA.

2.8.3 Genomic DNA isolation from Candida glabrata

Genomic DNA from *C. glabrata* was extracted from a 5 ml YPD over night culture using the Molzym Presto Spin D Fungi Kit (Omni Life Science, Bremen). The manufacturer's instructions were followed except that we used zymolyase instead of lyticase to digest the cell wall: cells were sedimented by centrifugation (4000 x g, 3 min) and the cell pellet washed with 1 ml 1 M sorbitol. Cells were then resuspended in 800 µl zymolyase solution (1 M sorbitol, 50 mM K_3PO_4, 10 mM DTT, 400 µg/ml zymolyase-20T) and incubated at 37 °C, 750 rpm for one hour. With the obtained spheroplasts we continued the DNA extraction according to the manufacturer's protocol. We used RNase I (0.1 µg/µl) for an "on column" digest (15 min, RT) of contaminating RNA. To obtain the DNA concentration of the samples the light absorbance was measured at 260 nm. The concentration was calculated according to the formula $c=OD_{260} * 50$ [µg] * dilution factor [1/ml] = x µg/ml

2.8.4 RNA isolation from Candida glabrata

To extract RNA from *C. glabrata*, 2 ml of a yeast culture were pelleted (4000 x g, 3 min) and resuspended in 300 µl lysis buffer (4 M guanidiniumisothiocyanat, 25 mM Na-citrat pH 7.0, 0.5% (w/v) sarcosyl, 25 µl β-mercaptothanol). To this mix we added 500 µl phenol/chloroform (1:1) and approximately ¼ volume glass beads. The tubes were vortexed at 4 °C for 5 min before they were centrifuged (17000 x g, 10 min, 4 °C) to separate the phases and supernatant was transferred to a new polypropylene tube. To the supernatant ½

volume isopropanol was added and RNA was precipitated at -70 °C for 15 min. The pellet was washed with ice cold 70% (v/v) ethanol, dried and resuspended in RNase free water.

2.8.5 DNA restriction enzyme digest

Restriction enzymes were used to generate and verify plasmid constructs as well as to cut genomic yeast DNA for southern blot analysis. The desired amount of DNA, varying from 200 ng (analytical scale) to 10 µg (preparative scale), was cut with the appropriate amount of restriction enzyme (1-5 U RE/µg DNA depending on the purpose of the digest). Most digests were incubated at 37 °C for 2-8 hours. Double digests were performed when possible. The DNA was purified between successive digestions using the DNA clean up kit (Qiagen).

2.8.6 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to analyze DNA according to its molecular weight/electro mobility. To analyse plasmids and PCR products samples were mixed with 1/10 vol of 10 x loading dye (Blue juice, Invitrogen) and run on a 1% (w/v) agarose (w/v). The gel was prepared with 1 x TAE buffer (40 mM Tris, 1% (v/v) acetic acid, 1 mM EDTA, pH 8.0), which was also used to run the gel at 90 V. For estimation of the size and amount of DNA in the applied sample, a marker with defined fragment sizes and DNA amounts was run in parallel. Bands were visualized by UV-light after staining the gel in an Ethidiumbromid bath (2 mg/L) for 20min.

2.8.7 DNA extraction from agarose gel

To extract DNA from agarose gels for further experiments (e.g. after restriction enzyme digestion, before progressing with ligations), the desired band was quickly cut from the ethidiumbromide stained gel under UV-light with a clean, sharp scalpel. The obtained gel slice was weighed and DNA was extracted using a gel extraction kit (Qiagen), following the manufacturer's instructions. DNA was eluted from the filter column with 20 µl H_2O. Probes were concentrated, if necessary, using a speedvac centrifuge.

2.8.8 DNA dephosphorylation

To prevent vector religation of the vector backbone in ligation reactions, linearized plasmid DNA was 5'-dephosphorylated prior to ligation. We used shrimp alkaline phosphatase (Roche) for dephosphorylation according to the instructions of the manufacturer.

2.8.9 Ligation

Vector backbones and the desired DNA fragments were ligated using T4-ligase or T4-quick ligase (NEB). For a standard reaction 50 ng of dephosphorylated vector DNA was incubated with a three fold molar excess of insert at 16 °C over night for T4-ligase or at 25 °C for 20 min for T4-quick ligase respectively.

2.8.10 Sequencing of plasmid DNA

To verify the sequence of plasmid constructs prior to further use, they were sequenced using a capillary electrophoresis sequencer (SeqLab, Göttingen). For a sequencing reaction 500 ng to 1 µg DNA was mixed with 20 pmol of the appropriate sequencing primer, filled up to 7 µl with 10 mM Tris-HCl (pH 7.5) and stored at -20 °C until the samples were processed. The obtained sequences were analysed using SeqMan from the DNASTAR Lasergene software package (DNASTAR Inc., Madison, USA).

2.8.11 Southern Blot

Solutions used for southern blotting:

20 x SSC buffer	**6 x SSC buffer**	**blocking buffer**
3 M NaCl	1 M NaCl	5% (w/v) dry milk
0.3 M Sodium citrate	0.1 M Sodium citrate	0.1% (v/v) Tween
in ddH_2O, pH 7.0	in ddH_2O, pH 7.0	in PBS, pH 7.4
washing solution 1	**washing solution 2**	**PBS**
0.3 M NaCl	15 mM NaCl	137mM NaCl
30 mM sodium citrate	1.5 mM sodium citrate	2.7mM KCl
0.5% SDS	0.5% SDS	4.3mM Na_2HPO_4
in ddH_2O	in ddH_2O	1.4mM NaH_2PO_4
		in ddH_2O, pH 7.4

2.8.11.1 DNA preparation

To verify that a knock out or complementation cassette had integrated at the right position, and only at this site, Southern blot hybridization experiments was performed. For that

purpose, 3 µg genomic DNA was digested with 30 U of a desired restriction enzyme at 37 °C over night. The digested DNA was run for 6-8 hours on a 0.7% agarose gel to obtain a good separation of fragments. Five µl marker (SMART ladder) and 5 µl of DIG-labelled DNA marker were run in parallel. After the run was completed the gel was agitated in 0.25 M HCl for 10 min to depurinate the DNA. Afterwards it was incubated twice in 1.5 M NaCl, 0.5 M NaOH for 20 min to separate the double stranded DNA and twice in 1.5 M NaCl, 0.5 M Tris-HCl pH 7.0, also for 20 min, to neutralize the base, which was used for the denaturation step.

2.8.11.2 Blotting procedure

DNA probes were transferred to a nitrocellulose membrane by capillary blotting. The set up was assembled as follows: a glass tray filled with 20 x SSC buffer was covered with a glass plate, carrying two whatman filter papers (3 mm), which dipped into the 20 x SSC buffer. The gel was placed on top of the filters and covered with a pre-soaked (20 x SSC) nitrocellulose membrane. Around the gel, the underlying filter papers were covered with Saran wrap, to ensure a liquid flux through the gel and not through the filter papers around. Two more whatman filter papers were placed on top of the nitrocellulose membrane. The stack was assembled taking care to avoid any air bubbles between the layers. Finally a pile of paper towels was placed on top and weighed down with a 1kg weight.
After 18-24 hours the set-up was disassembled, the nitrocellulose membrane was washed in 6 x SSC for 5 min to remove agarose and dried on air. Transferred DNA was cross linked to the membrane using a UV-crosslinker with a wavelength of 254 nm (Biometra, Göttingen) for 15 seconds.

2.8.11.3 Preparation of a DIG-labelled probe

DIG-labelled probes specific for the integrated cassettes were amplified from the plasmid carrying the desired DNA sequence (e.g. pSFS1A for the flipper construct or pTW23 for the HIS gene) with specific primers. PCR was performed with DIG-mix (0.2 mM dGTP, 0.2 mM dATP, 0.2 mM dCTP, 0.13 mM dTTP, 0.07 mM DIG-11-dUTP final concentrations) and Taq-polymerase in a standard PCR reaction.

2.8.11.4 Hybridization of the DIG-labelled probe to DNA

The nitrocellulose membrane was put in a hybridization bag, filled with 20ml prehybridization solution (50% formamide, 6 x SSC, 5x Denhardt's reagent, 0.5 % SDS, 100 µg/ml hering sperm DNA) and incubated for 2 hours at 37 °C. Four µl of the DIG-probe were boiled in 100 µl water for 5 min and 100 µl formamide were added before it was chilled on ice for 5 min. This mix was added to the membrane in the prehybridization solution, followed by incubation at 37 °C for 18-24 hours. The membrane was washed twice with 200 ml pre-warmed washing solution 1 for 15 min at 37°C. The third and fourth washing was done for 30 min at 68 °C with 200 ml pre-heated washing solution 2.

2.8.11.5 Detection of the hybridized DIG-labelled probe

The nitrocellulose membrane was equilibrated in PBS for 1 min at room temperature, before it was incubated in blocking buffer for 60 min to prevent unspecific binding of the antibody. A peroxidase labelled anti-DIG antibody (5 µl/10 ml blocking buffer) was used to detect the DIG labelled probes (2 hours incubation at RT). Afterwards the blot was washed with PBS + 0.1% Tween20 three times for 10 min and developed using the ECL-kit (pharmacia) according to the instructions of the manufacturer. An excess of the applied developing solution was removed and the membrane was covered with saran wrap before an X-ray film was exposed to the membrane. The film was developed using a photo developer kit GBX Developer/Fixer (Kodak, via Sigma) according to the manual (5 min developer, 2 min stop solution, 5 min fixation, 2 min washing).

2.8.12 Reverse transcription

To determine transcription levels of certain genes we used the omniscript RT-PCR kit (Qiagen) according to the manufacturer's instructions. RNA was isolated as described before and samples were digested with DNase to remove contaminating DNA in the probes. 1 µg RNA was digested with 2 units DNase in a final volume of 10 µl (in 1 x RT buffer). Samples were incubated at room temperature for 15 min before 1 µl STOP mix was added and incubation was continued for 10 min at 70 °C to inactivate the DNase. These samples were used to reverse transcribe mRNA with an oligo dT primer into cDNA. After the RT reaction cDNA was amplified by gene specific PCR.

2.8.13 Immunofluorescence microscopy

We used *C. glabrata* cells from mid logarithmic growth phase for microscopy. For this, cells were washed twice in PBS and then resuspended in PBS at a 1/20 dilution. 250 µl of this suspension was briefly centrifuged on top of round cover slips (2 min, 1000 x g) in a 24-well-plate. PBS was taken off and cells were fixed for 1 hour using 4% (v/v) paraformaldehyde (PFA) in Cacodylat-HCl. Cover slips were washed twice in PBS and aldehyde groups of PFA were quenched in 50 mM NH_4Cl (10 min, RT). Cells were washed again with PBS and then blocked with 1% (w/v) BSA in PBS for one hour at room temperature. After blocking, cover slips were washed (2 x in PBS) and the primary antibody (α-cwp1 serum) was applied in a 1:200 dilution for 90 min. After incubation, the primary antibody was thoroughly washed off (3 x in PBS) and the secondary antibody (Cy-3 labelled donkey anti-mouse antibody) was applied for one hour. After thorough washing (5 x in PBS) the cover slips were washed in ddH_2O and mounted on glass slides with Mowiol o/n at room temperature.

2.8.14 Electron microscopy

Cells of *C. glabrata* strains were grown to an OD_{600} of 1.0 (37 °C, YPD) and harvested by centrifugation (7000 x g, 2 min). They were first fixed in 3% (w/v) glutaraldehyde for 3 hours at RT followed by a post-fixation step using 1% (w/v) Osmiumtetroxide (OsO_4) at 4 °C. The samples were embedded in araldite after several washing and dehydration steps. Ultrathin sections (70-74 nm) were cut using an utramicrotome (Ultracut), contrasted with lead citrate and examined using a Zeiss EM 10 transmission electron microscope operating at 60 kV, at magnifications of 1.000 to 50.000 fold.

2.8.15 Time laps microscopy

To analyse the budding behaviour of the generated mutants, we performed time laps microscopy in collaboration with Andrea Walther and Jürgen Wendland, Friedrich Schiller University in Jena. *C. glabrata* wild type and mutant strains were inoculated in YPD medium and different dilutions were spread on an YPD plate. Using the microscope, an area with a single yeast cell was selected to be monitored during growth at 37 °C for six to eight hours.

2.9 Protein biochemical methods

2.9.1 Protein expression in Escherichia coli

For the generation of Cwp1p specific antibodies we generated a His_6-tagged Cwp1 protein. Therefore a 530 bp fragment of the coding sequence was cloned into pQE30 with the help of *Bam*HI and *Hind*III restriction enzyme recognition sites. When designing the primers (ExCgcwp1.1f/ExCgcwp1.1r) we took care to shorten the construct by the N-terminal signal peptide and the carboxy-terminal GPI-attachment domain to avoid problems in the protein expression. For the later purification of the generated antisera, we also constructed a GST-tagged Cwp1 protein using the same part of the coding sequence but cloning it into pGEX-KT expression vector (GE healthcare) with help of the *Hind*III and *Bam*HI restriction sites.
We used the *E. coli* protein expression strain BL21. 5 ml of an overnight culture were used to inoculate 500 ml LB (with 500 µg/ml amp). This culture was incubated at 37 °C and 190 rpm until it reached an OD_{600} of 0.6 and protein expression was induced by the addition of 0.5 mM IPTG. After expression of the protein at 30 °C and 190 rpm for 3 hours, the cells were centrifuged at 5000 x g and the cell pellet was frozen and stored at -20 °C until further use.

2.9.2 Protein purification from Escherichia coli

Buffers used for the purification of His_6-tagged proteins:

Binding buffer	**Wash buffer**	**Elution buffer D**	**Elution buffer E**
100 mM NaH_2PO_4	100 mM NaH_2PO_4	100 mM NaH_2PO_4	100 mM NaH_2PO_4
10 mM Tris-Cl	10 mM Tris-Cl	10 mM Tris-Cl	10 mM Tris-Cl
8 M urea	8 M urea	8 M urea	8 M urea
pH 8.0	pH 6.3	pH 5.9	pH 4.5

Proteins were purified from *E. coli* with the help of His_6- or GST-tags using the corresponding binding resins: Ni-NTA agarose (Qiagen) for His_6-tagged proteins and GST bind resin (Novagen) for GST-tagged proteins. For preparation of bacterial protein extracts, frozen cell pellets were thawed, resuspended in 20 ml PBS and immediately put on ice. Then 1 mM Phenylmethyl-sulphonylfluoride (PMSF) to irreversibly inhibit serine proteases and 1 mg/ml lysozyme to lyse the *E. coli* cells were added. After incubation for 10 min on ice sonification was performed using a micro-tip system for 3 times 30 seconds at an energy level of 60% of maximum and sonification cycle frequency of 50% of maximum. Afterwards we added 1% Triton and incubated the disrupted cells on ice for another 15 to 20 min. The suspension was centrifuged at 20000 x g, 4 ° C for 30 min.

The supernatant was applied to the appropriate binding resin and incubated on a rotary shaker for 2 hours at 4 °C. Generally 0.5 ml of binding resin slurry was used for a cell pellet of 500 ml culture. Proteins bound to beads were washed in 30 ml binding buffer 3 times for 5 min on the rotary shaker and washed with PBS (GST tag) or buffer C (His_6-tag) before elution. GST tagged Cwp1p was not eluted but cross linked to the beads (Please see 3.9.7. Antiserum purification). The elution of His_6-tagged Cwp1p was achieved with elution buffers D and E (8 M urea, 100 mM Na_2HPO_4, 10 mM Tris-Cl at pH 5.9 and 4.5 respectively).

2.9.3 SDS PAGE

Solutions used for SDS PAGE:

Stacking gel (3.8%)		**Separating gel (12%)**	
ddH_2O	3.25 ml	ddH_2O	5 ml
0.5M Tris pH 6.8	1.25 ml	1.5 M Tris pH 8.8	3.75 ml
10% SDS	50 µl	10% SDS	50 µl
30% acrylamide/0.8% bis-acrylamide	0.625 ml	30% AA/0.8% BAA	6 ml
10% ammonium persulfate (APS)	75 µl	10% APS	75 µl
TEMED	7.5 µl	TEMED	7.5 µl

Running buffer	**Coomassie staining solution**	**Destaining solution**
25 mM Tris pH 8.8	0.1% (w/v) Coomassie	10% (v/v) acetic acid
192 mM glycine	10% (v/v) acetic acid	25% (v/v) methanol
0.1% (w/v) SDS	25% (v/v) methanol	

To separate proteins according to their size denaturing sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed. The anionic detergent sodium dodecyl sulfate (SDS) denatures the proteins and provides the protein with a negative net charge. In order to completely denature secondary and also tertiary and quaternary structures the sample buffer contains reducing reagents, such as dithiothreitol (DTT) or β-mercaptoethanol (β-ME). Additionally, the samples are further denatured by boiling. The uniform mass to charge ratio and the complete elimination of secondary and tertiary structures assures that the distance, which the protein travels in the gel can be directly related to its size. For standard protein separation we used 12.5% acrylamide gels. Gels were poured using the BioRad mini protean device. Ammonium persulfate, which starts the polymerization (together with Tetramethylethylen-diamin [TEMED]) by radical formation, was added last just before pouring the gels. The stacking gel was poured on top of the separating gel when the latter had polymerized.

2.9.4 Gradient SDS PAGE

Solutions used for gradient SDS PAGE

Component	**20 % acrylamide**	**2.6 % acrylamide**
19% Acrylamide/1% Bisacrylamide	-	4.56 ml
27% Acrylamide/0.5% Bisacrylamide	17.55 ml	-
ddH_2O	3.13 ml	26.37 ml
Solution A	3.04 ml	4.53 ml
10 % ammonium persulfate	44.8 µl	287 µl

Solution A	**Running buffer**
3 M Tris	25 mM Tris pH 8.8
0.08 % (w/v) SDS	192 mM glycine
0.023 % (v/v) TEMED	0.1% (w/v) SDS
adjust to pH 8.9	in ddH_2O

In each case five sets of gel plates, combs and spacers were assembled in a Perspex holder to which a mixing chamber and a peristaltic pump was connected to. To minimise polymerisation the cold solutions were kept on ice as long as possible. The components for high and low percentage acrylamide were added to the two partitions of the mixing chamber and gels were poured by gradually mixing both components. Gels were left for polymerization for at least one hour and were stored in a wet chamber at 4 °C for no longer than three weeks.

2.9.5 Immunoblotting

Solutions used for Immunoblotting:

Transfer buffer	**TBST**	**Blocking buffer**
25 mM Tris	10 mM Tris-HCl pH 8.0	5% (w/v) milk in TBST
200 mM glycine	150 mM NaCl	
20% (v/v) methanol	0.05% (v/v) Tween	**Washing buffer**
in ddH_2O	in ddH_2O	1% (w/v) milk in TBST

Prior to immunoblotting proteins were separated by SDS PAGE and transferred to a nitrocellulose membrane using the wet blot system from BioRad. The gel size was measured and two whatman filter papers and one nitrocellulose membrane of the same size were prepared. The filter papers and nitrocellulose were soaked in transfer buffer. Then the soaked nitrocellulose and the polyacrylamide gel were placed on to one of the filter papers and covered with the second filter paper. This stack was placed between to sponge cloths before they were placed into the holding device of the blot chamber. Blotting was performed at 70 V for 4-6 hours. During the blotting procedure, the transfer buffer was cooled with an ice pack to avoid heating.

After disassembling, the membrane was soaked in blocking buffer for 1 hour at room temperature. It was then shortly rinsed with washing buffer and the primary antibody was applied in the same buffer for 2 hours at RT on a shaker. The blots were washed 3 times for 10 min in washing buffer before the secondary horseradish peroxidase labelled goat-anti-rabbit antibodies were applied in the same buffer at a dilution of 1:10000 for 2 hours at RT. The membranes were washed three times in TBST and once in PBS before the blots were developed using the ECL Immunoblotting Detection Kit from pharmacia. An excess of the applied developing solution was removed and the membrane was covered with saran wrap before an X-ray film was exposed to the membrane. The film was developed using a photo developer kit GBX Developer/Fixer (Kodak, via Sigma) according to the instructions of the manufacturer (5 min developer, 2 min stop solution, 5 min fixation, 2 min wash).

2.9.6 Antiserum production

The His_6-tagged recombinant Cwp1 protein was dialysed against PBS (twice for three hours and once over night at 4 °C) to eliminate the high urea concentration in the elution buffer. Two New Zealand White rabbits were immunized with the protein using the polyclonal antibody facility from Eurogentec (Seraing, Belgium). Following the immunization protocol, the rabbits were challenged with 200 µg antigen and an appropriate adjuvant per injection. The injections occurred on days 0, 14, 28 and 50. Antibody production was checked with help of a small bleeding on day 35 and the final bleeding was done on day 65. The serum was enriched in α-Cwp1 specific antibodies and was used in immunoblotting in a 1:5000 dilution.

2.9.7 Antiserum purification

When released GPI-anchored proteins of *C. glabrata* were blotted and probed with the produced antiserum we observed bands in addition to the expected Cwp1 protein band. In order to exclude unspecific antibodies (e.g. antibodies against other fungal surface molecules such as glucan or mannan) we decided to purify the serum.

Therefore, GST fusion proteins were expressed and purified from *E.coli* as described above and cross-linked to the beads using the GST Orientation Kit from Pierce (Perbio Science, Bonn). The applied cross-linker disuccinimidyl-suberate (DSS) is a water-insoluble, non-cleavable, homobifunctional *N*-hydroxysuccinimide ester (NHS-ester). Targets of the cross linker are primary amines. Thus, accessible α-amine groups present on amino-termini of proteins and peptides but also the ε-amine of lysine react with the NHS-ester. This reaction between primary amines and NHS-esters produces a covalent amide bond and thus fixes the

protein to the beads. For covalent fixation of the protein 3.5 mg DSS were dissolved in 250 µl DMSO and added to the bead bound proteins. Cross linking was allowed during 1 h incubation at RT, before the beads were blocked with 50 mM Tris/150 mM NaCl pH 7.3 (10 min at RT) and then three times washed with 50 mM Tris/150 mM NaCl/50 mM glutathione (10 min at RT). Finally the beads were washed twice with 50 mM Tris/150 mM NaCl pH 7.3. To the beads 5 ml rabbit antiserum was applied before they were washed five times with 50 mM Tris/150 mM NaCl pH 7.3. Afterwards, the α-cwp1 specific antibodies were eluted with 2 x 2 ml 4 M $MgCl_2$ and dialysed against PBS at 4 °C.

The purified serum was tested for and approved of Cwp1 specificity by immunoblot analysis with recombinant Cwp1 protein as well as GPI-protein fractions of *C. glabrata* cell wall material. After the purification, the antibody solution could be used at 1:500 dilutions for immunoblotting and 1:50 for immunofluorescence microscopy.

2.9.8 Protein extraction from Candida glabrata

Solutions used for protein extraction:

Lysis buffer 1	**Lysis buffer 2**	**10x protease inhibitor**
0,45 g urea	0,1 g CHAPS	0,027 g DTT
0,19 g thiourea	125 µl ddH_2O	70 µl 1M Tris pH 10.8
6,3 µl 0.2M EDTA	250 µl glycerol	165 µl ddH_2O
420 µl ddH_2O	125 µl ampholyte	1 mini EDTA free protease
→ 100 µl 10 x prot. inhib.	carrier pH 4-7	inhibitor cocktail tablet
was added prior to use		4,9 µl pepstatin (1 mg/ml in MetOH)

We extracted soluble intracellular proteins from *C. glabrata* for proteomic analysis of the pH response. Cells were harvested in Falcon tubes by centrifugation (4000 x g, 10 min, 4 °C) and pellets were washed twice with ice cold ddH_2O, before transferred into 1.5ml polypropylene tubes. Cells were centrifuged again (17000 x g, 5 min, 4 °C) and the supernatant was discarded except a few micro litres which were used to resuspend the pellet. Cells were transferred into prechilled 2 ml screw caps (70-100 mg of "wet pellet" per cap) and previously weighted 0.4 nm glass beads (0.25 g per cap) were added. Caps were kept on ice the whole time. 160 µl of lysis buffer 1 was added to each pellet and beads and bead beaten 12 times for 30 seconds on medium speed (or 12 x 30 seconds, level 4 in a fast prep machine) with 1 min on ice in between the beating. Samples were spun (17000 x g, 5 min, 4 °C), before 40 µl of lysis buffer 2 was added and samples were gently mixed with the pipette tip. Proteins were kept on ice for 1 h with gentle shaking. Then the mix was bead beaten 6 times for 1 min on low speed (about one fifth of maximum power, or 6 x 1 min, level 1 in fast prep machine) with 1 min on ice in

between the beating. Finally samples were centrifuged (17000 x g, 5 min, 4 °C) and the supernatant was transferred into a sterile 1.5 ml cap. Samples were stored at -20 °C for use within the next days or at -80 °C for long term storage.

2.9.9 Candida glabrata cell wall preparation

SDS extraction buffer (pH 7.8)
50 mM Tris-HCl
2 % SDS
100 mM Na-EDTA
in ddH_2O
3.2 µl β-ME per ml extraction buffer was added prior to use

From a yeast over night culture in YPD 300 ml of fresh YPD were inoculated at an OD_{600} of 0.1 and grown to an OD_{600} of 2.0 (37 °C, 200 rpm). Cells were then harvested in 50 ml polypropylene tubes and washed once in 300 ml ddH_2O. Throughout the whole procedure the samples were kept on ice. Cell pellets were resuspended in 100 ml sterile and ice cold 10 mM Tris-HCl, pH 7.5 and centrifuged at 3000 rpm (5 min, 4 °C). Pellets were frozen in liquid nitrogen and stored at -20 °C if necessary.

Pellets were carefully resuspended in 12 ml Tris-HCl and the suspension was divided into six 2ml screw-cap tubes. Tubes were centrifuged for 1 min at 4000 x g, 4°C. Each Pellet was then resuspended in 200 µl Tris-HCl and shaken for several minutes to ensure complete suspension. Cold glass beads (500 nm diameter) were added to the tubes (excess, dry beads were tipped off). After the addition of 30 µl protease inhibitor (protease inhibitor mixture for yeast, Sigma) the tubes were transferred to a fast prep machine (Fastprep FP120, Bio101, Savant) and shaken on speed level 6 for 20 seconds. This shaking was repeated to a total of three rounds with cooling on ice (5 min) in between the shaking.

Cold sterile 1 M NaCl was used to rinse the tubes and transfer the contents (including beads) into a cold 50 ml tube. The six identical tube contents of one sample were pooled in one 50 ml tube. The supernatant of the pooled samples were transferred to a second large tube taking care not to transfer any beads. The remaining beads were repeatedly and extensively washed with 1 M NaCl until the supernatant of the beads was very clear (approximately 15 times and a total of 35ml). The cell wall suspension was centrifuged for 3 min at 800 x g and 4

°C. A sample of the supernatant was kept as an example of intracellular proteins and stored at -20 °C.

The pellet was then washed once in 25 ml ice cold 1 M NaCl and twice in ice cold ddH_2O before it was resuspended in 6 ml ice cold ddH_2O. This suspension was transferred into six pre-weighed 2 ml tubes. The 50 ml tubes were washed with another 6 ml ice cold ddH_2O to remove all cell wall material and the washes were added to the 2 ml tubes. Tubes were centrifuged for 1 min at 17000 x g and once more washed with 1 ml ddH_2O. The tubes were weighed with the pellet to determine the pellets' wet weight.

SDS extraction buffer was added to the pellets (1 ml per 200 mg wet weight) and pellets were completely resuspended in a shaker. The tubes were boiled on a hot-block at exactly 100 °C for 5 min and then cooled at room temperature but not on ice to avoid SDS precipitation. After centrifugation (1 min, 17000 x g) samples of the supernatant were kept as the SDS-extractable fraction (non-covalently bound wall proteins). The pellet was then again boiled with SDS extraction buffer to remove all non-covalently bound proteins from the cell wall material. The pellets were washed three times with ice cold ddH_2O before they were frozen in liquid nitrogen and freeze-dried over night. The dry weight of each sample was determined and lyophilised cell walls were stored at -20 °C until use.

2.9.10 Enzymatic release of GPI-anchored cell wall proteins

Recombinant β-1,6-glucanase was used for the specific release of GPI-anchored proteins from purified cell wall material as described before (BOM *et al.*, 1998). Therefore, 4 mg cell wall material (dry weight) was resuspended in 200 µl of 50 mM phosphate buffer pH 5.5, before 2.5 µl β-1,6-glucanase and 2 µl protease inhibitor (Sigma) were added. The samples were incubated at 37°C over night (shaking at 750 rpm) for glucanase digestion.

The next day 100 µl of 3 x sample buffer (SB) was added and the tubes were boiled at 100 °C for 3 min. After centrifugation (2 min, 17000 x g) the supernatant containing the GPI-anchored cell wall proteins was taken off and stored at -20 °C until use in SDS PAGE. If the pellet (cell wall without GPI-proteins) was of interest, it was boiled twice in SB and washed three times with ddH_2O.

2.9.11 Chemical release of GPI-anchored cell wall proteins

To chemically release GPI-anchored proteins from purified cell wall material 4 mg of lyophilised cell walls were incubated with 300 µl hydrofluoric-acid-pyridine-complex for 3

hours on ice. Afterwards, the hydrofluoric acid was carefully quenched with 300 µl ddH_2O before the samples were further diluted with 1.4 ml ddH_2O. To entirely remove the hydrofluoric acid from the samples, they were dialysed in ddH_2O for one hour, followed by a second dialysis step in fresh ddH_2O at 4 °C over night.

2.9.12 Release of mild alkali extractable cell wall proteins

Pir proteins can be released from the cell wall by mild alkali treatment. Therefore, 4 mg of lyophilized cell walls were weighed out and 91 µl of cold 30 mM NaOH was added, gently resuspending the cell wall material, using cold pipette tips and working in the cold room. The samples were incubated at 4 °C over night in a bench top shaker gently shaking. After the mild alkali treatment, the suspension was neutralized by adding 109 µl cold 30 mM acetic acid again using cold pipette tips. Then, 100 µl of 3 x sample buffer were added and samples were boiled for 3 min. Tubes were centrifuged (17000 x g, 1 min) and the supernatant containing the mild alkali soluble proteins was transferred to a fresh tube. Pellets were boiled a second time in 0.4% (w/v) SDS and the supernatant was pooled with the supernatant of the first boiling step. If further digestion of the cell walls was required, the pellets were washed in 1 ml ddH_2O, freeze dried and stored at -20 °C.

The pooled supernatants were dialysed overnight against ddH_2O with repeated change of the water. The dialysed supernatants were concentrated using Centricon tubes. Pir protein samples were stored at -20 °C until required.

2.9.13 Protein quantification

We used the BCA™ Protein Assay Kit from Pierce (Perbio Science, Bonn) for protein quantification according to the manufacturer's instructions as the Bradford assay is not compatible with higher concentrations of detergents and chaotropic reagents present in our samples. This performed assay is based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. Cu^{2+} is reduced to Cu^{1+} by protein in an alkaline medium (biuret reaction). These Cu^{1+} ions are chelated by the BCA reagent forming purple-coloured complexes. Light absorbance of this complex was measured at 595 nm.

2.9.14 Two dimensional SDS PAGE

After protein extraction and quantification the first dimension of the 2D SDS PAGE was performed on isoelectric focussing (IEF) strips pH 4 to 7 (Immobiline™ DryStrip, IPGphor

4-7, 24 cm from Amersham). At first, the strips were rehydrated over night in rehydration solution (RHS: 4.204 g urea, 1.522 g thiourea, 0.4 g CHAPS, 0.04 g DTT, 7 µl pepstatin, 200 µl carrier ampholyte 4-8 filled up to a volume of 10 ml with ddH_2O) and covered with cover fluid (Amersham). After rehydration, 500 µg of proteins were applied onto IPGphor strips and proteins were separated in the first dimension according to the following protocol: 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, followed by a gradient switch of 1000 to 8000 V for 0.5 h and 8000 V for 12 h, which adds to a total of 80.000 Vh.

After isoelectric focussing the IPG strips were equilibrated in a two step equilibration protocol:

The equilibration buffer contained 50 mM Tris-Cl pH 8.8, 6M urea, 30% (v/v) glycerol and 2% (w/v) SDS. For the first equilibration step 10 mg/ml DTT and 1.25 mg/L bromphenolblue were added to the EQ buffer. For the second step 25 mg/ml iodoacetamide and 1.25 mg/L bromphenolblue were added. Equilibration steps were done at room temperature for 25 min each.

Strips were then embedded into low melting agarose on top of SDS polyacrylamide gels. For the second dimension precast 12.5% (w/v) SDS polyacrylamide gels (DALT Gel 12.5, Amersham) and the anode/cathode buffer set as provided by Amersham were used. The four replicate biological samples of three different pH growth conditions were run in parallel to get comprehensive results. The twelve gels were run at 2.5 W per gel for one hour, followed by 4 to 4 ½ hours at 180 W.

2.9.15 Preparation of the gels for image analysis

After completion of the run, gels were fixed in 2% (v/v) H_3PO_4, 50% (v/v) ethanol in ddH_2O over night. Gels were then washed with ddH_2O three times for 30 min before they were equilibrated in 1.25 M $(NH_4)_2SO_4$, 2% (v/v) H_3PO_4, 34% (v/v) methanol for at least 2 hours.

Following the equilibration, colloidal coomassie was added at an amount of 0.67 g/L to stain the separated proteins. Staining of the gels was stopped by washing with ddH_2O after three days. To avoid artefacts on the gels, the colloidal coomassie was completely removed by extensive washing.

Gels were scanned using a HP Scanjet 5370C, before they were dried for storage during the time of image analysis. Gels and cellophane sheets were soaked in 1.5 % (v/v) glycerol, before the first piece of cellophane was placed on top of a glass plate. The gel was laid on top avoiding any air bubbles. The second foil piece covered the gel and appearing air bubbles were removed. The gels were allowed to dry for two to three days at room temperature.

2.9.16 Image analysis

Images were analysed using the Phoretix 2-D Expression software (Nonlinear Dynamics, Newcastle upon Tyne, UK). To identify spots that were reproducibly up- or down-regulated spots were matched by automatic matching and a manual correction of mismatched spots. Spot volumes were normalised against total spot volume and total spot area after background subtraction (mode of non-spot). Features that displayed statistically significant changes in their normalised spot volume on at least three of the four replicate gels (p value ≤ 0.05; Student's t-test), or that were reproducibly present in one condition but not the other, were selected for protein identification. A spot was defined as absent in one experimental condition if it was not detectable in at least three of the four replicate gels from that set, but was detectable in at least three gels from the other experimental condition.

2.9.17 Protein preparation for mass spectrometry

Selected spots were cut from gels after gel rehydration in ddH_2O (30 min) and transferred to a 96-well microtitre plate using an Investigator ProPic robotic workstation (Genomic Solutions, Huntingdon, UK). The picked proteins were digested with trypsin using an Investigator ProGest robot (Genomic Solutions) and the tryptic peptides were extracted and purified using $ZipTip_{\mu\text{-}C18}$ tips (Millipore, Watford, UK). Peptides were eluted from the ZipTips with 1% (w/v) α-cyano-4-hydroxycinnamic acid (CHCA) using the Investigator ProMS robotic workstation (Genomic Solutions) and crystallized on the mass spec plate.

2.9.18 Mass spectrometric analysis of proteins

We obtained peptide mass fingerprints by MALDI-TOF MS using a Voyager DE-STR biospectrometry workstation with Voyager 5.1 software (Applied Biosystems, Foster City, CA, USA). Analysis of mass spectra was done using the Data Explorer software from Applied Biosystems.

The spectra were calibrated by internal standardisation using the trypsin autolysis peaks at mass to charge ratio (m/z) 842.5 and 2211.1. After the calibration we removed trypsin autodigestion and keratin peaks from the spectrum before database searches were started.

The database searches were performed using MASCOT for searches at www.matrixscience.com or MS-Fit for searches on local databases. These include one

database built from the annotated *C. glabrata* open reading frame set in the Génolevures database (http://cbi.labri.fr/Genolevures/C_glabrata.php).

We set the mass tolerance at 50 ppm, allowed a maximum of one missed cleavage site per peptide and determined a minimum of four matched masses for a positive identification.

2.10 Microbiological methods

2.10.1 Heat shock transformation of Escherichia coli

A single fresh colony of *E. coli* DH5α cells was inoculated into 50 ml LB medium and grown over night at 37 °C with shaking (220 rpm). Five ml of the starter culture was used to inoculate 500 ml LB medium, which was then grown at 37 °C, 220 rpm to an OD_{600} of ~0.4. After cooling on ice the cells were harvested by centrifugation (15 min, 4 °C, 2500 x g), the pellet was resuspended in 250 ml ice cold 100 mM $CaCl_2$, centrifuged again and then resuspended in 50 ml 100 mM $CaCl_2$. After another centrifugation, cells were resuspended in 5 ml 100 mM $CaCl_2$, 20% (w/v) glycerol and 100 µl aliquots were transferred into prechilled, sterile polypropylene tubes. Cells were frozen immediately in liquid nitrogen and stored at -70 °C until use. One aliquot (100 µl) of chemically competent DH5*α*-cells was thawed on ice and gently mixed with 5-15 ng of DNA in prechilled polypropylene tubes. After incubation on ice for 10 min, cells were incubated at 42 °C for 1 min, resuspended in 900 µl dYT-medium and incubated at 37 °C for 45 min to recover. Afterwards cells were plated on selective media (LB + antibiotics) and incubated at 37 °C over night. Transformants were picked the next day.

2.10.2 Electrotransformation of Escherichia coli

Electrotransformation was used to introduce large plasmids (e.g. pSFS1A complementation constructs) into *E.coli*. 500ml dYT medium were inoculated with 5 ml of an overnight culture of TOP10 cells and grown until mid-log phase (OD_{600} ~0.6) at 37°C, 220 rpm. The culture was cooled on ice for about 30 min before the cells were pelleted in the centrifuge (20 min, 4000xg, 4°C). The pellet was washed once with 200 ml ice-cold sterile ddH_2O and once with 40 ml ice-cold 10% (v/v) glycerol. Cells were finally resuspended in 2 ml ice-cold 10% (v/v) glycerol and 50 µl aliquots were frozen in liquid nitrogen and stored at -80°C until use. For electroporation cells were thawed on ice before they were gently mixed with the purified plasmid DNA and transferred into a prechilled electroporation cuvette (2 mm). The electroporation was performed at 1.6 kV, 186 Ω, 25 µF. Immediately after the pulse 950 µl

SOC medium was added and bacteria were recovered at 37 °C for 30 min before plating on selective media.

2.10.3 Escherichia coli colony PCR

To screen transformants for successful insert integration a colony PCR was performed using material of a freshly grown colony directly in the PCR mixture. PCR was performed as described in 3.8.1.

2.10.4 Heat shock transformation of Candida glabrata

We used a transformation protocol based on a method that was described previously (WALTHER and WENDLAND, 2003). In brief: an overnight culture was used to inoculate 50 ml YPD at an OD_{600} of 0.2. Cells were grown at 37 °C, 220 rpm until they reached an OD_{600} of 1.0. Cells were pelleted (4000 x g, 4 °C) and were washed twice with 30 ml ice cold, sterile water and once with 30 ml ice cold TEL (10 mM Tris, 1 mM EDTA, 100 mM lithium acetate, pH 7.5). The cell pellet was resuspended in 1.5 ml TEL and used for transformation. The transformation mixture contained 100 µl cells, 100 µg (10 µl) Yeast Maker Carrier DNA (Clontech), 600 µl 40% (w/v) Polyethylene glycol 4000 in TEL and 2-5 µg DNA. After mixing, the transformation mixture was incubated overnight at 30 °C. Heat shock was performed at 44 °C for 15 min. Cells were pelleted at 800 x g and were subsequently resuspended in 200 µl TE (10 mM Tris, 1 mM EDTA, pH 7.5). Cells were plated on selective plates and were incubated at 37 °C. Transformants could be picked after one to two days.

2.10.5 Electrotransformation of Candida glabrata

Solutions used for the electrotransformation :

10 x TE	**10 x LiAc**
100 mM Tris-HCl pH 8	1 M lithiumacetate pH 7.5
10 mM EDTA	

To improve transformation efficiency for larger DNA fragments, electro transformation was performed as an alternative. *C. glabrata* cells were grown in 200 ml YPD at 37 °C, 220 rpm to mid log phase (OD_{600} ~0.6). The cells were pelleted at 5000 x g and resuspended in 40 ml water before 5 ml 10 x TE and 5 ml 10 x LiAc were added. The suspension was incubated for 45 min at 30 °C, shaking with 150 rpm prior to the addition of 1.25 ml 1 M DTT. The incubation was continued for another 15 min. Afterwards, 200 ml water was added to wash

the cells. Following the washing, cells were sedimented at 5000 x g and washed with 125 ml of ice cold water. Cells were then washed with 20 ml of ice cold 1 M sorbitol. After pelleting at 5000 x g, the cells were resuspended in 250 µl of 1 M sorbitol. Freshly prepared competent cells were used for transformation by electroporation.

For the transformation of *C. glabrata* with constructs for gene knock out or complementation the corresponding cassettes were mobilized from the plasmid with restriction enzymes. The DNA was purified and used for transformation. In prechilled polypropylene tubes 40 µl of freshly prepared competent cells were mixed with 3 to 5 µg DNA. This mixture was transferred to prechilled 2 mm electroporation cuvettes and incubated on ice for 30 min. Transformation was performed in a BTX electroporation system at 186 Ω, 1.6 kV and 25 µF. After the pulse cuvettes were immediately rinsed with 950 ml YPD and cells were incubated for 2 hours at 37 °C, 220 rpm to recover before they were plated on selective plates, which were either YNB drop out plates for (non-) auxotrophic mutants or YPD plates with nurseothricin for transformations with the flipper-construct. Plates were incubated at 37 °C for 1-3 days until colonies appeared.

2.10.6 Yeast colony PCR

To screen for positive transformants a colony PCR was performed with a crude cell extract of the transformants. For this, a medium sized colony was taken off the plate with a pipette tip and the tip was rinsed in 20 µl zymolyase solution (2.5 mg/ml zymolyase in 1.2 M sorbitol, 0.1 M Na_3PO_4). The suspension was incubated at 37 °C for 30 min and boiled at 95 °C for 10 min. The prepared cell extract was chilled on ice, before 2 µl were used in the PCR reaction.

2.10.7 Culturing of Candida glabrata for proteomic analysis

Single colonies of *C. glabrata* ATCC2001 were inoculated into YPD medium and grown for 24 h at 37 °C. Four single clonal cultures were pooled, washed and resuspended in sterile water and used to inoculate 600 ml of Pan Fungal Minimal Media (PFM) pH 7.4 at a density of 8 x 10^5 cfu/ml. *C. glabrata* PFM cultures were grown at 37 °C, 220 rpm for 16-18 hours until they reached an OD_{600} of 0.5. Cells were collected by centrifugation (5 min, 800 x g) and shifted to PFM at pH 4.0, 7.4 or 8.0 at the same optical density. Cultures were grown at 37 °C, 220 rpm for 2 hours and harvested by centrifugation (5 min, 800 x g, 4 °C). Pellets were washed twice with ice cold water and stored at -80 °C until used for protein extraction.

2.11 Phenotypic analysis

2.11.1 Growth rate determination

C. glabrata wild type and knock out strains were grown in 5 ml YNB to an OD_{600} of 2. These cultures were used to inoculate 50 ml fresh YNB media (or YNB + 0.7 M NaCl respectively) at OD_{600}=0.1. The cultures were incubated at 30 °C or 37 °C, shaking at 150 rpm, until stationary phase was reached (approximately 24 hours). During the incubation time, samples were taken in time intervals of 30 min to 2 hours to observe the increase in cell number by determining the optical density at 600 nm over time.

We also compared the growth rate of ATCC 2001 wild type strain and different *CWP1* knock out mutants in liquid cultures that were not shaken. Therefore, cell suspensions at OD_{600} of 0.05 and 0.1 were prepared in different media (YPD, YPD pH 8.0, YNB) and 200 µl were transferred into the wells of a 96 well plate. Every growth curve set up was prepared in triplicate to obtain comprehensive results. The plates were incubated in the plate reader at 37 °C and increase in OD was monitored over a time period of 24 hours with measurements every 7 minutes.

2.11.2 Microdilution spot assay

Over night cultures were used to inoculate fresh medium at an OD_{600} of 0.5-0.6. These cultures were incubated for 2 hours so that they reached an OD_{600} ~1. Cells were washed and resuspended at a cell density of $1x10^5$ cells/ml in YPD or YNB media, depending whether they were later spotted onto YPD or YNB plates. Serial 1/5 dilutions of cells were prepared in the same medium, 3 µl of these dilutions were spotted on the media plate surface (Figure 2-6) and plates were incubated at 25 °C, 30 °C, 37 °C, 40 °C and 42 °C. Serial dilutions of cells were also grown under stress conditions like acidic pH (pH 4.5 and pH 3.5), high osmolarity (0.7 M NaCl, 1.2 M NaCl), cell wall perturbing gents (Calcofluor white, Congo red), detergent additives (SDS) or antibiotics (Caspofungin).

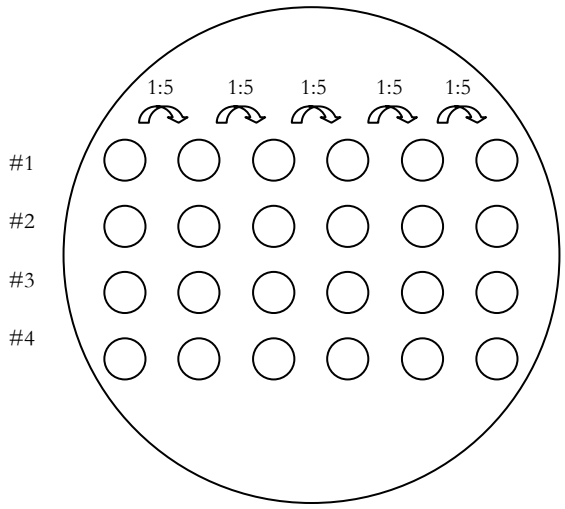

Figure 2-6 Scheme of the microdilution spot assay
Serial 1:5 dilutions of cell suspensions from different strains were prepared and spotted on the surface of agar plates.

In order to test sensitivity of the mutants to the cell wall perturbing compound calcofluor white (CFW), which binds to nascent chitin chains and thus inhibits correct cell wall formation, cells were spotted on to the surface of YPD plates containing different amounts of CFW. The stock solution was prepared by dissolving 100 mg/ml Calcofluor (Fluorescent brightener 28; Sigma) in 250 mM NaOH. YPD plates containing various concentrations of CFW (50 μg/ml to 600 μg/ml) were buffered to pH 7.5 with 150 mM HEPES as Calcofluor tends to precipitate at more acidic pH. Congo red, which binds to the cell wall glucan and thus perturbs correct assembly, was added to the media agar after autoclaving and cooling just before pouring the plates (at concentrations of 100 μg/ml to 600 μg/ml).

The detergent SDS disturbs the cell membrane organization and can be used to reveal cell wall weakening. Therefore, the growth of mutants and wild type cells was tested on YNB plates containing different amounts of SDS. For the preparation of plates containing SDS, a 10% (w/v) SDS stock solution was prepared and appropriate amounts were added to the autoclaved media before plates were poured with final concentrations of 0.01%, 0.012%, 0.015% and 0.02% (w/v) SDS.

2.11.3 Quantazym sensitivity assay

To specifically analyse β-1,3-glucanase sensitivity of the generated mutants, quantazym, a recombinant β-1,3-glucanase was used to lyse cells. For that purpose, cells from a 5 ml over night culture were harvested by centrifugation (4000 x g, 2 min), washed with and then resuspended in YNB minimal media and used to inoculate 30 ml YNB media at an optical density of OD_{600} of 0.5. Cells were grown (30 °C, 220 rpm) until they reached an OD of 2. They were then pelleted, washed with 50 mM Tris-HCl pH 7.5 and resuspended in the same

buffer. Subsequently they were used to inoculate 50 ml of 50 mM Tris-HCl pH 7.5 at an optical density of 1.0 and β-mercaptoethanol (β-ME) was added to a final concentration of 40 mmol/L. This set up was incubated at room temperature for 1 hour, before quantazym was added (5 U/ml) and the decrease in OD was measured over a time period of 1 hour.
For large scale screening of mutants, we inoculated 5ml YPD at an OD_{600} of 0.2 to 0.3 from an over night culture. Cells were grown for 3 hours and harvested in mid log phase. After washing and resuspending in 50 mM Tris-Cl pH 7.5, OD in this buffer was adjusted to 1.0. We added β-ME to a final concentration of 40 mmol/L, incubated the mixture at RT for 1 hour, before quantazym was added (5 U/ml) and the decrease in OD was measured at RT. This smaller set up allowed the parallel measurement of up to 15 strains in triplicate.

2.11.4 Susceptibility test towards antimycotic agents – E-test

The sensitivity of the generated mutant as well as the wild type strain towards the antifungal agents Fluconazole, Amphotericin B, Flucytosin and Voriconazole was tested using the E-Test® (Inverness, Cologne) format. For the assay, about $3*10^9$ cells were resuspended in water spread on Sabouraud plates with a cotton tip and E-test strips containing a concentration gradient of the different agents was laid on top. The plates were incubated at 37°C and the assay was read out after 24 or 48 hours depending on growth of the strains.

2.11.5 Peptide killing assay

It has been shown that certain small peptides as they are present e.g. in the oral cavity are able to kill fungi by integrating into the membrane, formation of a pore and thus killing the fungus (SHAI, 2002). We tested two of these peptides for their antifungal activity: Histatin-5 and LL-37. It was shown that LL-37 disintegrates the membrane bilayer into discrete vesicles, and thus induces an instantaneous efflux of small molecules (such as ATP) as well as larger molecules (such as proteins) from the cell. The effects of Histatin-5 on the membrane morphology are less pronounced, but are efficient to induce an efflux of nucleotides, which also kills the attacked fungus (DEN HERTOG *et al.*, 2005).
In the assay, the two peptides were used at the following concentrations:
His-5: 1 μM/250 nM/62.5 nM
LL-37: 500 nM/125 nM/31.2 nM
For the preparation of peptide solutions, the freeze dried peptides were first dissolved in 15 μl DMSO. They were then diluted in 1 mM K_3PO_4 to give a final concentration of 1 mg/ml.

These solutions were used to further dilute the peptides to the final concentrations mentioned above.

For the killing assay, an over night culture was used to inoculate fresh YPD medium at an OD_{600} of 0.2 - 0.3. Cells were grown to mid logarithmic phase, washed and resuspended in 1 mM K_3PO_4 at a density of 3.2 x 10^6 cells per ml, as determined by cell count in a Neubauer chamber. To 50 µl of these cell suspensions, 50 µl of the different peptide dilutions were added and the mixes were incubated at 37 °C for 30 min. Suspensions were then diluted 1:200 in 1 mM K_3PO_4 and 25 µl of these dilutions were plated on Sabouraud plates in triplicate. After two days of incubation at 37 °C colonies were counted and the survival ratio of yeasts with/yeasts without peptide was calculated.

2.11.6 Determination of the chitin content of Candida glabrata cell walls

Solutions used for chitin content determination:

Solution A	**Solution B**
1.5 M Na_2CO_3	2.7% (w/v) (4)-dimethyl-aminobenzaldehyde
4% (v/v) acetylacetone	50% (w/v) HCl
in ddH_2O	in ethanol

Besides the great amounts of glucan and mannan, chitin is the third polysaccharide, which is found in the fungal cell wall in remarkable amounts. The chitin content of fungal cell walls can be determined by hydrolyzing the chitin into its N-acetylglucosamine monomers followed by colorimetric analysis. Therefore, 4 mg freeze dried cell wall material was resuspended in 200 µl 1M NaOH and boiled for 10 min to hydrolyse the glucan structures. After cooling down to room temperature, the suspension was neutralized with 200 µl 1 M HCl and centrifuged (17000 x g, 5 min). Supernatant was taken off and the remaining pellet was hydrolysed in 1 ml 6 M HCl at 100 °C for 18 hours. In parallel, dilutions of N-Acetyglucosamine (Glc-NAc) at various concentrations (0/2/5/10/20…. 100 µg/ml) in 6 M HCL was prepared for the generation of a standard curve. These standards were treated the same way as the hydrolyzed cell wall material.

After the hydrolysation, the acid was completely evaporated at 60 °C under a constant stream of nitrogen gas. The dried pellet was resuspended in 1ml ddH_2O and then centrifuged (17000 x g, 5 min) to remove remaining debris from the Glc-NAc solution. 100 µl of the supernatant was mixed with 100 µl solution A and incubated at 100 °C for 20 min. When the samples had cooled down to room temperature, 700 µl of pure ethanol and 100 µl solution B were added. After one hour of incubation at room temperature, the light absorbance was measured at 520

nm. For the standard curve we prepared two samples of each dilution and the analysed cell wall samples were prepared in triplicate.

2.11.7 Macrophage uptake assay

In the immune competent host, *C. glabrata* cells are phagocytosed by macrophages followed by lysis of the yeasts within the phagolysosomes. We tested the ability of wild type and mutant *C. glabrata* strains to withstand the killing by macrophages in a cell culture assay. Therefore, we cultivated J774A.1 mouse macrophages in RPMI medium (supplemented with 1% (v/v) streptomycin/penicillin, 1% (v/v) Na-pyruvate, 10 mM HEPES and 10% (v/v) FCS) at 37 °C and 5% CO_2. When they reached confluency, cells were harvested, counted and 2 x 10^4 cells (in 2 ml medium) were seeded in each well of a 12-well cell culture plate and incubated as before for 24 h. After the incubation, cells of one well were harvested and counted to determine cell numbers in the wells. *C. glabrata* cells were inoculated into YNB medium from an over night liquid culture (YNB, 37 °C, 220rpm) at OD_{600} 0.5 and grown for 2 hours. After harvesting the yeast cells, they were added to the macrophages at a ratio of 1:1, briefly centrifuged (1 min, 800 x g) to initiate cell contact and incubated at 37 °C, 5% CO_2 for 1 h. Afterwards, cytochalasin D was added to the cells to stop phagocytosis of the macrophages and well contents were harvested. The cell suspension was centrifuged (1000 x g, 2 min) and the pellet was washed with PBS, before it was resuspended in 1 ml PBS with 1% (w/v) BSA and incubated at RT for 20 min. Pellets were washed twice with PBS and the primary antibody (mouse anti-β-1,3-glucan) was applied for 20 min at a 1:300 dilution to label yeast cells by the detection of exposed β-1,3-glucan. Cells were washed twice with PBS, before the secondary antibody (FITC- labelled goat anti mouse) was applied at a 1:200 dilution for 20 min at RT in the dark. Cells were washed with PBS and macrophages were lysed with 1% (v/v) Triton-X, before dead cells were labelled by the addition of propidium-iodid (25 µl per suspension). The stained cells were examined by fluorescence activated cell sorting (FACS).

2.11.8 Adhesion assay

We tested the ability of *C. glabrata* wild type cells and the generated mutants to adhere to different mammalian cells in a cell culture assay.

The following immortal cell lines were used:

- Vero (kidney epithelial cells, African Green Monkey)
- HeLa (human cervix carcinoma epithelial cells)
- EA926 (human endothelial cells)
- HaCaT (human keratinocytes)
- HEp2 (human laryngeal carcinoma)

For culturing Hep2 and HeLa cells we used Dulbecco's modified Eagle's medium (DMEM) containing 1% glutamine, sodium pyruvate and glucose, supplemented with heat inactivated (30 min at 56°C) fetal calf serum (10% (w/v) FCS), antibiotics (50 units/ml Penicillin, 50 µg/ml Streptomycin, 2.5 µg/ml Vancomycin) and 1% non essential amino acids. Vero, HaCaT and EA926 cells were cultured in RPMI with 1% glutamine, 5% FCS and antibiotics (50 units/ml Penicillin, 50 µg/ml Streptomycin, 2.5 µg/ml Vancomycin).

Cells were grown until confluent and taken off the bottom of the cell culture dish by trypsination. After resuspension of cells in 40 ml fresh media 200 µl of the cell suspension were seeded into each well of a flat bottom 96-well-plate. Cells were incubated at 37 °C and 5% CO_2 until they were confluent in the 96-well-plates, which took between one and four days depending on the cell line.

C. glabrata cells were grown in 5% SC medium as it was shown to maximize adhesion protein expression (Epa1p). After inoculation of the yeast at OD_{600} of 1.0, they were grown at 37 °C, 220 rpm shaking for 30 min. They were then harvested, washed twice with sterile PBS and resuspended in 4 ml RPMI or DMEM without FCS, as it inhibits adhesion, at a cell density of $2x10^7$ c/ml. Afterwards a 1:2 dilution series of the cell suspensions was prepared in the appropriate media. The medium of the confluent 96-well-plate was taken off and 200 µl of one cell suspension was added into the "columns" of the plate. Additionally, we added 200 µl RPMI or DMEM media in the first (1) and last (12) column of the plate as a medium control. Thus, the experiment included eight replicates of each strain on every plate. The 96-well-plate was briefly centrifuged (2 min, 500 x g) to initiate cell contact between yeasts and cell culture and then incubated at 37 °C, 5% CO_2 for two to six hours. After the adherence Calcoflour white was added to a final concentration of 20 µg/ml and staining of the fungal cells was allowed during 30 min of incubation at 37 °C. Non adherent cells were removed by repeated washing (three times) with 200 µl PBS. Fluorescence of the adherent calcofluor white stained yeast cells was measured in a plate reader (excitation 360 nm, emission 460 nm). We repeated each experiment at least twice to receive comprehensive results.

3 RESULTS

The aims of this study were (1) to elucidate the function of the *C. glabrata* cell wall protein 1 family in the architecture of the fungal cell wall and (2) to analyse the proteomic changes, which occur in response to ambient pH.

The first aim was approached by molecular cell wall analysis and successive targeted gene knock out of all three members of the *CWP1* family, followed by phenotypic analysis of the generated mutants. To achieve the second goal, we examined protein expression of *C. glabrata* by 2D gel electrophoresis and identified pH regulated proteins by mass spectrometry.

3.1 Cwp1p is anchored in the cell wall via GPI and Pir like linkages

In silico analysis of the *C. glabrata* genome identified the Cwp1 proteins as putative GPI-anchored cell wall proteins. Immunofluorescence microscopy using α-cwp1.1 specific antibodies demonstrated that Cwp1.1p localizes to the cell surface (Figure 3-1 A). This indicates that Cwp1.1p is localized either to the plasma membrane or the fugal cell wall. We therefore released GPI-anchored proteins from cell wall material using β-1,6-glucanase and subjected them to SDS PAGE and immunoblotting (Figure 3-1 B). Cwp1.1p has a theoretical molecular weight of 21.3 kDa but exhibits less electrophoretic mobility (apparent molecular weight of 38 kDa) when separated on SDS gels due to post-translational glycosylations. As seen in Figure 3-1 B, Cwp1.1p is a highly abundant GPI-protein in the cell wall of *C. glabrata.* Interestingly, the sequence of Cwp1.1p also contains the characteristic repetitive motifs found in Pir proteins and might thus be linked to the cell wall via an alkaline sensitive linkage. Analysis of the *C. glabrata* cell wall structure by fractionated digestion followed by immunoblotting revealed that Cwp1.1p is indeed linked to the cell wall either via the GPI-linkage or an additional Pir like linkage. Depending on the pH of the growth medium, one or the other linkage is predominant (WEIG, unpublished data). The GPI-attachment is favoured during growth at pH 7.5, whereas the additional Pir like linkage is strongly increased when cells were grown at pH 4.

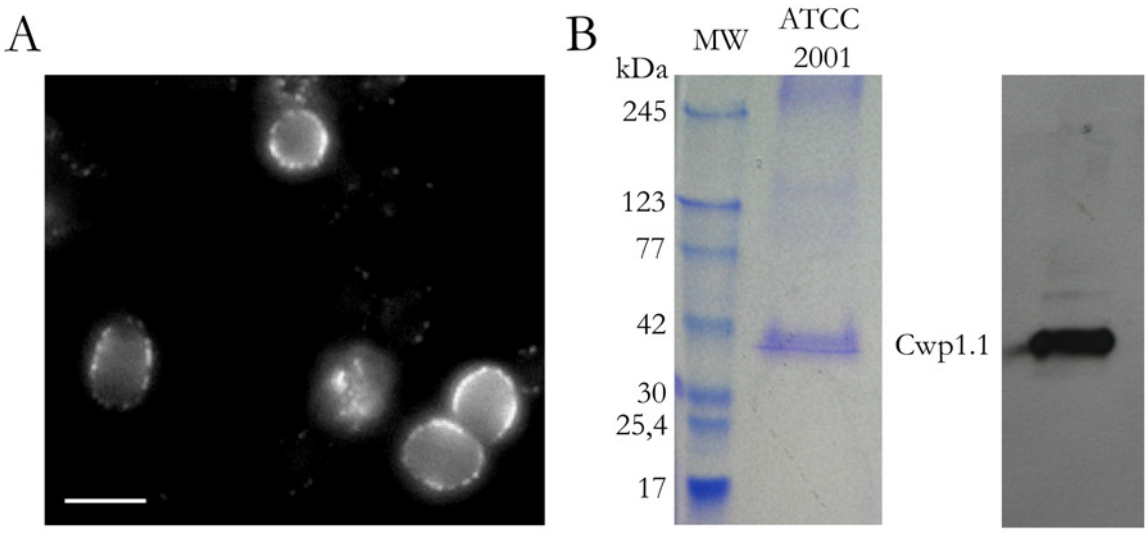

Figure 3-1 Cwp1.1p is localized to the cell wall
(A) *C. glabrata* cells were fixed with 4% PFA and processed for indirect imunofluorescence microscopy using affinity purified antibodies directed against Cwp1.1. Scale bar represents 5 μm. **(B)** β-1,6-glucanase extracted GPI-anchored cell wall proteins were separated on SDS polyacrylamide gels and stained with coomassie (left panel) or subjected to immunoblotting using α-cwp1.1 specific antibodies (right panel)

3.2 Generation of knock out and complemented strains

For the generation of cassettes for gene knock out as well as gene complementation, the necessary fragments were amplified by PCR and cloned into the corresponding vectors (pSFS1A or pCgACT14, see materials and methods), which were then used for yeast transformation. The correct orientation of fragments used for the generation of mutants was achieved by the use of two different restriction enzymes when cloning the fragments. Additionally, we confirmed the correct orientation by analytical restriction enzyme digests. One example of a test digest is shown in Figure 3-2.

The constructs used for gene re-integration were sequenced to verify the correct amplification, orientation and thus correct gene expression.

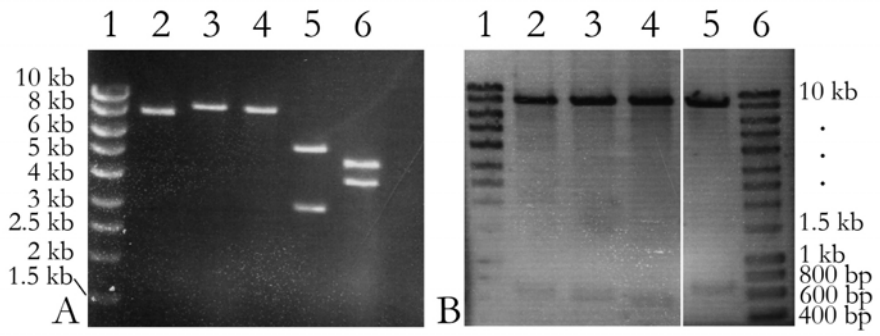

Figure 3-2 Test digests of flipper constructs for the generation of *CWP1.1/1.2* knock out strains
As an example for the test digests, the restriction pattern of plasmids pSFS1A-F2 (A) and pSFS1A-F1-F2 (B) for the knock out of *CWP1.1/1.2* are shown. **(A)** lane 1: molecular weight standard; lane 2: *Sac*II linearized pSFS1A as a control; lane 3: *Apa*I linearized pSFS1A-F2; lane 4: *Xho*I linearized pSFS1A-F2; lane 5: *Hind*III/*Sac*II digest (*Hind*III cuts within caFLP, *Sac*II cuts 3' of F2) and lane 6: *Xho*I/*Not*I digest (mobilizing the selection cassette with FRT sequences) both to confirm the correct orientation of the cloned F2 fragment **(B)** lanes 1 and 6: molecular weight standard; lane 2: *Apa*I/*Bam*HI digest (as confirmation for correct orientation of F1, mobilizes F1+FRT); lane 3: *Apa*I/*Xho*I digest (mobilizes F1); lane 4: *Sac*II/*Not*I digest (mobilizes F2), lane 5: *Pst*I/*Sac*II digest (as confirmation for correct orientation of F2, mobilizes F2+FRT

After transformation of *C. glabrata* wild type strains (ATTC 2001, ATTC 90876, ΔHT6 and ΔH1) with the corresponding knock out cassettes, colonies that grew on selective plates were picked and subjected to colony PCR to confirm the integration of the knock out cassette at the correct locus. The PCR was performed using primers, annealing in the genomic sequence up or down stream of the integrated cassette and a corresponding primer, which anneals within the cassette (e.g. Cgcwp1.1 ver f/HIS ver r and HIS ver f/Cgcwp1.1 ver r for *CWP1.1* knock out with the *HIS3* marker or Cgcwp1.1 ver f/FLP ver r and FLP ver f/Cgcwp1.1 ver r for knock out using the flipper cassette, see appendix for primer sequences, Primers used for verification PCRs can be recognized by the "ver" abbreviation). Using these sets of primers amplification of fragments occurs only in clones, which integrated the knock out cassette at the correct locus.

To verify the correct integration of the knock out cassette at the target locus and only there, positive clones were subjected to southern blot hybridization. Digestion of the genomic DNA yielded fragments of specific size, which were then detected using fragment specific DIG-labelled probes, i.e. a FLP specific probe for knock out with the flipper cassette and a HIS specific probe for knock out with the *HIS3* marker (see appendix for primer sequences. Primers used for the preparation of DIG labelled probes to be used in southern blotting can be recognized by the "SOU" abbreviation.). The southern blot for the *CWP1.1/1.2* knock out using the flipper cassette (pSFS1A-F1-F2) is shown (Figure 3-3) as an example.

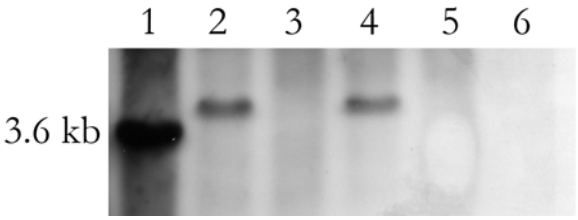

Figure 3-3 Southern blot of *CWP1.1/1.2* knock out using the flipper cassette (pSFS1A-F1-F2)
Genomic DNA of generated mutants was digested with BspH I, which cuts within the knock out cassette and in the genomic sequences downstream of it, generating a fragment of 3886bp. The blot was probed with a FLP specific DIG-labelled probe, which was generated using the primers SOU FLP f and SOU FLP r. Clones, which still carry the flipper construct (FLP) as well as the corresponding clones, where the cassette was recycled by SAP2 promoter induction of the flippase (ΔFLP) were included in the blot. Lane 1: DIG-labelled molecular weight marker; lane 2: 2001⊿*cwp1.1/1.2* (*FLP*), lane 3: 2001⊿*cwp1.1/1.2* ⊿*FLP*; lane 4: 90876⊿*cwp1.1/1.2* (*FLP*), lane 5: 90876 ⊿*cwp1.1/1.2* (⊿*FLP*), lane 6: ATCC 2001 wt.

We performed immunoblots and RT-PCR to demonstrate gene deletion in the knock out strains and to prove correct gene transcription and protein expression in the complemented strains. Gene transcription of the three *CWP1* genes was examined using gene specific primers to amplify cDNA after RNA isolation and reverse transcription. Primer pairs RT Cgcwp1+2 f/RT Cgcwp1.1 r, RT Cgcwp1+2 f/RT Cgcwp1.2 r and RT Cgcwp1.3 f/RT Cgcwp1.3 r were used for gene specific fragment amplification from *CWP1.1* (246 bp), *CWP1.2* (389 bp) or *CWP1.3* (417 bp) genes respectively (see appendix for primer sequences). In the wild type strain the mRNA of all three *CWP1* genes was detected, whereas the signals of the deleted genes were absent in the respective knock out strains (Figure 3-4).

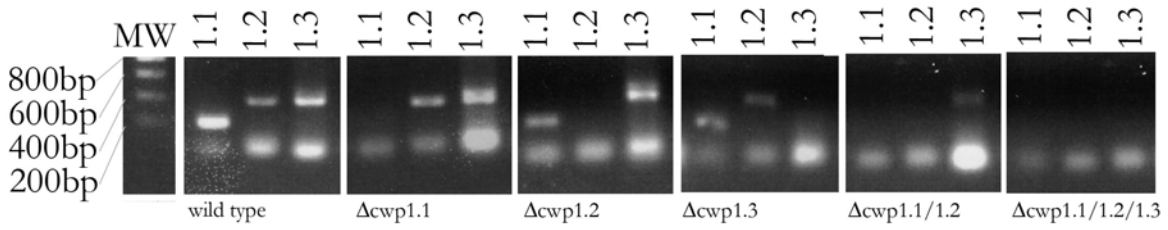

Figure 3-4 Gene specific RT-PCR for control of gene knock out in the deletion strains
Reverse transcription of mRNA followed by gene specific PCR was used to demonstrate *CWP1.1, CWP1.2* and *CWP1.3* deletion in *C. glabrata* wild type strain ATCC 2001. MW: molecular weight marker; 1.1: *CWP1.1* specific PCR; 1.2: *CWP1.2* specific PCR; 1.3: *CWP1.3* specific PCR; strains: ATCC 2001 (wild type) strain and indicated *CWP1* deletion strains thereof

In the case of *CWP1.3* gene knock out and complementation, RT-PCR was pivotal as, lacking a Cwp1.3p specific antibody, we could not analyse protein expression in immunoblots. Transcription of the *CWP1.3* gene was gone in ⊿*cwp1.3* knock out strains and restored after gene complementation (Figure 3-5).

For *CWP1.1* and *CWP1.2* deletion and reconstituted strains, Cwp1.1p expression was examined with the help of an α-cwp1.1 antibody. The immunoblot analysis verified the knock out of *CWP1.1* and *CWP1.2* (as the antibody cross reacts with Cwp1.2p) and it demonstrates the high expression levels of Cwp1.1p in non mutant strains (wild type and complemented strains, Figure 3-6).

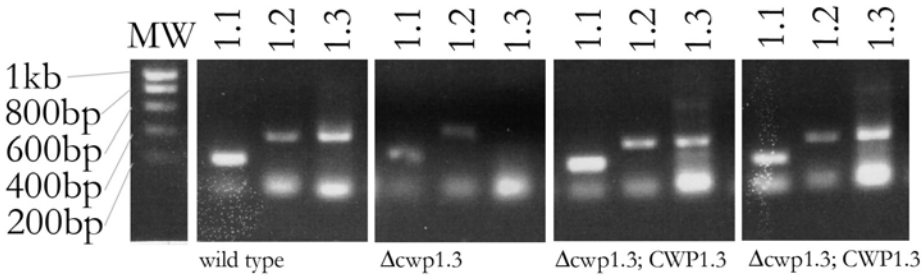

Figure 3-5 *CWP1.3* specific RT-PCR for control of gene knock out and gene complementation
Reverse transcription of mRNA followed by gene specific PCR was used as a control for *CWP1.3* deletion in ATCC 2001 and restored gene function in *CWP1.3* reconstituted strains (2001⊿*cwp1.3*+*CWP1.3*). MW: molecular weight marker; 1.1: *CWP1.1* specific PCR; 1.2: *CWP1.2* specific PCR; 1.3: *CWP1.3* specific PCR; 1) ATCC 2001; 2) 2001Δ*cwp1.3*; 3) 2001Δ*cwp1.3*+*CWP1.3*#1; 4) 2001Δ*cwp1.3*+*CWP1.3*#2.

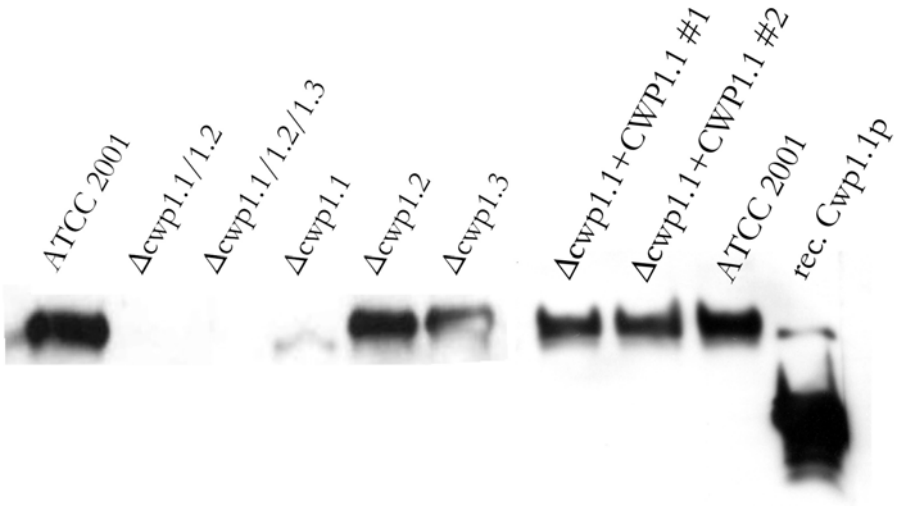

Figure 3-6 Immunoblot of *Candida glabrata* wild type strain, *Δcwp1* mutants and complemented strains
For immunoblotting, 4mg cell wall material was digested with β-1,6-glucanase to release GPI-anchored cell wall proteins. Cwp1.1p is expressed at high levels in the wild type strain ATCC 2001 and in the ⊿*cwp1.2* and ⊿*cwp1.3* mutant (left panel). In the ⊿*cwp1.1* knock out strain the lower expression of Cwp1.2p can be detected as the α-cwp1 antibody cross reacts with Cwp1.2p and the weak signal is not overlaid by the stronger Cwp1.1p signal. The knock out of *CWP1.1* was complemented using the plasmid pCgACT14-CWP1.1 for gene reintegration. Protein expression of Cwp1.1p from the autonomously replicating plasmid was comparable to wild type levels in *CWP1.1* complemented strains (⊿*cwp1.1*+*CWP1.1*, right panel). The recombinant Cwp1.1 protein produced in *E. coli* runs at lower molecular weight because it is not glycosylated as in the yeast cell wall.

3.3 Cell wall architecture

3.3.1 CWP1 deletion alters the structure of the fungal cell wall

The fungal cell wall of ascomycetous yeasts appears as a layered structure when visualized by electron microscopy (EM). The outermost, electron dense, layer represents the mannoproteins. It is underlined by a less electron dense but thicker layer made of β-glucan and chitin. The cell wall is separated from the cytosol by the plasma membrane, which also appears electron dense. EM images of wild type strains used in this study show the anticipated structures (Figure 3-7), whereas the simultaneous deletion of *CWP1.1*, *CWP1.2* and *CWP1.3* results in a rather diffuse cell wall where layers are not as distinct as in wild type cells (Figure 3-7). We did not observe any differences in the intracellular structures of *C. glabrata* wild type and ⊿*cwp1* mutant strains.

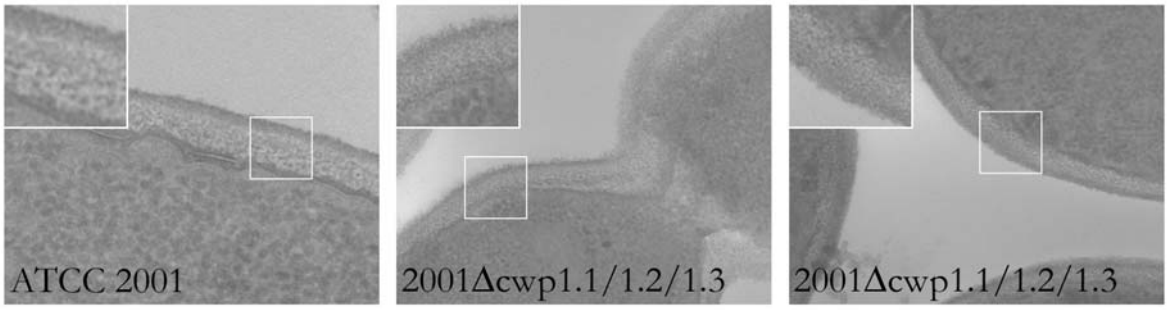

Figure 3-7 Cell wall structure of *Candida glabrata* wild type and *Δcwp1.1/1.2/1.3* mutant strains
Electron microscopic images of *C. glabrata* wild type (ATCC 2001) and ⊿*cwp1.1/1.2/1.3* deletion strains show that the triple knock out mutant appeared to have a less dense mannoprotein layer than the wild type. Insets show two fold magnifications of boxed areas.

3.3.2 Deletion of CWP1.1/1.2/1.3 leads to increased calcofluor white sensitivity

Electron microscopy revealed that the cell wall structure of ⊿*cwp1.1/1.2/1.3* seems altered in comparison to wild type cells. Therefore, we tested the strains' ability to resist cell wall stress by the use of the cell wall perturbing agents calcofluor white (CFW) and congo red (CR). CFW binds to nascent chitin chains and CR to newly synthesized glucan, thus inhibiting correct cell wall assembly.

In previous studies we found that the ΔHT6⊿*cwp1.1/1.2* double mutant showed an increased sensitivity to calcofluor white when compared to the wild type (KAPLANEK, 2004). The comparison of the wild type strains ATCC 2001, ΔH1 and ΔHT6 however showed that the observed cell wall phenotype was in fact not caused by the *CWP1.1* and *CWP1.2* deletion but

was also present in the ΔHT6 wild type strain (KAPLANEK, 2004). A similar effect has been recognized in *C. albicans*, where ectopic expression of the *URA3* marker influences virulence phenotypes (BRAND *et al.*, 2004). Due to the observation of a cell wall related phenotype in the ΔHT6 wild type strain and the limitations regarding the numbers of possible subsequent gene deletions in auxotrophic strains, we changed our knock out system from the use of auxotrophic strains to the flipper construct, which uses a recyclable antibiotic resistance marker for the selection of transformants. The triple knock out mutant (*⊿cwp1.1/1.2/1.3*) generated in the ATCC 2001 wild type strain using the flipper construct showed an increased CFW sensitivity (Figure 3-8). The observed phenotype was rescued by reintegration of the *CWP1.3* gene at its original locus.

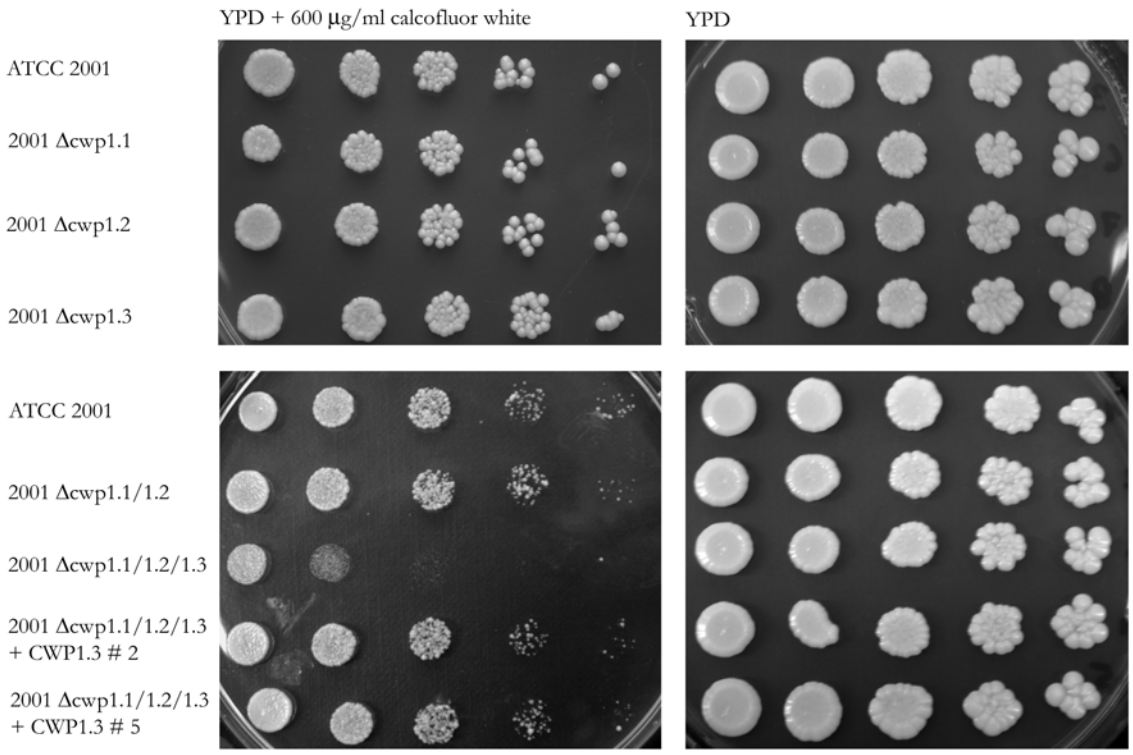

Figure 3-8 Calcofluor white sensitivity of wild type and mutant strains
Serial dilutions of *C. glabrata* wild type and different *CWP1* knock out strains were spotted on YPD plates containing 600µg/ml calcofluor white (left panel). The *⊿cwp1.1/1.2/1.3* triple knock out mutant showed an increased sensitivity towards the cell wall perturbing agent. Reintegration of the *CWP1.3* gene at its original locus (using pSFS1A-CWP1.3-F6) rescued the observed cell wall phenotype. The right panel shows the same cell suspensions spotted on YPD plates as a growth control.

Another cell wall perturbing agent, which is frequently used to reveal cell wall weakening, is congo red, which binds to glucan structures inhibiting correct cell wall assembly. It has been shown before that *S. cerevisiae* as well as *C. albicans* strains, which are more sensitive to

calcofluor white, might also display an increased congo red sensitivity (GARCERA *et al.*, 2003; VAN DER VAART *et al.*, 1995). However, in our analysis we found no increased congo red sensitivity in the generated mutants (Figure 3-9) as compared to the wild type strain.

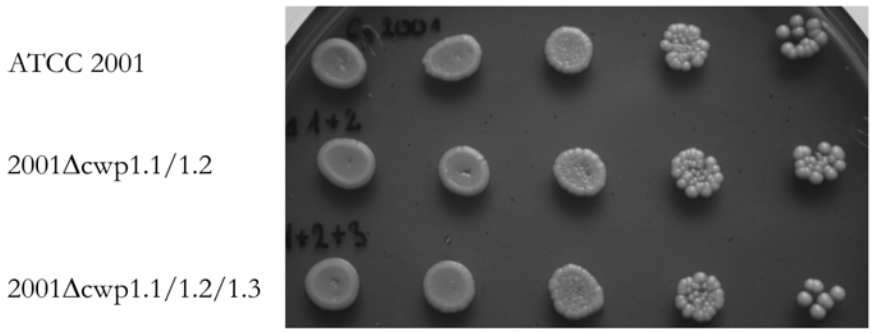

Figure 3-9 Congo red sensitivity of wild type and mutant strains
Serial dilutions of *C. glabrata* wild type ATCC 2001, 2001 *⊿cwp1.1/1.2* and 2001 *⊿cwp1.1/1.2/1.3* mutants were spotted on YPD plates containing 100µg/ml congo red. The *⊿cwp1.1/1.2* and *⊿cwp1.1/1.2/1.3* knock out mutants showed no difference in sensitivity towards the cell wall perturbing agent.

We also tested the sensitivity of all mutants and wild type strains (ATCC 2001, ΔH1 and ΔHT6) towards the detergent SDS, which perturbs the cell membrane integrity and might thus reveal cell wall weaknesses in an indirect manner, as it has been demonstrated before (BADER *et al.*, 2001). We were able to show that SDS sensitivity of the generated knock out strains was identical with the wild type (Figure 3-10). Also different incubation temperatures (30°C or 37°C) did not cause any differences in the ability to grow (data not shown).

The wild type and knock out strains were also tested for their ability to grow on YPD plates containing 1.2 M NaCl, as hyperosmolaric stress challenges the cell wall to resist the osmotic pressure. We found no differences in growth between wild type and mutants strains.

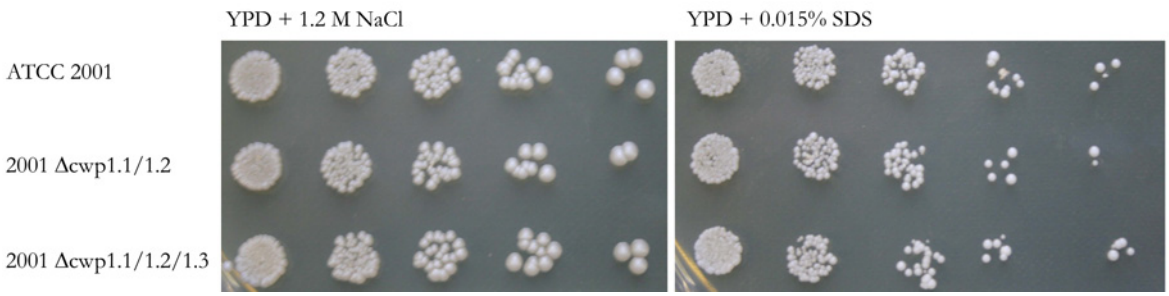

Figure 3-10 Hyperosmolaric stress and SDS sensitivity of wild type and mutant strains
Serial dilutions of ATCC 2001 wild type, *⊿cwp1.1/1.2* and *⊿cwp1.1/1.2/1.3* knock out strains were spotted on YPD plates containing 1.2M NaCl or 0.015% (w/v) SDS. No difference in growth between the wild type and the knock out strains was detectable.

3.3.3 Chitin content of ΔCWP1 mutant cell walls is unchanged

Cell wall weakening brought about for example by deletion of cell wall proteins might be compensated by upregulation of chitin to enhance robustness of the polysaccharide network. The observed increased sensitivity of the *⊿cwp1.1/1.2/1.3* triple mutant to calcofluor white, an agent which binds to nascent chitin chain, indicated that chitin contents or chitin incorporation of the mutant cell walls might be altered.

Analysing the chitin contents of wild type and *CWP1* mutant cells, we found that the cell wall chitin of all examined strains was around one percent of the total cell wall dry weight. Only the *⊿cwp1.1/1.2/1.3* triple knock out mutants showed a marginally lower chitin content (0.89% of cell wall dry weight). When rising the growth temperature or applying cell wall stress during growth (600µg/ml CFW), the proportion of chitin in the cell wall increases. The increase of chitin at 42°C or calcofluor white stress conditions appears slightly more pronounced in the wild type than in the *⊿cwp1.1/1.2* and *⊿cwp1.1/1.2/1.3* mutant strains (Figure 3-11).

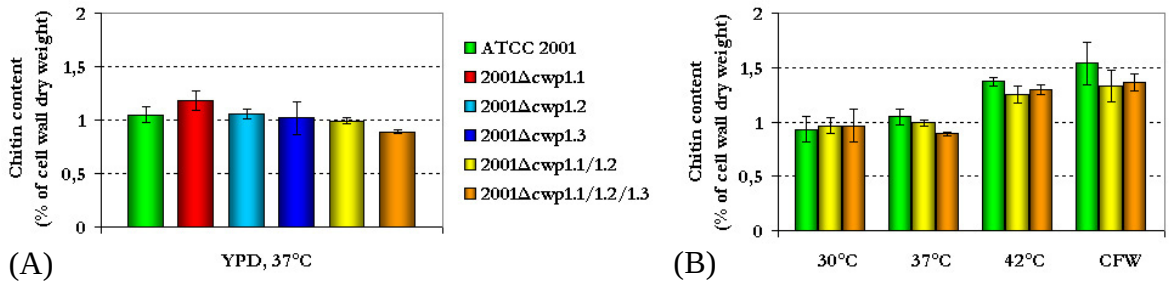

Figure 3-11 Chitin content of *Candida glabrata* cell walls
Hydrolysis of freeze dried cell wall material in HCl released glucoseamine monomers from chitin, which were detected by light absorption at 520 nm after a colorimetric reaction. **(A)** The knock out of *CWP1* genes did not significantly alter chitin content of the fungal cell walls when cells were grown under standard conditions (YPD, 37°C). **(B)** With higher growth temperatures the chitin content increases in mutant as well as wild type cells. Growth under calcofluor white mediated cell wall stress conditions (600µg/ml CFW), induced a further increase in chitin content of the cell walls. Error bars represent standard deviation.

3.3.4 Δcwp1.1 mutants show an increased quantazym sensitivity

The β-1,3-glucan network is the backbone of the fungal cell wall. Since we expected structural changes in the cell wall structure caused by *CWP1* deletion, we tested the ability of *C. glabrata* wild type and mutant cells in regard to their ability to withstand the digestion by β-1,3-glucanase. The analysis revealed that *CWP1* mutants display increased quantazym sensitivity. This increased sensitivity is mainly mediated by *CWP1.1* deletion as the differences in sensitivity between *⊿cwp1.1* knock out strain and the double (*⊿cwp1.1/1.2*) as well as triple

(⊿*cwp1.1/1.2/1.3*) knock out mutant were only marginal. Additionally, the ability to resist β-1,3-glucanase digestion to a certain extend was not altered in the ⊿*cwp1.2* and ⊿*cwp1.3* single knock out mutants compared to the wild type strain (Figure 3-12).

In order to correct variations in the efficiency of cell lysis using different β-1,3-glucanase batches, we calculated quantazym sensitivity of the mutants in relation to the wild type's sensitivity (Figure 3-12).

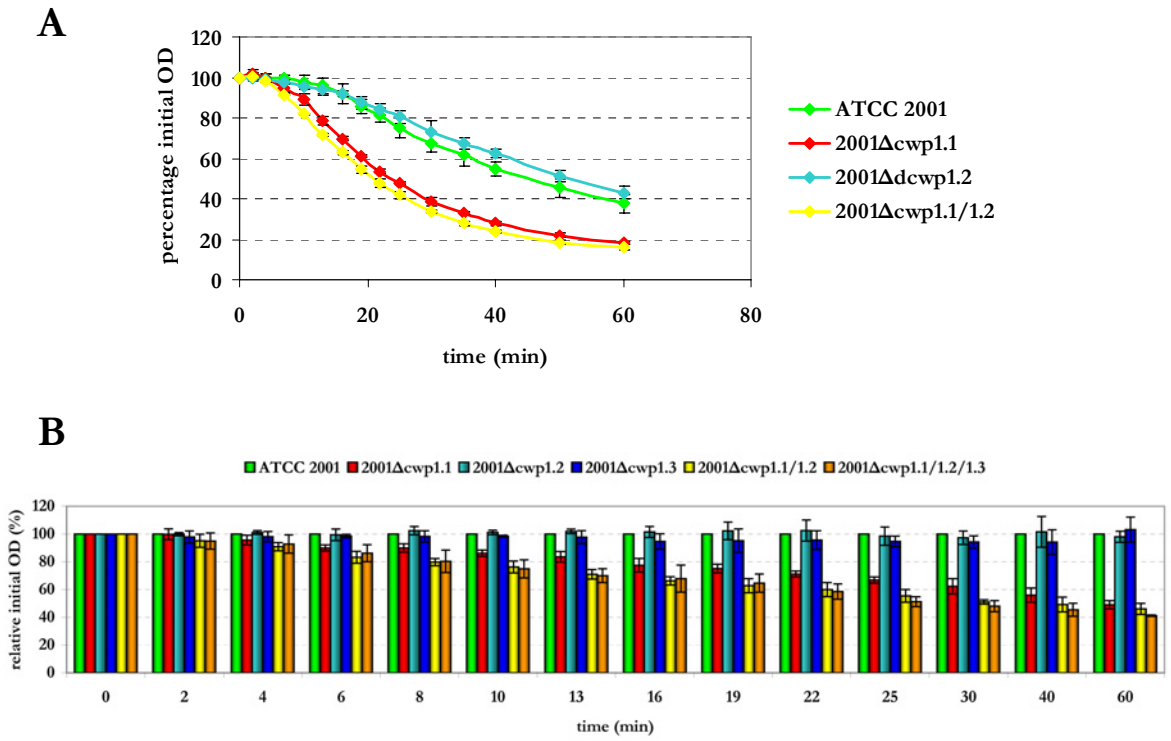

Figure 3-12 Quantazym sensitivity of wild type and mutant strains
Quantazym lyses *C. glabrata* cells by digestion of the β-1,3-glucan backbone of the fungal cell wall. Cell lysis was monitored by the decrease in OD_{600}. **(A)** The initial experiment demonstrates that enhanced quantazym sensitivity is mediated by *CWP1.1* deletion as the ⊿*cwp1.2* mutants shows the same sensitivity as the wild type strain and additional deletion of this gene in the ⊿*cwp1.1* background does not further increase β-1,3-glucanase sensitivity. **(B)** For a clearer data presentation including all deletion strains and error bars, bar charts are used to show sensitivities of the deletion strains relative to wild type sensitivity. Error bars represent standard deviations.

We generated ⊿*cwp1* mutants in various wild type strains (ATCC 2001, ΔH1, ΔHT6, ATCC 90876) and examined these mutants and their corresponding parental wild type strains in regard to their β-1,3-glucanase sensitivity. In all examined strains, the ⊿*cwp1.1* knock out mutants were significantly more sensitive to β-1,3-glucanase digestion. After 60 min of incubation with β-1,3-glucanase, the amount of non-lysed ⊿*cwp1.1* cells was around 50% of the wild type. Reintegration of *CWP1.1* with the plasmid pCgACT14-CWP1.1 reversed the

observed phenotype of enhanced β-1,3-glucanase sensitivity almost to wild type levels (Figure 3-13).

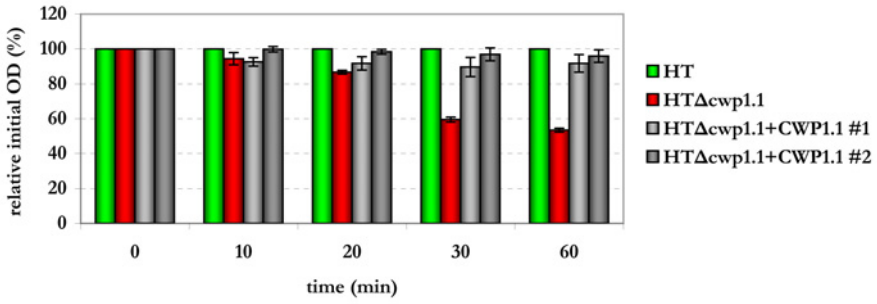

Figure 3-13 Quantazym sensitivity of wild type, *Δcwp1.1* knock out and complemented strains
Cell lysis of *C. glabrata* cells by quantazym was monitored by the decrease in OD_{600}. Sensitivities of the deletion and complementation strains are shown relative to wild type sensitivity. Deletion of the *CWP1.1* gene results in enhanced β-1,3-glucanase sensitivity. The *⊿cwp1.1* knock out (HT*⊿cwp1.1*) was complemented using pCgACT14-CWP1.1. Reintegration of the *CWP1.1* gene reversed the observed phenotype of enhanced quantazym sensitivity.

3.3.5 Protein content of CWP1 mutant cell walls is unchanged

Deletion of the gene encoding for one of the most abundant proteins in the cell wall of *C. glabrata* we might induce changes in the cell wall composition, with special regard to protein contents. However, when analysing the cell walls of generated *CWP1* mutants we found no significant changes in the total amount of protein in relation to cell wall dry weight. The protein contents of all examined strains were around 6% of the cell wall dry weight (Figure 3-14), independent of *CWP1* deletion and growth conditions like heat (42°C) as well as cell wall stress (600µg/ml calcofluor white).

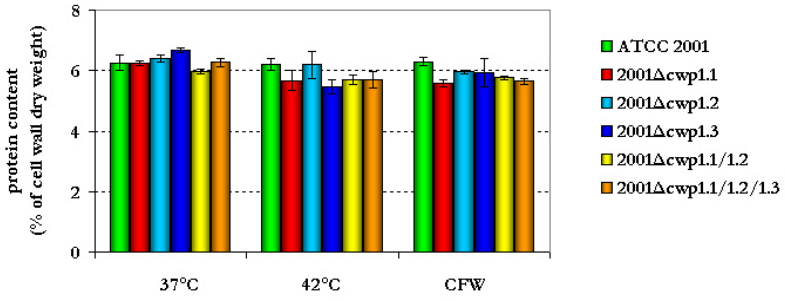

Figure 3-14 Protein content of *Candida glabrata* cell walls
Cell wall proteins were released from freeze dried cell wall material using β-1,6-glucanase and mild alkali. The protein content of *C. glabrata* cell walls is shown as percentage of cell wall dry weight. Deletion of *CWP1* genes did not significantly alter protein content of the fungal cell walls. Also an increase in growth temperature (42°C) or cell wall stress during growth (600 µg/ml calcofluor white) did not change the protein contents.

3.4 Growth inhibitory effect of CWP1 deletions

3.4.1 Δcwp1 mutants exhibit longer generation times at alkaline pH

We have shown before that *CWP1.1/1.2* knock out does not alter the growth rate of *C. glabrata* when grown in YNB and hypertonic YNB (0.7 M NaCl) at 30°C (KAPLANEK, 2004). Using these media, the wild type strain ATCC 2001, *⊿cwp1.1* single and *⊿cwp1.1/1.2* double knock out mutants showed comparable growth rates at any given time point of the measurement. They all reached stationary phase after approximately 23 hours at almost identical OD_{600} values.

However, when grown at alkaline pH (YPD pH 8.0), the *⊿cwp1.1/1.2* and *⊿cwp1.1/1.2/1.3* mutants showed slightly decreased growth rates. They reached stationary phase at approximately the same optical density (ATCC 2001: 1.70, *⊿cwp1.1/1.2*: 1.64 and *⊿cwp1.1/1.2/1.3*: 1.63) but the mutants reached stationary phase about one hour later than wild type. The lag phase was noticeably prolonged during growth in alkaline YPD and doubling times in the mid logarithmic growth phase prolonged as well: 140 minutes in YPD (all three strains) versus 171 minutes (ATCC 2001), 185 minutes (2001 *⊿cwp1.1/1.2*) and 207 minutes (2001 *⊿cwp1.1/1.2/1.3*) in YPD pH 8.0 (Figure 3-15).

We also monitored growth of the ATCC 2001 wild type and *⊿cwp1.1/1.2/1.3* mutant on YPD plates using time laps microscopy. We found no differences in the procedure of budding in respect to bud formation, bud growth and bud release from the mother cell (data not shown).

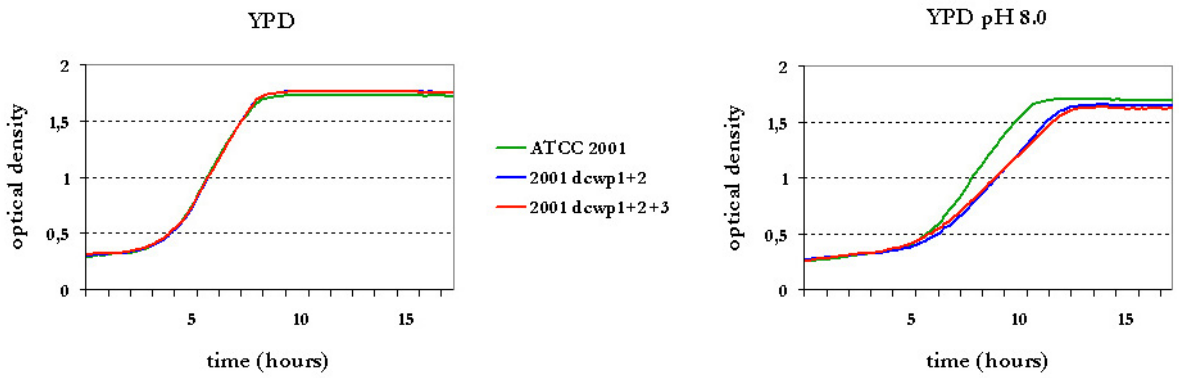

Figure 3-15 Growth rates of *Candida glabrata* wild type strain and *CWP1* mutants
C. glabrata wild type and *CWP1* deletion strains were inoculated in YPD and YPD pH 8.0 at an optical density of 0.2 and increase in OD_{600} was monitored over time. Growth rates of the three examined strains (ATCC 2001, 2001 *⊿cwp1.1/1.2* and 2001 *⊿cwp1.1/1.2/1.3*) were identical in YPD but not at alkaline conditions. The knock out strains displayed an extended lag phase as well as longer doubling times in YPD pH 8.0.

3.4.2 CWP1 deletion does not alter antifungal susceptibility

We tested the generated mutants in regard to their sensitivity to antimycotic drugs. *C. glabrata* strains often display reduced sensitivity or even resistance to Fluconazole, as shown for the ATCC 2001 wild type strain (Figure 3-16). The performed E-tests for Fluconazole, Amphotericin B, Flucytosin, Voriconazole and Caspofungin showed no differences in the susceptibility of wild type and mutants strains.

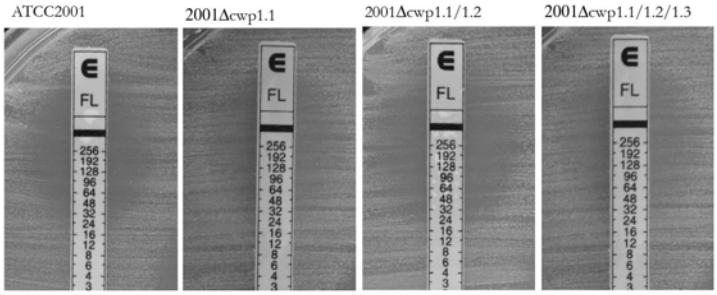

Figure 3-16 E-test for Fluconazole susceptibility
CWP1.1 deletions did not alter Fluconazole sensitivity. The four tested strains showed a comparable degree of Fluconazole resistance.

3.5 The role of CWP1 for pathogenicity

3.5.1 CWP1 deletion influences the ability to adhere to epithelial cells

When testing the adhesion properties of *C. glabrata* to different mammalian cell lines, we observed that *C. glabrata* wild type cells adhered better to HeLa and HEp2 cell lines than the examined *Δcwp1.1/1.2/1.3* deletion strain. All tested strains showed only a limited ability to adhere to Vero, EA926 and HaCaT cell lines. However, the adhesion capacity of the *Δcwp1.1/1.2/1.3* knock out mutant towards laryngeal (HEp2) and cervical (HeLa) carcinoma cells was significantly reduced compared to the corresponding wild type strain (Figure 3-17). Depending on the number of cells, the amount of adherent mutant cells was about 30% to 27% lower than the wild type in the case of HEp2 cells and about 40% to 32% lower in the case of HeLa cells.

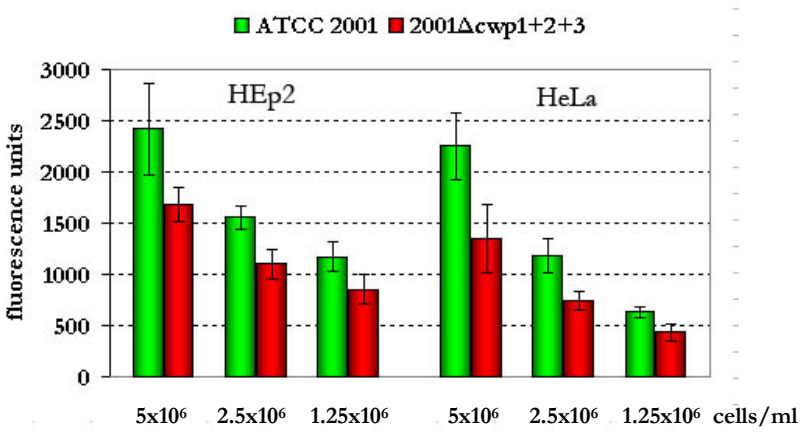

Figure 3-17 Adhesion of *Candida glabrata* to human epithelial cells
The ability of *C. glabrata* wild type and *⊿cwp1.1/1.2/1.3* mutant cells to adhere to human laryngeal (HEp2) and cervical (HeLa) carcinoma cells was tested in a cell culture assay. Three different cell dilutions were tested on a confluent layer of human epithelial cell. *C. glabrata* cells were stained with calcofluor white and fluorescence was detected at 460 nm. Adherence of mutant cells is significantly reduced compared to the wild type. Error bars represent standard deviations.

3.5.2 The ability to withstand killing by macrophages is unaltered in Δcwp1 deletion strains

The recognition of yeast cells by macrophages is mediated by the cell surface receptor dectin-1, which recognizes β-glucans in the yeast cell wall (BROWN *et al.*, 2002; TAYLOR *et al.*, 2002). Upon co-cultivation of *C. glabrata* and J774A.1 mouse macrophage cell line, yeast cells are taken up by the macrophages and are eventually lysed in the phagolysosome (Figure 3-18). As the previously performed quantazym assay demonstrated that *⊿cwp1.1* mutants are more β-1,3-glucanase sensitive than the wild type, we speculate that β-1,3-glucan might be more exposed in the mutants than in the wild type strain possibly leading to a better recognition by macrophages. We therefore analysed the exposure of β-1,3-glucan and rate of phagocytosis by macrophages using FACS analysis. Staining of *C. glabrata* cells with a β-1,3-glucan specific antibody and detection of the same with a FITC-labelled secondary antibody showed no differences in glucan exposure between wild type and mutant strains (Figure 3-19 A). The anti-glucan antibody was applied after co-cultivation with macrophages, therefore staining only the extracellular, non phagocytosed yeast cells. This allowed to compare the amount of non phagocytosed wild type and mutant fungal cells and thus the extend of phagocytosis. Staining with propidium-iodid (PI) allowed the discrimination between living and dead cells, as healthy cells stay impermeable for the intercalating dye. Thus, the analysis allowed to distinguish between four different cell populations: phagocytosed/dead (stained with PI),

phagocytosed/living (not stained), non phagocytosed/dead (stained with PI and FITC-labelled antibody) and non phagocytosed/living (stained only with FITC-labelled antibody).

Wild type and mutant cells were stained with α-glucan antibody to the same extend. Also the survival of fungal cells (propidium-idodid staining) was identical for wild type and knock out strains (Figure 3-19 B). The distribution of fungal cells among the four cell populations described above were comparable for wild type and knock out strain (Figure 3-19 C).

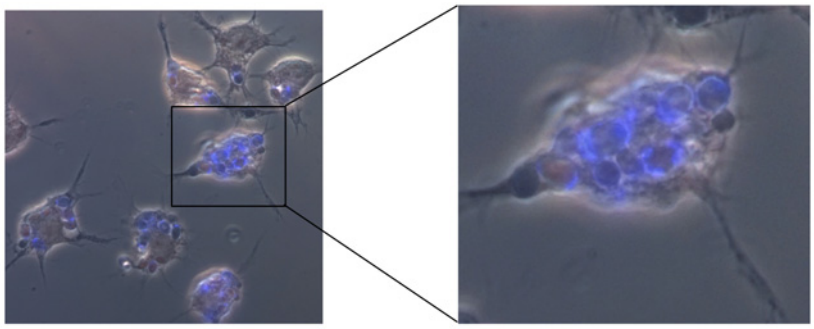

Figure 3-18 *Candida glabrata* cells are taken up by macrophages via phagocytosis
C. glabrata cells (stained with calcofluor white) were co-incubated with macrophages. After rapid uptake by phagocytosis they will later be killed by lysis in the phagolysosomes.

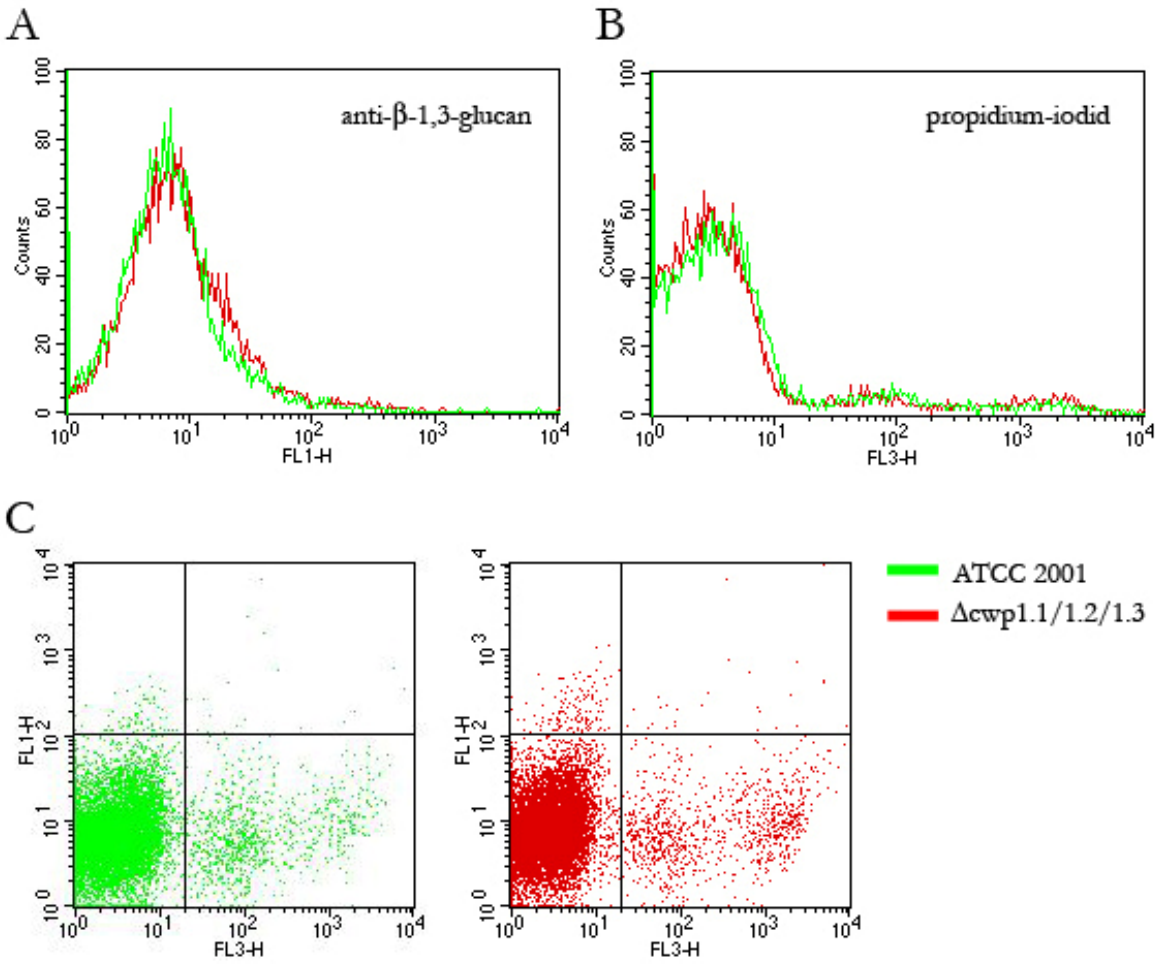

Figure 3-19 The exposure of β-1,3-glucan and killing by macrophages is unaltered in *Δcwp1.1/1.2/1.3* mutant strains

C. glabrata cells were co-incubated with J774A.1 mouse macrophages and stained with β-1,3-glucan directed antibodies and a FITC-labelled secondary antibody for detection by FACS analysis. Macrophages were lysed with Triton-X and dead fungal cells were stained with propidium-iodid. **(A)** The exposure of β-glucan is identical in wild type and *Δcwp1.1/1.2/1.3* deletion strains. **(B)** The number of detected dead cells was identical for both examined strains. **(C)** The cell populations of living/phagocytosed and dead/phagocytosed cells were comparable in the wild type and *Δcwp1.1/1.2/1.3* deletion strain.

3.6 Proteomic changes in Candida glabrata caused by the ambient pH

3.6.1 Spot detection and matching

In order to characterise the global proteomic response of *C. glabrata* to changes in ambient pH, cells were grown to mid-exponential phase in Pan Fungal Medium (PFM) at pH 7.4, a growth medium that has been developed by the Haynes laboratory (London, UK) for the standardisation of experiments involving the analysis of pH responses in bakers yeast and filamentous fungi (personal communication). Cells were harvested and transferred to fresh PFM at pH 4.0, 7.4 or 8.0. Total soluble protein extracts were prepared and subjected to 2D-gel electrophoresis to examine proteins with pIs between 4.0 and 7.0. Four independent experiments were performed resulting in quadruplicate gels for each pH condition.

After scanning of the coomassie stained gels, the images were processed using the Phoretix 2D software for spot analysis. Automatic spot detection identified about 1500 spots per gel. Detected artefacts were removed and spots were edited manually (e.g. when two very close spots were identified as a single spot by the software), resulting in approximately 800-900 spots per gel, which were used for matching (Figure 3-20). For each condition (pH 4.0, pH 7.4 and pH 8.0) the gel containing the most spots of the four replicates was chosen as the average gel. We first matched the three other replicates to the average gel and later the average gels to the reference gel, which was one of the pH 4.0 condition gels, as it had the highest number of detected spots.

Before the automatic matching of spots, around 40 to 50 representative "seed spots" were set across the gel, which served the software as anchor and orientation points. Afterwards, the automatic spot matching was refined manually. We then checked the correct matching of all "slave spots" (spots on the replicate gels) to the corresponding "reference spots" (spots on the average or reference gel) by analysing the "match vectors" (Figure 3-21). The vector lines always show a certain directive in their orientation as the replicate gels are not identical in their protein separation but are usually shifted or warped into one direction. Thus, the size and direction of the match vectors can be used to find mismatched spots. After this final step of correction, we analysed the spot volume data sets to find proteins that are pH regulated. We selected spots with statistically significant ($p \leq 0.05$) changes of more than two-fold, relative to the expression levels at pH 4.0.

To test the reproducibility of the replicate experiments, the spot patterns on all of the gel images were subjected to Principle Components Analysis using the Progenesis software (Figure 3-22). The four independent replicates for each experimental condition clustered

closely together, with the pH 7.4 gels lying between the pH 4.0 and pH 8.0 replicates. Furthermore, the *C. glabrata* gels from this experiment clustered separately from *C. albicans* and *S. cerevisiae* 2D-gels (Yin *et al.*, 2004). This analysis demonstrated that the *C. glabrata* gels of the four independent pH experiments were highly reproducible.

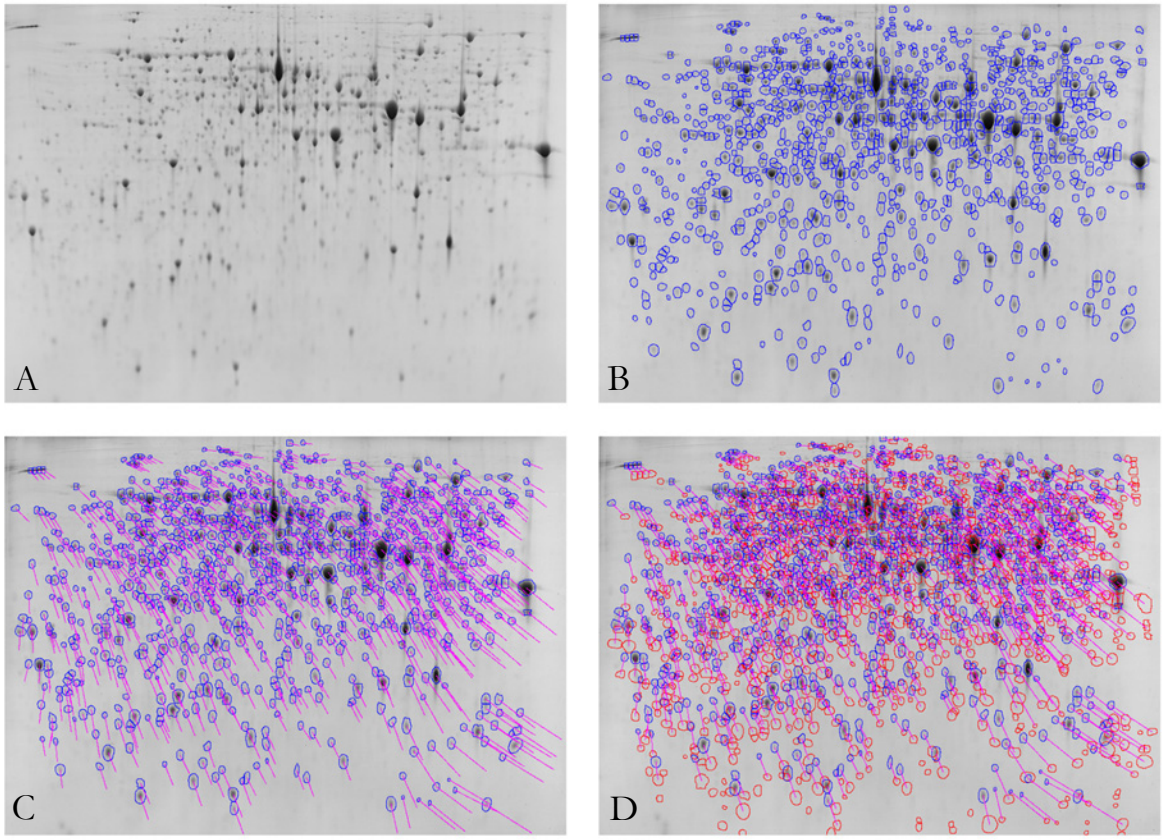

Figure 3-20 Working steps of gel analysis
An example for the spot analysis: **(A)** The scanned picture of a pH 4.0 gel was imported into Phoretix 2D software. Picture **(B)** shows the slave spots (blue), which were used for the spot analysis. **(C)** The slave spots and their match vectors (pink) are shown. Picture **(D)** shows the slave spots, their match vectors and the corresponding reference spots (red) of the reference gel.

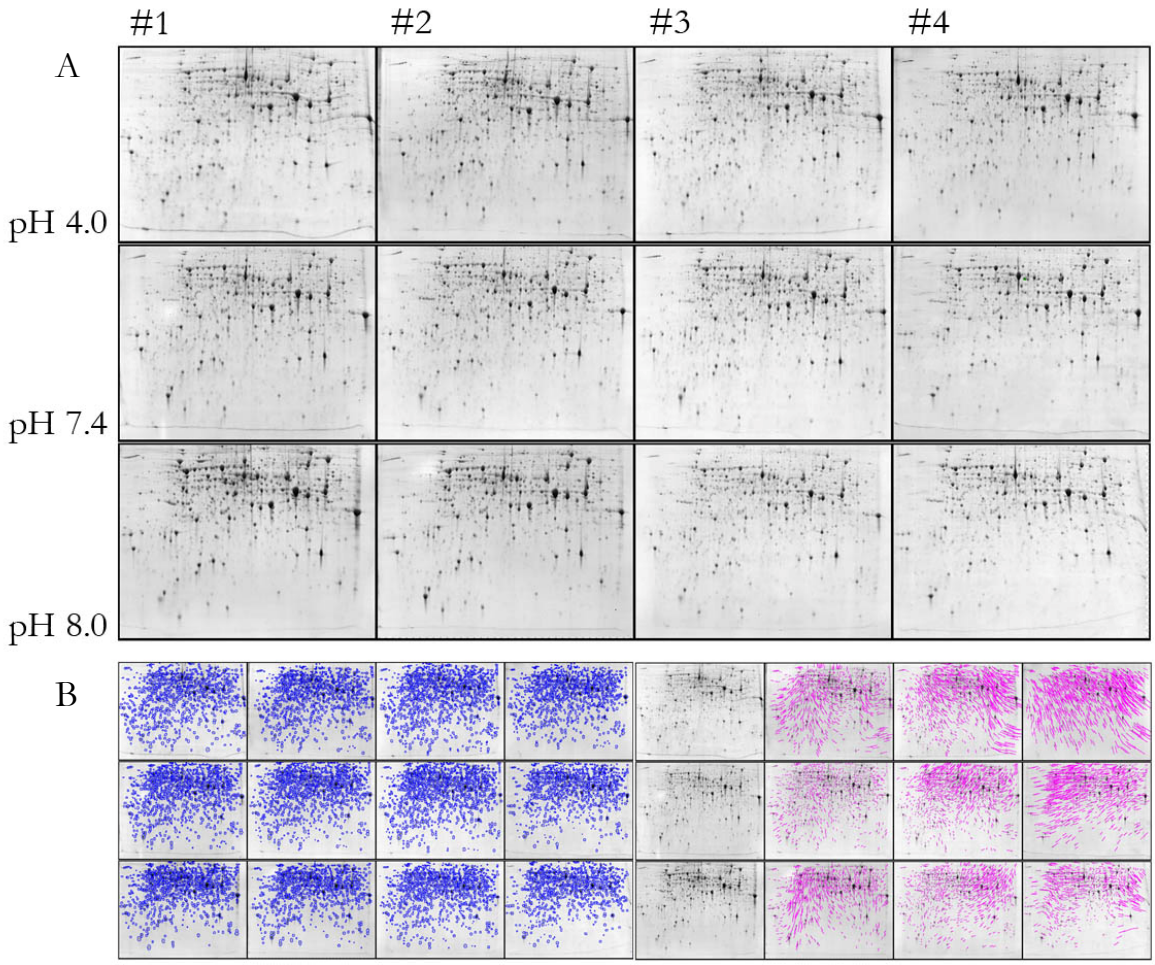

Figure 3-21 Analysed 2D gels
(A) For proteome analysis four replicate gels for each pH condition were prepared and used for spot analysis. **(B)** After spot filtering, about 800 to 900 spots per gel were analysed (slave spots are depicted in blue in the left panel). The same directive and length of the match vectors (pink, right panel) of neighbouring spots indicate identical protein spots. These vectors were used after the first spot matching to correct for mismatched spots.

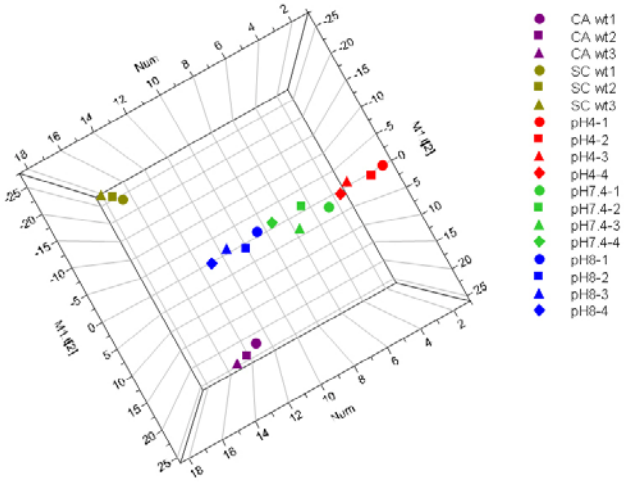

Figure 3-22
Principle Components Analysis of the 2D gels
The Principle Components Analysis of independent quadruplicate *C. glabrata* 2D-gels at pH 4.0 (red symbols), pH 7.4 (green symbols) and pH 8.0 (blue symbols) cluster together with the pH 7.4 gels lying between the pH 4.0 and pH 8.0 gels. The replicate 2D gels of *S. cerevisiae* (brown symbols) and *C. albicans* (purple symbols), taken from a different experiment (YIN *et al.*2004) and used for comparison, cluster in different areas of the coordination system.

3.6.2 Spot volume analysis

After matching of all spots, the normalized spot volumes of quadruplicate gel images for each pH condition were analysed quantitatively using Phoretix 2D™ software. We selected statistically significant ($p \leq 0.05$) changes of more than two-fold, relative to the expression levels at pH 4.0 as proteins which are pH regulated. Two examples of proteins that are pH-regulated in *C. glabrata* are illustrated in Figure 3-23. Tdh3, a glyceraldehyde-3-phosphate dehydrogenase, was reproducibly found to be down-regulated at pH 7.4 and pH 8.0 relative to pH 4.0. In contrast, the vacuolar endopeptidase Pep4 was up-regulated at both pH 7.4 and 8.0.

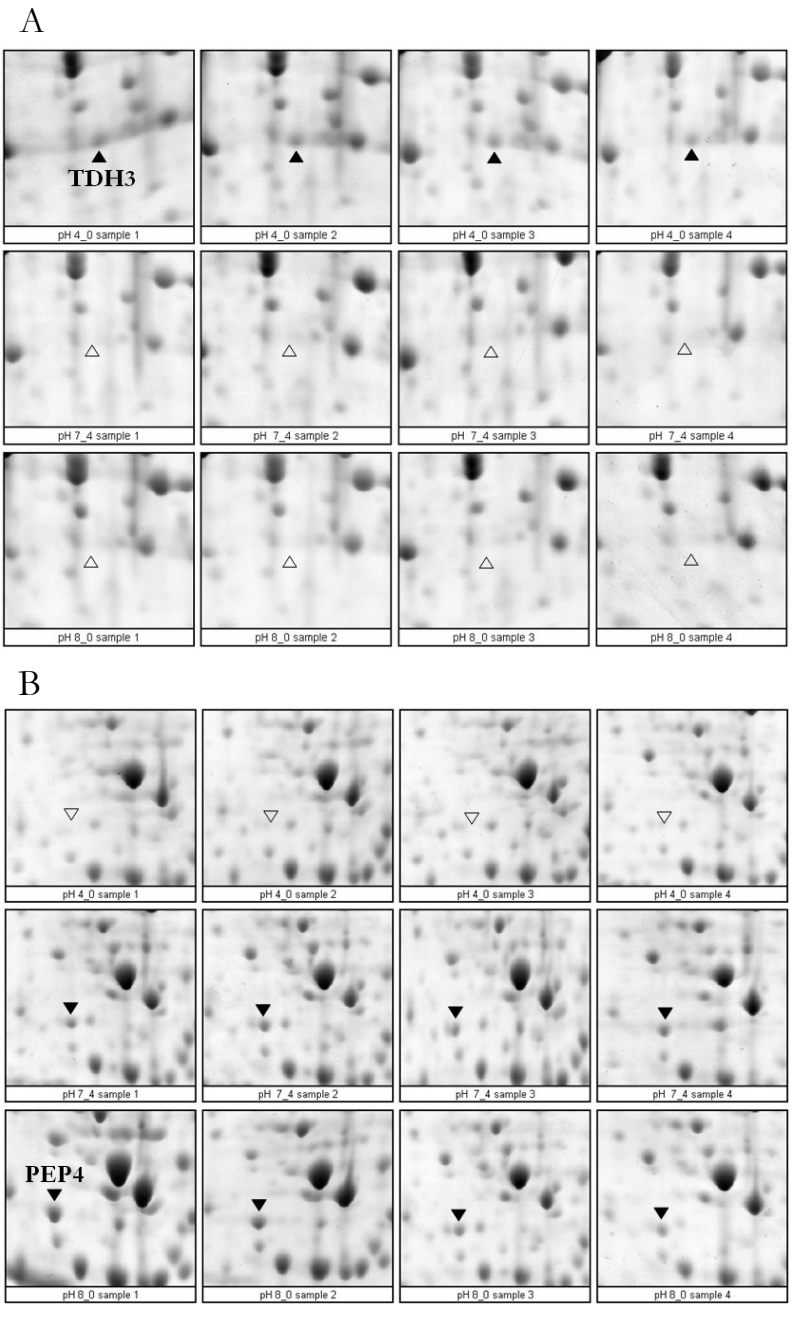

Figure 3-23 pH dependent protein expression
Expression of the glyceraldehyde-3-phosphate dehydrogenase Tdh3 and the vacuolar endopeptidase Pep4 was reproducibly identified up- or downregulated on the quadruplicate gels. Tdh3 was more than 100 fold downregulated at basic pH whereas Pep4 showed the inverse expression pattern and was upregulated more than 60 fold at pH 7.4 and 8.0 as compared to pH 4.0. Arrows indicate the positions of the Tdh3 and Pep4 spots in **(A)** and **(B)**, respectively.

After thorough spot analysis, we selected 174 spots of interest, which where regulated in a pH dependent manner. Expression levels of theses spots were represented graphically using the GeneSpring software (Figure 3-24). The spots of interest were cut from the gels and containing proteins were digested with trypsin. After extraction and purification of the tryptic peptides, they were eluted and crystallized on a "mass spec plate". Peptide mass fingerprints of the peptides were obtained by MALDI-TOF analysis and proteins were identified by database search using MASCOT (www.matrixscience.com).

Of the 174 spots that were picked for analysis, identifications were obtained for 154 (88.5%) by peptide mass fingerprinting. Multiple hits were obtained for a number of proteins, yielding a total of 87 distinct full-length proteins (red lines in Figure 3-24).

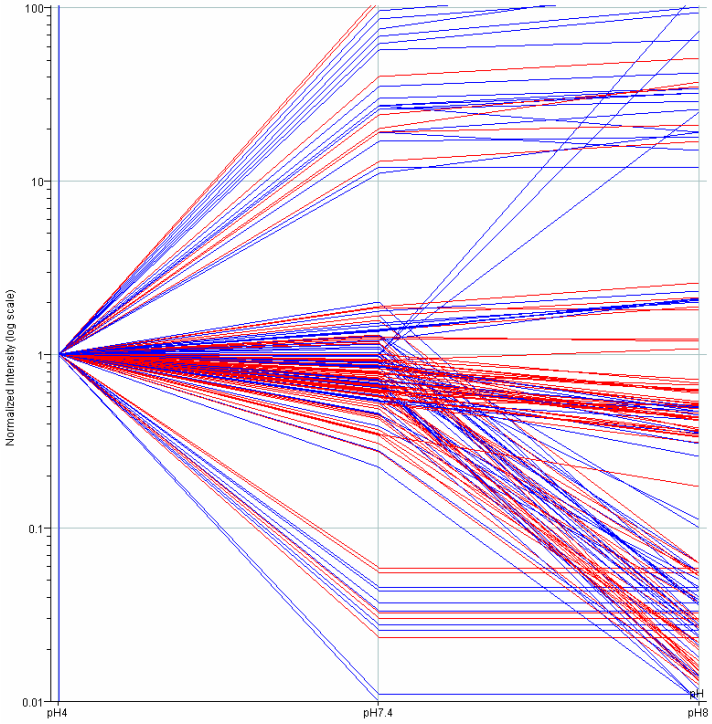

Figure 3-24 Cluster graph of all picked and identified spots
We picked 174 spots which appeared to be pH regulated (red and blue lines in this graph). 154 of these proteins were identified by peptide mass fingerprinting. The analysis included a number of "multiple hits" yielding 87 distinct protein IDs (red colour) at the end. Expression levels of the depicted proteins are shown as multiples of the expression level at pH 4.0.

3.6.3 pH regulated proteins in Candida glabrata

3.6.3.1 pH-regulated clusters in Candida glabrata

The pH-regulated proteins are grouped in four main clusters. Cluster 1 contained thirteen proteins that are expressed at significantly lower levels at pH 7.4 and 8.0 compared to pH 4.0 (Table 3, Figure 3-25 A). Five of these proteins (ACO1, TDH3, KRS1, MDH1, PDC1) are

involved in organic acid metabolism, mainly carbon metabolism (glycolysis or gluconeogenosis and TCA cycle). Also, RIP1, an ubiquinol-cytochrome-c reductase, which transfers electrons from ubiquinol to cytochrome c during respiration, was downregulated at pH 7.4 and 8.0. Additionally we identified proteins involved in protein folding (SSE1, HSP82) and protein complex assembly (HSP82, COX12) being expressed at lower levels at the given condition.

Cluster 2 contained twenty-nine proteins that were expressed at significantly lower levels at pH 8.0 compared to pH 4.0 (Table 4, Figure 3-25 B). To assure that this cluster contains only proteins that are specifically downregulated at pH 8.0 and not at pH 7.4, the proteins' expressions levels at pH 7.4 had to be above a 0.6667 threshold when compared to pH 4.0. A number of proteins identified in this cluster play important roles in energy derivation. Especially enzymes involved in glucose catabolism (TKL1, FBA1, CDC19, PGI1) are represented in this group as well as proteins that take part in the TCA-cycle (LSC1) or fermentation processes (ADH3, DLD1, PDC1). Moreover, the cell redox homeostasis seems to be profoundly constricted by the downregulation of key enzymes and regulators (TRR1, AHP1, TSA1). The microtubular cytoskeleton and thus chromosomal segregation (NUF2) as well as cell polarization and endocytosis (ACT1) is also influenced by the downregulation of participating proteins.

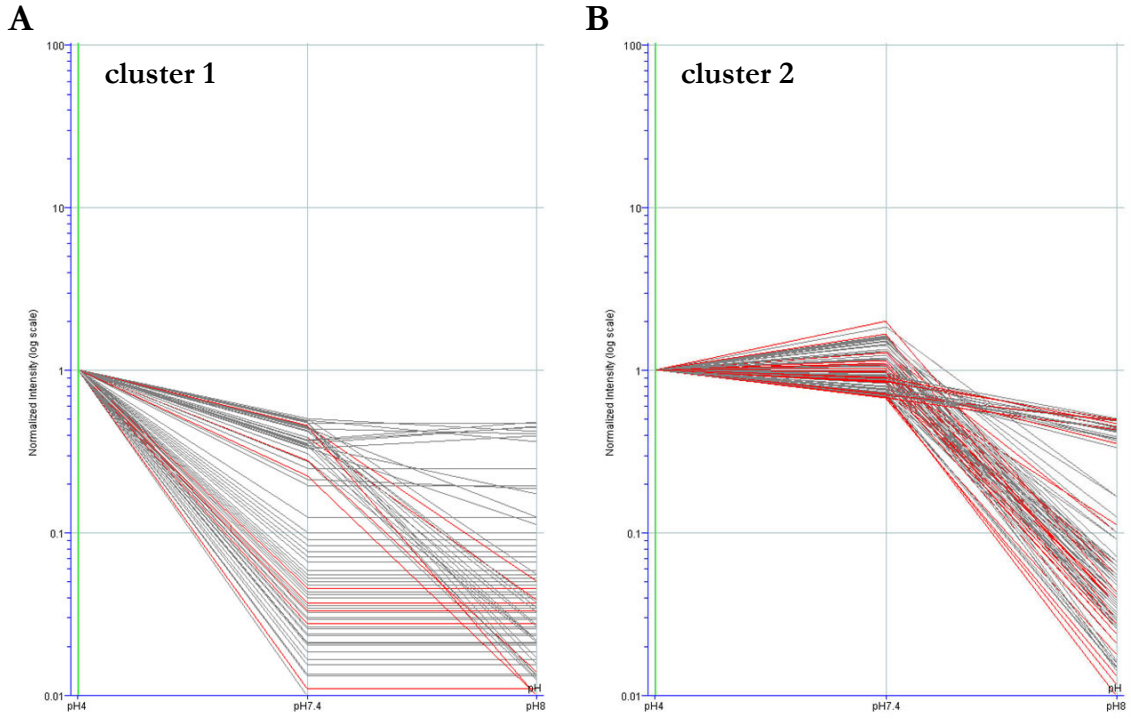

Figure 3-25 Cluster graphs of proteins downregulated at pH 7.4 and/or 8.0
(A) Protein spots, which were found to be downregulated at pH 7.4 and 8.0, are grouped in cluster 1. The spot volume intensities at pH 7.4 and 8 are shown as multiples of pH 4 intensity. The cluster contained a number of unmatched spots and also "multiple hits" when analysed by peptide mass finger printing (grey lines). Eventually, 13 single protein IDs (red lines) were obtained from this group of pH regulated proteins. **(B)** Cluster 2 shows the expression levels of proteins specifically downregulated at pH 8.0. We identified 29 of the shown proteins (red lines). Unmatched spots or "multiple hits" are depicted in grey.

Table 3 - pH regulated proteins in cluster 1
Proteins, which were downregulated at pH 7.4 and 8.0 were grouped in cluster 1.

Protein Name		Fold-Regulation		Function
Biological	Formal	pH 7.4	pH 8.0	
ACO1	CAGL-CDS0863.1	-35,97	-35,97	aconitate hydratase
COX12	CAG57814**	-3,60	-71,94	cytochrome-c oxidase
GET3	CAGL-CDS3157.1	-90,91	-90,91	homolog of YDL100c (ATPase)
HSC82	CAGL-CDS1077.1	-27,03	-27,03	heat shock protein
KRS1	CAGL-CDS1555.1	-22,99	-22,99	lysyl-tRNA synthetase
MDH1	CAGL-CDS3282.1	-39,06	-39,06	malate dehydrogenase
NFU1	CAGL-CDS3894.1	-21,98	-21,98	iron homeostasis
PDC1	CAGL-CDS1705.1	-4,50	-100,00	pyruvate decarboxylase
RIP1	CAGL-CDS4323.1	-2,19	-100,00	Ubiquinol-cytochrome C reductase iron-sulfur protein
SSE1	CAGL-CDS1116.1	-2,60	-25,97	heat shock protein of HSP70 family
TDH3	CAGL-CDS3304.1	-100,00	-100,00	glyceraldehyde-3-phosphate dehydrogenase
TPD3	CAGL-CDS1444.1	-30,03	-30,03	ser/thr protein phosphatase 2A, regulatory chain A
YMR315W		-2,22	-20,00	hypothetical protein

Table 4 - pH regulated proteins in cluster 2
Proteins, which were downregulated at pH 8.0 were grouped in cluster 2.

Protein Name		Fold-Regulation		Function
Biological	Formal	pH 7.4	pH 8.0	
ACT1	CAGL-CDS2867.1	-1,44	-84,75	actin
ADH1	CAGL-CDS3139.1	-1,18	-2,02	alcohol dehydrogenase I
AHP1	CAGL-CDS4636.1	-1,49	-2,32	AHP1 alkyl hydroperoxide reductase
ARG1	CAGL-CDS2634.1	-1,49	-2,32	argininosuccinate synthetase
ATP2	CAGL-CDS2034.1	-0,60	-34,01	F1F0-ATPase complex, F1 beta subunit
CDC19	CAGL-CDS2071.1	-1,42	-2,04	pyruvate kinase
DLD1	CAGL-CDS1640.1	-1,49	-75,76	D-lactate ferricytochrome C oxidoreductase
EFT2	CAGL-CDS0703.1	-1,25	-25,00	Elongation factor 2
FBA1	CAGL-CDS3065.1	-1,25	-2,84	fructose-bisphosphate aldolase
GLK1	CAGL-CDS2099.1	-0,78	-17,99	aldohexose specific glucokinase
HIS4	CAGL-CDS0806.1	-1,12	-9,00	phosphoribosyl-AMP cyclohydrolase/phosphoribosyl-ATP pyrophosphatase/histidinol dehydrogenase
HSP60	CAGL-CDS1672.1	-1,38	-17,99	heat shock protein 60, mitochondrial precursor
IPP1	CAGL-CDS3706.1	-1,47	-55,87	inorganic pyrophosphatase, cytoplasmic
LSC2	CAGL-CDS2643.1	-0,78	-28,01	succinate-CoA ligase beta subunit
MUK1	CAGL-CDS1670.1	-0,93	-27,03	heat shock protein
NAB1A	CAGL-CDS4484.1	-0,90	-27,03	ribosomal protein p40
NUF2	CAGL-CDS2318.1	-1,00	-16,00	NUF2 spindle pole body protein
PDC	CAGL-CDS1705.1	-1,11	-2,13	pyruvate decarboxylase
PDR13	CAGL-CDS1860.1	-1,08	-42,02	regulator protein involved in pleiotropic drug resistance
PGI1	CAGL-CDS1740.1	-0,87	-28,01	glucose-6-phosphate isomerase
PST2	CAGL-CDS4456.1	-1,11	-42,02	unknown function
SAM2	CAGL-CDS2881.1	-1,20	-48,08	S-adenosylmethionine synthetase
SCP160	CAGL-CDS3486.1	-0,77	-10,00	required for maintenance of exact ploidy
SSB2	CAGL-CDS1430.1	-1,40	-100,00	heat shock protein of HSP70 family, cytosolic
TEF1	CAGL-CDS2328.1	-1,03	-35,97	translation elongation factor eEF1 alpha-A chain
TIF1	CAGL-CDS2789.1	-1,17	-2,26	translation initiation factor
TKL1	CAGL-CDS1156.1	-0,50	-22,99	transketolase
TRR1	CAGL-CDS3413.1	-0,97	-35,97	thioredoxin reductase (NADPH)
TSA1	CAGL-CDS4485.1	-1,03	-39,06	thioredoxin peroxidase

Cluster 3 contained twenty proteins that were expressed at significantly higher levels at pH 7.4 and 8.0 compared to pH 4.0 (Table 5, Figure 3-26 A). In this cluster, we observed a significantly enhanced expression of proteins known to be involved in stress responses (BCY1, LSP1, PIL1, RFA1, PEP4). We also observed a strong induction of the two subunits of the phosphofructo-kinase 1 and 2 (PFK1, PFK2), which are indispensable for glycolysis.

Cluster 4 contained ten proteins that were expressed at significantly higher levels at pH 8.0 compared to pH 4.0 (Table 6, Figure 3-26 B). To assure that this cluster contains only proteins that are specifically upregulated at pH 8.0, expressions levels at pH 7.4 had to be below a 1.5 fold threshold when compared to pH 4.0. Strikingly, most proteins identified in this cluster are involved in the closely related processes of cytoskeleton organization (AIP1, PPH21) and intracellular transport (SEC28, VMA2, SSA3). Other proteins, which were expressed at higher levels, are involved in protein catabolism (LAP4, RPT3).

In addition, we identified fifteen *C. glabrata* proteins that did not belong to one of the clusters discussed above but nevertheless displayed significant pH regulation (Table 7). One of these proteins is catalase A (CTA1), which showed a 0,6579 fold expression level at pH 7.4 and a 0,0263 fold expression at pH 8.0. Thus, it is 38 fold downregulated at pH 8.0 compared to pH 4.0, but was not picked up in cluster 2 because it was below the critical threshold of 0.667 at pH 7.4. The clustering also did not include proteins, which were slightly upregulated at pH 7.4 (e.g. YEF3, 1,5625 fold up at pH 7.4) but significantly downregulated at pH 8.0 (YEF3 was 16 fold downregulated at pH 8.0). These 15 proteins did not show a significant enrichment in certain functional categories.

Among the proteins, which were downregulated at pH 8.0, we identified proteins involved in protein (CPR1, ILV5), lipid (CTA1, INO1) and glucose metabolism (ENO1, PGK1, RHR2, TPI1) as well as translational regulators (YEF3, GCN3).

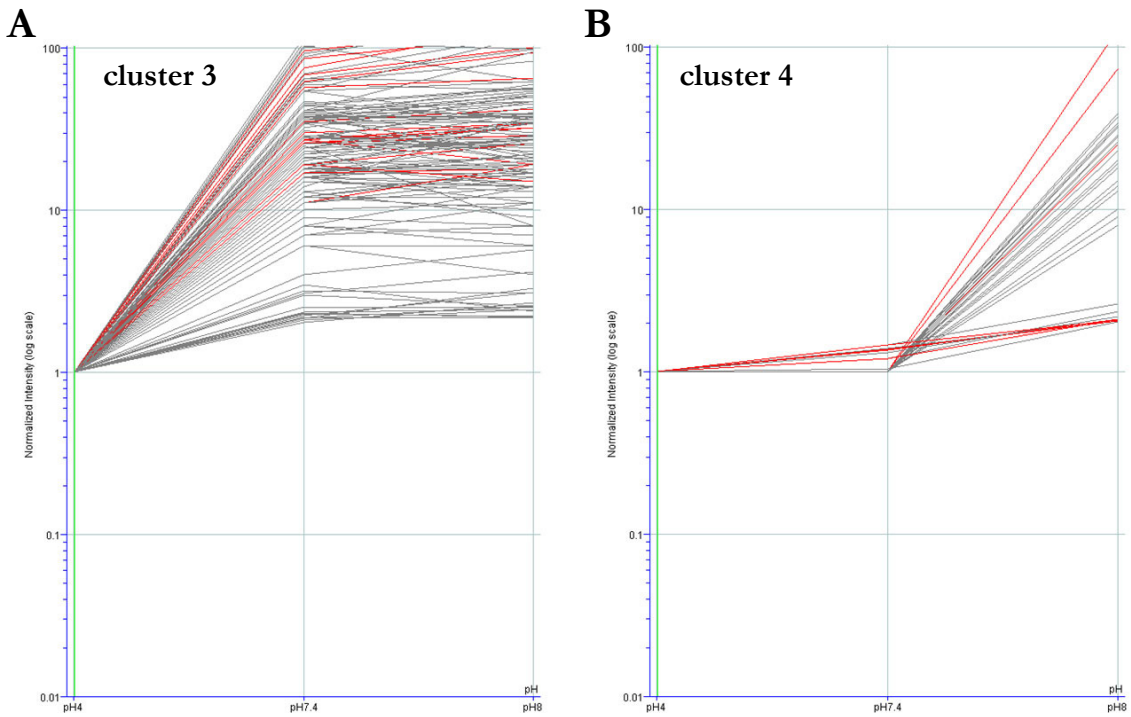

Figure 3-26 Cluster graphs of proteins upregulated at pH 7.4 and/or 8.0
(A) Proteins in cluster 3 are upregulated at pH 7.4 and 8.0. Of this cluster, which included a number of "multiple hits" and unmatched spots (grey lines), we obtained 20 single protein IDs (red lines). **(B)** Cluster 4 shows the expression levels of proteins specifically upregulated at pH 8.0. We obtained 10 single protein IDs (red lines) from these proteins. The cluster contained a number of unmatched spots and "multiple hits" (grey lines).

Table 5 - pH regulated proteins in cluster 3
Proteins, which were upregulated at pH 7.4 and 8.0 were grouped in cluster 3.

Protein Name		Fold-Regulation		Function
Biological	Formal	pH 7.4	pH 8.0	
ANC1	CAGL-CDS4480.1	19,00	15,00	transcription initiation factor
CDC48	CAGL-CDS0734.1	17,00	18,00	microsomal protein of CDC48/PAS1/SEC18 family of ATPases
DOS1	CAGL-CDS3022.1	27,00	19,00	involved in genome stability
FMP29	CAGL-CDS1287.1	27,00	32,00	homolog of YER080w
LSP1	CAGL-CDS3497.1	86,00	119,00	long chain base-responsive inhibitor of protein kinases Phk1p and Phk2p, acts along with the Pil1p
MET6	CAGL-CDS0893.1	12,00	12,00	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase
NAS6	CAGL-CDS4155.1	11,00	19,00	regulatory, non-ATPase subunit of the 26S proteasome
PDI1	CAGL-CDS1942.1	62,00	94,00	protein disulfide-isomerase
PEP4	CAGL-CDS2671.1	68,00	101,00	aspartyl protease Saccharopepsin precursor (EC 3.4.23.25)
PFK1	CAGL-CDS0454.1	26,00	32,00	6-phosphofructokinase, alpha subunit
PFK2	CAGL-CDS0484.1	19,00	26,00	6-phosphofructokinase, beta subunit
PIL1	CAGL-CDS3503.1	96,00	133,00	long chain base-responsive inhibitor of protein kinases Phk1p and Phk2p, acts along with Lsp1p
PRT1	CAGL-CDS1029.1	26,00	29,00	translation initiation factor eIF3 subunit
RFA1	CAGL-CDS1363.1	27,00	32,00	DNA replication factor A, 69 KD subunit
SRA1	CAGL-CDS2738.1	57,00	65,00	cAMP dependent protein kinase, regulatory subunit
SUN1	CAGL-CDS3844.1	27,00	34,00	26S proteasome regulatory subunit
TAL1	CAGL-CDS3297.1	30,00	34,00	transaldolase
TIF5	CAGL-CDS2734.1	75,00	138,00	translation initiation factor eIF5
YLL029W	CAGL-CDS2738.1	12,00	12,00	homolog of YLL029w
YNL134C		35,00	42,00	possible alcohol dehydrogenase

Table 6 - pH regulated proteins in cluster 4
Proteins, which were upregulated at pH 8.0 were grouped in cluster 4.

Protein Name		Fold-Regulation		Function
Biological	Formal	pH 7.4	pH 8.0	
AIP1	CAGL-CDS1453.1	1,00	73,00	actin cytoskeleton component
APE1	CAGL-CDS2014.1	1,00	73,00	Vacuolar aminopeptidase I
PPH21	CAGL-CDS3003.1	1,39	2,06	protein ser/thr phosphatase
SEC28	CAGL-CDS3645.1	1,00	25,00	epsilon-COP coatomer subunit
SOL3	CAGL-CDS4048.1	1,00	127,00	Probable 6-phosphogluconolactonase
SSA3	CAGL-CDS1280.1	1,45	2,07	heat shock protein of HSP70 family
VMA2	CAGL-CDS2020.1	1,36	2,09	H+-ATPase V1 domain 60 KD subunit
YER175C	CAGL-CDS3673.1	1,20	2,10	hypothetical protein
YJL123C		1,36	2,09	hypothetical protein
YTA2	CAGL-CDS2557.1	1,37	2,05	26S proteasome regulatory subunit

Table 7 - Other pH regulated *Candida glabrata* proteins

Protein Name		Fold-Regulation		Function
Biological	Formal	pH 7.4	pH 8.0	
ALD5	CAGL-CDS2056.1	1,51	2,01	aldehyde dehydrogenase
ATP1	CAGL-CDS1821.1	1,78	2,31	F1F0-ATPase complex, F1 alpha subunit
COX6	CAGL-CDS4818.1	-1,85	-2,39	cytochrome-c oxidase subunit VI
CPR1	CAGL-CDS4714.1	-1,52	-38,00	cyclophilin (peptidylprolyl isomerase)
CTA1	CAGL-CDS2032.1	-1,54	-2,15	catalase A
ENO1	CAGL-CDS2501.1	-1,57	-3,28	enolase
GCN3	CAGL-CDS3563.1	-1,72	-3,86	translation initiation factor eIF2B
GIN3	CAGL-CDS1983.1	-1,59	-43,00	Hypothetical protein
ILV5	CAGL-CDS2775.1	-1,83	-22,00	ketol-acid reducto-isomerase
INO1	CAGL-CDS1849.1	-1,86	-2,86	myo-inositol-1-phosphate synthase
PGK1	CAGL-CDS2665.1	-1,92	-71,00	phosphoglycerate kinase
RHR2	CAGL-CDS4010.1	1,29	-66,00	DL-glycerol phosphatase
SSA2	CAGL-CDS1311.1	-1,54	-2,15	heat shock protein of HSP70 family
TPI1	CAGL-CDS4014.1	1,56	-16,00	triose-phosphate isomerase
YEF3	CAGL-CDS0384.1	1,37	2,05	translation elongation factor eEF3

3.6.3.2 Specific functional categories are enriched in pH-regulated expression clusters

We observed a distinct influence of external pH conditions on Glucose catabolism. This functional group was enriched in clusters 2 and 3 (Table 8), where we identified GLK1, FBA1, PGI1, CDC19, LSC2 and TKL1 to be downregulated at pH 8.0 (probability $5.5x10^{-6}$), whereas PFK1 and 2 as well as TAL1 are expressed at much higher levels at pH 7.4 and 8.0 compared to pH 4.0 (probability $6.8x10^{-5}$).

Some of these genes are also included in the group of organic acid metabolism, which shows enrichment in pH regulated clusters 1 and 2. All representatives of this functional group identified in our screen, ACO1, TDH3, KRS1, MDH1, PDC1, (probability $1.1x10^{-4}$) and ARG1, SAM2, FBA1, HIS4, CDC19, PGI1, PDC1 (probability $1.4x10^{-4}$) are expressed at lower levels at pH 7.4 and/or pH 8.0. The same holds true for amine metabolism (ACO1, KRS1, HIS4, ARG1, SAM2) and cellular respiration (ACO1, RIP1, MDH1, DLD1, LSC2) which are also downregulated at basic pH.

Table 8 - Enrichment of functional categories in differentially regulated protein clusters

GO ID	GO term	cluster 4 ▼7A &8D	cluster 5 ▼8D	cluster 6 ▲7A &8D	cluster 7 ▲8D
226	microtubule cytoskeleton organization and biogenesis		3,7E-02		
6007	glucose catabolism		5,5E-06	6,8E-05	
6082	organic acid metabolism	1,1E-04	1,4E-04		
6098	pentose-phosphate shunt		6,0E-04		
6412	protein biosynthesis		8,8E-02		
6413	translational initiation			7,6E-03	
6457	protein folding	6,7E-03			
6461	protein complex assembly	2,9E-02			
6796	phosphate metabolism	4,5E-02			
6950	response to stress			4,1E-03	
7059	chromosome segregation		6,5E-02		
7569	cell aging		1,2E-02		
9308	amine metabolism	5,7E-02	5,5E-02		
9408	response to heat			1,4E-05	
15980	energy derivation by oxidation of organic compounds		7,6E-07		
30036	actin cytoskeleton organization and biogenesis				8,6E-03
30163	protein catabolism			7,5E-03	1,7E-02
45333	cellular respiration	5,0E-04	5,1E-02		
45454	cell redox homeostasis		9,2E-06		
46907	intracellular transport				2,6E-02

4 DISCUSSION

4.1 Cwp1p-family mediates cell wall integrity in Candida glabrata

The cell wall is the outermost protective layer of fungi and has been shown to mediate various important functions. It is responsible for maintaining cell shape as well as limiting permeability. Cell wall proteins determine surface properties like adhesion, hydrophobicity or the ability to remodel the cell wall architecture. Thus, they appear to be crucial for many aspects that contribute to the biological fitness of fungi. *S. cerevisiae* cell wall functions are very well examined and also the cell wall of *C. albicans* is more and more being understood. However, a comprehensive analysis of *C. glabrata* cell wall proteins is still missing. The modest number of cell wall related proteins, which have been characterized so far are the adhesins Epa1-5 (CORMACK *et al.*, 1999; DE LAS PENAS *et al.*, 2003; FRIEMAN *et al.*, 2002), the Gas/Phr orthologues Gas1 and Gas2 (WEIG *et al.*, 2001), which are putative 1,3-β-glucanosyl transferases and thus important for cell wall biosynthesis and a family of yapsins (KAUR *et al.*, 2007), extracellular aspartyl proteases, which play an important role in cell wall remodelling by processing GPI-anchored cell wall proteins (e.g. Epa1). We therefore aimed at characterizing the *C. glabrata* cell wall architecture and examined the function of the highly abundant cell wall protein Cwp1.1p as well as its homologues Cwp1.2p and Cwp1.3p.

4.1.1 Towards a cell wall architectural model for Candida glabrata

We investigated the molecular organization of the *C. glabrata* cell wall to elucidate whether it conforms to the current model of cell wall architecture built on data obtained from *S. cerevisiae* and *C. albicans* experiments. We analysed the incorporation of mannoproteins into the cell wall of *C. glabrata* and were able to release GPI-anchored proteins using β-1,6-glucanase, demonstrating that these proteins are connected to the β-1,6-glucan moiety of the cell wall (Figure 3-1). The most abundant protein released by β-1,6-glucanase was identified as Cwp1.1p. Furthermore, we found that this mannoprotein can be linked to the cell wall via two different covalent linkages, a GPI-anchor and a Pir protein like mild alkali sensitive linkage (Weig, unpublished data). This demonstrates that proteins with sequence prerequisites for both covalent attachments can indeed use the two possibilities of anchorage at the same time.

We also analysed protein and chitin contents of the *C. glabrata* cell wall. Protein content was found to be around 6% of the cell wall dry weight. Thus, pure protein contents are up to 50% higher than in *S. cerevisiae* (4-5%, KLIS *et al.*, 2006)) and *C. albicans* (4% DE GROOT *et al.*,

manuscript in preparation) cell walls and indicate that mannoproteins might play an important role in the *C. glabrata* cell wall architecture. In contrast, we found chitin contents of the *C. glabrata* cell wall to be around 1% of cell wall dry weight and thus lower than those of *S. cerevisiae* and *C. albicans* cell walls. In the case of *C. albicans*, chitin contents vary considerably depending on growth conditions and growth form. Hyphal cells display approximately two to three times higher chitin contents than yeast cells of the same strain varying between 2-6% and 1-2% respectively (MASUOKA, 2004; ROWBOTTOM *et al.*, 2004). Also in the baker's yeast chitin contents range from 1.5% to 6% of cell wall dry weight depending on the growth conditions (AGUILAR-USCANGA and FRANCOIS, 2003). In our analysis, chitin contents of *C. glabrata* cell walls varied little and increased to only 1.3% when growth temperature was risen to 42°C or 1.5% when cell wall stress (calcofluor white) was applied. Thus, chitin might possibly play a minor role in *C. glabrata* cell walls than it does in *S. cerevisiae* or *C. albicans*.

Our data demonstrate that the principal structure of the *C. glabrata* cell wall is very similar to that of *C. albicans* and *S. cerevisiae*. It consists of β-1,3- and β-1,6-glucan, chitin and differently attached cell wall proteins. Considering the quantitative contribution of the different components however, the molecular architecture of the cell walls differs notably. Therefore, even though closely related, the three ascomycetous yeasts are prone to display different cell wall properties.

Thus, together with previous analysis of protein-polysaccharide linkages performed in our lab (WEIG *et al.*, 2004), we were able to build a structural cell wall model for *C. glabrata*, which is comparable to but not identical with that of *S. cerevisiae* and *C. albicans* (see Figure 1-8 in the Introduction).

4.1.2 Cwp1.1p is a major cell wall protein with possible structural functions

In silico analysis of Cwp1.1p suggested localization of this mannoprotein to the cell wall. We generated α-cwp1.1 specific antibodies in order to analyse the actual molecular integration of Cwp1.1p. Using these antibodies, immunofluorescence microscopy showed that the protein localizes to the cell periphery, which indicates that it is either bound to the cell membrane or the cell wall of the fungus.

Subsequently, we demonstrated that Cwp1.1p and its homologue Cwp1.2p are indeed abundant GPI-anchored cell wall proteins in *C. glabrata*. Both proteins were identified in mass spectrometric analysis after 2D gel electrophoresis of HF-released GPI cell wall proteins (WEIG *et al.*, 2004). Immunoblot analysis confirmed that Cwp1.1p is expressed at much higher levels than its homologue Cwp1.2 (Figure 3-6) and the release of CWPs by β-1,6-glucanase treatment followed by immunoblot analysis verified the cell wall localization and nature of linkage to the

cell wall polysaccharide backbone. Consecutive treatment of cell wall material with β-1,6-glucanase and mild alkali revealed that Cwp1.1p is in fact bound to the fungal cell wall via two different linkages - the GPI-anchor and the Pir protein like mild alkali sensitive linkage (WEIG, unpublished data). In *S. cerevisiae*, the structural GPI-anchored cell wall proteins Tir1, Tir4 and Dan4 also show the internal tandem repeats indicating a possible double attachment to the cell wall (VERSTREPEN *et al.*, 2005). Our investigations showed that, as expected, the Cwp1 proteins are GPI anchored. However, with a decrease in pH of the culture medium the additional Pir like linkage to the cell wall can be observed to more extend. Cwp1.1p is the first *C. glabrata* cell wall protein, which has been shown to be connected to the cell wall via both forms of covalent linkage. The use of this possibility might ensure a particular solid attachment of Cwp1.1p within the glucan network, highlighting its importance in strengthening the cell wall architecture.

Sequence analysis of the three *CWP1* family members *CWP1.1*, *CWP1.2* and *CWP1.3* did not reveal any sequence motifs, which would suggest an enzymatic function or a role in cell adhesion. We therefore aimed at characterizing the function of these cell wall proteins. The Cwp1 proteins of *C. glabrata* are highly similar to Cwp1 in *S. cerevisiae*, for which a structural role has been suggested. Deletion of the encoding gene in baker's yeast resulted in an increased sensitivity of the generated mutants towards calcofluor white and congo red (VAN DER VAART *et al.*, 1995). A recent study investigating Cwp1 localization in *S. cerevisiae* using a GFP-tagged version of the protein showed that it localized to bud scars, which suggests a role in cell division (SMITS *et al.*, 2006). However, the *S. cerevisiae ⊿cwp1* deletion strains analysed in the mentioned study did not show an altered growth rate, a change in morphology or a delay in cell separation, leaving the actual function of Cwp1 at the site of budding unclear.

The fact that the protein can be rigidly linked to the cell wall via two different covalent linkages as also seen *S. cerevisiae* structural cell wall proteins and that deletion of its homologue in *S. cerevisiae* renders the cells more sensitive to cell wall perturbing agents, one might assume a structural function of Cwp1.1p within the fungal cell wall of *C. glabrata*. This possible role in architecture was supported by our finding that the cell walls of the mutant strain appeared to be different in morphology than the wild type cell wall in electron microscopic analysis. The mannoprotein layer of the *⊿cwp1.1/1.2/1.3* mutant strain appeared rather diffuse. This contrasts the wild type situation and might indicate that Cwp1 proteins influence compactness of this outermost protective layer. Interestingly, this phenomenon occurs only in the triple knock out mutant (*⊿cwp1.1/1.2/1.3*) but not the mutant strains missing only *CWP1.1* and/or *CWP1.2*.
One possibility might be that Cwp1.3p incorporation into the cell wall is upregulated to compensate for *CWP1.1/1.2* deletion. However, semi quantitative RT-PCR did not show

enhanced *CWP1.3* transcription in *⊿cwp1.1/1.2* mutant strains (Figure 3-4). Thus, incorporation of Cwp1.3p into the cell wall rather than transcription of *CWP1.3* might be enhanced in the double knock out mutant. As the generation of a Cwp1.3p specific antibody is still pending, we have not yet analysed Cwp1.3p incorporation into the cell wall as it was done for Cwp1.1p and Cwp1.2p. In fact, it is still unclear whether Cwp1.3p is at all localized to the fungal cell wall under the examined conditions and the mode of attachment to the glucan backbone remains to be investigated. Thus, its specific role in the cell wall architecture remains unclear.

4.1.3 CWP1 deletion causes growth defects at alkaline pH

It has been shown before that gene deletion of different or multiple genes of the same family might result in different phenotypes. In *C. albicans* for example, the single deletion of secreted aspartyl proteinases *SAP1, SAP2* or *SAP3* resulted in attenuated virulence of the mutants in a murine infection model, whereas gene deletion of the three proteinases *SAP4-6* was necessary to cause a similar effect (HUBE *et al.*, 1997; NAGLIK *et al.*, 2003; SANGLARD *et al.*, 1997). Additionally, differences in growth of the *SAP1*, *SAP2* and *SAP3* mutants were observed. While growth of the *SAP2* deleted strain in medium containing BSA a the sole nitrogen source was strongly inhibited, *SAP1* and *SAP3* deletion mutants were slower in growth than the wild type strain but reached the same optical density about 24 hours later (HUBE *et al.*, 1997; NAGLIK *et al.*, 2003; SANGLARD *et al.*, 1997).

Therefore, we examined the growth rates of generated single (*⊿cwp1.1*, *⊿cwp1.2*, *⊿cwp1.3*), double (*⊿cwp1.1/1.2*) and triple knock out mutants (*⊿cwp1.1/1.2/1.3*). When grown under alkaline conditions (YPD pH 8.0) the generation times of the *⊿cwp1.1/1.2/1.3* and *⊿cwp1.1/1.2* knock out mutants were longer than those of wild type strains (Figure 3-15). This growth defect at alkaline pH indicates that cells depleted of *CWP1.1* and *CWP1.2* are diminished in their ability to adapt effectively to environmental pH. It has been observed that the cell wall protein population is significantly altered depending on environmental pH (WEIG, personal communication). Immunoblot analysis of GPI-dependent cell wall proteins with concanavalin A, which recognizes the glycosylations groups of mannoproteins, showed that the amount of released GPI-proteins in *C. glabrata* increases when cells are grown at alkaline pH as compared to acidic pH, whereas the population of Pir proteins is reduced at alkaline pH. It seems possible that Pir proteins are increasingly incorporated in the cell wall of mutant strains to compensate *CWP1.1/1.2/1.3* deletion, but growth at alkaline pH removes these protective proteins from the cell wall, leaving the fungus more exposed to the environmental impacts. Interestingly, *CWP1* transcription in *S. cerevisiae* has been shown to be induced when cells were shifted to more acidic

pH (from pH 5.5 to pH 3.5) (KAPTEYN *et al.*, 2001). However, in the study, it was neither shown whether the protein arrives at the cell wall nor how it is linked to the glucan backbone. Nevertheless, the results of the analysis support our findings that Cwp1 might be involved in the pH dependent remodelling of the fungal cell wall.

4.1.4 CWP1.1/1.2/1.3 deletion mutants show an increased calcofluor white sensitivity

As Cwp1.1p was identified as one of the most abundant cell wall proteins of *C. glabrata* we expected the deletion of *CWP1.1* to result in a classical cell wall phenotype such as increased calcofluor white, congo red or zymolyase sensitivity as it was observed for *CWP1* deletion in *S. cerevisiae* (VAN DER VAART *et al.*, 1995). These phenotypical screens have been used successfully to demonstrate gene deletion induced cell wall weaknesses in *S. cerevisiae* and *C. albicans* (DE NOBEL *et al.*, 2000a; POPOLO and VAI, 1998; VAN DER VAART *et al.*, 1995). Although we observed an increased β-1,3-glucanase sensitivity in the *⊿cwp1.1* deletion mutant indicating cell wall defects, which will be discussed later on, the calcofluor white, congo red and zymolyase sensitivities of the strain were unaltered. This observation could be due to an upregulated Cwp1.2p or Cwp1.3p expression or increased incorporation of the proteins into the fungal cell wall. However, transcription of *CWP1.2* and *CWP1.3*, controlled by RT-PCR, as well as protein expression, examined by immunoblot analysis of incorporated GPI-proteins, showed that the transcript and protein levels of the two homologous proteins were unaltered in the *⊿cwp1.1* mutant. Thus, compensatory effects might rather be mediated by increased expression or incorporation of other cell wall proteins or a molecular restructuring of cell wall polysaccharides.

Interestingly, the calcofluor white sensitivity of the generated *⊿cwp1.1/1.2/1.3* triple knock out mutant was increased in the phenotypic analysis, indicating that the fungus can rather well compensate single *⊿cwp1.1* induced cell wall weaknesses but not as well those induced by the deletion of all three homologous proteins. The fact that triple mutants were more sensitive towards calcofluor white but not congo red and zymolyase demonstrates that very specific cell wall alterations were induced. Their possible nature will be discussed later on.

4.1.5 Cwp1.1p mediates β-1,3-glucanase protection

As described above, deletion of *CWP1.1* did not result in an increased calcofluor white, congo red or zymolyase sensitivity. However, further analysis of the generated mutants showed that *⊿cwp1.1* cells were more sensitive to β-1,3-glucanase digestion than wild type cells. Approximately

twice as many cells of the *⊿cwp1.1* deletion strain were lysed within 60 minutes of digestion with quantazym, a recombinant β-1,3-glucanase, as compared to the wild type strain. Enhanced β-1,3-glucanase sensitivity was neither induced by *CWP1.2* or *CWP1.3* knock out in the wild type strain nor enhanced by the same gene deletions in the *⊿cwp1.1* knock out background. Therefore, we conclude that the observed increase in β-1,3-glucanase sensitivity is based solely on *CWP1.1* deletion. The observed increase in β-1,3-glucanase sensitivity seems to contradict the fact that the mutant strains were not more sensitive towards zymolyase digestion than the wild type strain. However, this can be explained by the composition of these cell wall-active enzymes. While quantazym is a purified specific β-1,3-glucanase, zymolyase preparations also contain protease, cleaving not only the glucan backbone of the cell but also the protective mannoprotein layer. Thus, the specific incidence of β-glucanase sensitivity indicates that the glucan backbone of the cell wall might be affected by *CWP1.1* deletion.

As Cwp1.1p was identified as one of the most abundant cell wall proteins in *C. glabrata*, we assumed that *CWP1.1* deletion may either cause a noticeable depletion of the protective mannoprotein population in the cell wall or induce an altered cell wall polysaccharide architecture. Diminishing this outermost protective layer, the altered β-1,3-glucan backbone could be easier accessible or more susceptible to enzymatic degradation. Further analyses showed that the protein contents of the cell walls of *⊿cwp1* strains are not significantly reduced. Protein contents were roughly 6% of the cell wall dry weight for all tested strains, wild type as well as all knock out strains. These measures of pure protein content showed that *CWP1.1* deletion did not reduce total mannoprotein content. We also analysed β-1,3-glucan exposure using a β-1,3-glucan specific antibody in FACS analysis. No differences of glucan exposure were detected between the wild type and *CWP1* deletion strains (Figure 3-19). The recognition of yeast cells by macrophages is mediated by binding of β-glucan through the dectin-1 receptor. Therefore, we examined whether mutant cells are phagocytosed by J774A.1 mouse macrophages more efficiently than wild type cells, which was not the case.

Therefore, it seems also feasible that other structural GPI-dependent cell wall proteins, for example proteins of the Tip1p family (WEIG *et al.*, 2004) or Pir proteins, might be incorporated in the cell wall of the *CWP1* deletion strains to compensate the induced cell wall weakening, thereby protecting the β-1,3-glucan backbone from degrading enzymes such as β-1,3-glucanase.

Thus it seems that the displayed β-1,3-glucanase sensitivity of *⊿cwp1.1* mutants is not caused by a reduced amount of covalently bound protein but possibly by an altered amount, composition or architecture of cell wall β-glucans and mannoproteins.

4.1.6 Δcwp1.1/1.2/1.3 knock out mutants compensate cell wall weakening by increased β-1,3-glucan crosslinking

The integrity of the fungal cell wall is crucial for the survival of the fungus and depends on the correct assembly of all its components. This important process of cell wall assembly can be impaired by deletion of cell wall related genes or environmental stress conditions such as heat or the treatment with cell wall perturbing agents. Thus, weakening of the fungal cell wall has to be counterbalanced. Indeed, it has been demonstrated that fungi use a salvage pathway to remodel the cell wall in order to guarantee survival (reviewed by POPOLO *et al.*, 2001). In *S. cerevisiae* and *C. albicans* it has been shown that the deletion of cell wall related genes induces characteristic alterations of the cell wall composition and architecture. Compensatory elevation of chitin content or the increased connection of chitin directly to β-1,6-glucan are observed mechanisms to counterbalance cell wall weaknesses (FONZI, 1999; KAPTEYN *et al.*, 1997; LAGORCE *et al.*, 2002; POPOLO *et al.*, 1997). Studies in *S. cerevisiae* showed that cell wall weakening induced chitin upregulation can lead to an increase of chitin contents from normally 1-2% to 15% of the cell wall dry weight (KAPTEYN *et al.*, 1997; LAGORCE *et al.*, 2002). We observed this compensatory mechanism also in *C. glabrata*, but only to less extend. When cells were grown at higher temperature (42 °C) or under cell wall perturbing conditions (calcofluor white), we observed a slight increase in cell wall chitin content from one percent of cell wall dry weight to 1.3% and 1.5%, respectively (Figure 3-11). Furthermore, the generated *Δcwp1* mutants did not show an increased chitin upregulation when compared to wild type cells. We found no significant differences in chitin levels between single (*Δcwp1.1*, *Δcwp1.2*, *Δcwp1.3*), double (*Δcwp1.1/1.2*) and triple knock out mutants (*Δcwp1.1/1.2/1.3*) (Figure 3-11). Interestingly, the triple knock out mutant (*Δcwp1.1/1.2/1.3*) showed an increased sensitivity to calcofluor white, which binds to nascent chitin chains and perturbs cell wall assembly. This indicates that changes in the cell wall architecture do occur in this particular deletion strain, but can not be explained by the chitin content of the cell wall. Possibly, chitin-glucan or glucan-glucan linkages are increased in the *Δcwp1.1/1.2/1.3* deletion mutant to secure cell wall robustness and the applied calcofluor white prevents correct assembly of this counterbalancing interconnection of cell wall polysaccharides.

Proteomic analysis, performed in our laboratory to compare protein expression of *C. glabrata* wild type cells and the generated *Δcwp1.1/1.2/1.3* triple knock out mutant supported this idea (ARLT, 2007). Besides other proteins, the glycosyltransferase Bgl2 was expressed at higher levels in the knock out mutant as compared to the wild type. Bgl2, first described as an exoglucanase (KLEBL and TANNER, 1989), then as an endoglucanase (MRSA *et al.*, 1993) shows transglycosylase activity, which catalyses strand-rejoining of 1,3-β-glucan strands using a free reducing and a free nonreducing end of the glucan (GOLDMAN *et al.*, 1995). Thus it is implicated in the important

function of glucan cross linking to enhance robustness and physical strength of the fungal cell wall. It has been observed that deletion of *BGL2* in *C. albicans* renders the mutants hypersensitive towards nikkomycin Z, an inhibitor of chitin synthase (SARTHY *et al.*, 1997). Thus, it seems that cells which have lost glucosyltransferase function are more dependent on chitin as a structural element in the cell wall, since the glucan network is not as robust anymore. This means that chitin and glucan complement each other's function in the cell wall architecture and that one mechanism might be used to compensate the impairment of the other. This parallels our observation that deletion of *CWP1.1/1.2/1.3* leads to an increased calcofluor white sensitivity and concurrent enhanced Bgl2 expression, indicating that the mutant strain compensates the induced weakness by enhanced glucan cross linking and not by chitin upregulation. Interestingly, congo red, a dye which binds to hydroxyl groups of glucose molecules in the β-glucan structures, did not lead to growth inhibition in the triple mutant. Possibly, the β-glucan, although tagged with congo red molecules, still forms a three dimensional resilient network in the fungal cell wall as different hydroxyl groups might be occupied with the dye than that are necessary for glucan crosslinking.

We conclude that the cell wall weakening induced in the *⊿cwp1.1/1.2/1.3* deletion strain is compensated by enhanced β-1,3-glucan cross linking and possibly also by increased linkage of β-glucan to chitin, but not by chitin upregulation.

4.1.7 CWP1 deletion induced cell wall alterations reduce the ability of Candida glabrata to adhere to human epithelial cells

Adhesion of *C. glabrata* to human epithelium is mainly mediated by the lectin Epa1, which binds to N-acetyl lactosamine on the host cells. It has been shown that deletion of the encoding gene reduced adherence of fungal cells to HEp2 cells in culture by 95% (CORMACK *et al.*, 1999). Strikingly, the adhesin's C-terminal Ser/Thr-rich region is essential to localize the protein to outer layers of the cell wall enabling the protein to extend its N-terminal lectin domain into the external environment (FRIEMAN *et al.*, 2002). Only at the surface of the fungal cell it can bind to its ligand and mediate adhesion.

In our study *⊿cwp1.1/1.2/1.3* strains showed a reduced adhesion to human epithelial cells (HEp2 and HeLa) in culture. At first this might seem inconsistent with the described adhesion potency of Epa1, which mediates about 95% of the adhesion properties. However, the observed reduction in adherence might be brought about by the *CWP1.1/1.2/1.3* deletion induced cell wall alteration rather than Cwp1 being absent from the cell surface. As discussed before, our analyses

showed that the cell wall β-1,3-glucan structure of mutant cells might be significantly altered. Increased crosslinking of β-1,3-glucan or an enhanced connection to chitin or β-1,6-glucan could modify Epa1 linkage and location within the fungal cell wall, reducing its ability to bind to epithelial cells' surfaces as the lectin domain does not reach the external environment. Additionally, we found *⊿cwp1.1/1.2/1.3* mutant cell walls to be less compact than in the wild type, which might keep Epa1 hidden in internal cell wall layers, further contributing to reduced adherence. Cwp1p does not have the sequence prerequisites of an adhesin. Thus, it does not directly mediate adhesion, but deletion of the *CWP1* genes reduces the adhesion properties of *C. glabrata* in an indirect manner, possibly due to changes in the cell wall architecture.

4.2 Candida glabrata prefers acidic environments

In our analysis of the *C. glabrata* cell wall architecture and phenotypic characterisation of *CWP1* deletion mutants, we recognized the influence of environmental pH on a number of important cell wall related processes. At first, incorporation of GPI-dependent proteins is increased at alkaline pH, whereas Pir protein incorporation is favoured under acidic growth conditions (WEIG, unpublished data). Secondly, we found the major cell wall protein Cwp1.1p to be connected to the cell wall glucan structure via two different linkages (GPI-anchor and Pir like linkage) at acidic, but not at alkaline pH (WEIG, unpublished data). Thirdly, previous studies done in our laboratory demonstrated that β-1,3-glucanase sensitivity of *C. glabrata* strains is lower when cells were grown in acidic medium (pH 3.5) prior to challenge with quantazym (KAPLANEK, 2004). Fourthly, the generated *⊿cwp1.1/1.2* and *⊿cwp1.1/1.2/1.3* knock out mutants showed reduced growth at pH 8.0 (Figure 3-15). Therefore, the environmental pH influences a number of cell wall structural events and properties but this influence will most probably be more global and not only limited to the cell surface. In its human host *C. glabrata* survives in the relatively acidic environments of the oral cavity, stomach, duodenum and vaginal tracts, but also in more basic niches such as the bloodstream and kidneys. In this respect, *C. glabrata* is similar to *C. albicans*, which occupies connatural niches.

For *C. albicans* it has been shown that pH signalling is essential for virulence (DAVIS *et al.*, 2000a; DAVIS *et al.*, 2000b) and the same is true for the filamentous fungal pathogen *Aspergillus fumigatus* (BIGNELL *et al.*, 2005). Additionally, differential pH regulation of essential cell wall functions affects the ability of *C. albicans* to infect vaginal tissue and internal organs (DE BERNARDIS *et al.*, 1998) and pH sensing appears to be critical for tissue invasion during systemic infections (THEWES *et al.*, 2007). In a detailed transcriptional screen Fradin and co-workers

identified more than 500 *C. albicans* genes to be up or downregulated, following exposure to human blood (FRADIN *et al.*, 2005; FRADIN *et al.*, 2003). Expression of *PHR1* and *PRA1*, two genes which encode for cell wall proteins and are known to be upregulated at alkaline pH, were constantly expressed at high levels during the incubation. However, the expression of *PHR2*, which encodes for a GPI-anchored cell wall protein and is usually repressed at alkaline pH was also induced throughout the experiment. This paradox might be explained by the uptake of fungal cells by leucocytes followed by their exposure to the acidic phagolysosome.

To obtain comprehensive results in our pH response examinations, we tried to avoid similar complex and intransparent interactions by choosing a well defined minimal medium (Pan Fungal Minimal Medium). We have examined the global response of *C. glabrata* to changes in ambient pH using a proteomic approach. Even though *C. glabrata* and *C. albicans* are evolutionarily divergent, we reasoned that they might display some similarities in their global responses to ambient pH as they are both prevalent opportunistic fungal pathogens and occupy similar niches in their human host.

4.2.1 Acidic ambient pH induces expression of carbon catabolic proteins

C. glabrata must respond effectively to changes in ambient pH if it is to thrive in the diverse niches it occupies. The same holds true for another fungal pathogen, *C. albicans*, for which global pH response has been examined by transcript profiling (BENSEN *et al.*, 2004). Bensen and co-workers observed that *C. albicans* genes involved in a number of functional categories were regulated in response to ambient pH. Besides the cell wall structure and hyphal development, these functional categories include carbohydrate, amino acid and lipid metabolism, electron transport, signal transduction, ion transport as well as protein synthesis, folding and degradation. For several reasons a number of these functional categories are likely to be underrepresented in our proteomic analysis of pH responses in *C. glabrata*. Firstly, cell wall proteins and integral membrane proteins such as ion transporters would not have been extracted under the lysis conditions we used, and would not have been resolved well on our 2D gels. Secondly, although *C. glabrata* can undergo morphogenesis (CSANK and HAYNES, 2000), it does not form hyphae in response to ambient pH. Therefore we would not have expected (pseudo)hyphal proteins to be highlighted in our analyses. Despite the significant differences between transcript profiling and proteomics, and despite the evolutionary divergence of *C. albicans* and *C. glabrata*, some common themes have emerged from these global analyses.

Clearly carbon metabolism is regulated in response to changing ambient pH in both *C. glabrata* and in *C. albicans* (BENSEN *et al.*, 2004). It has been shown that central carbon metabolism is regulated in a niche-specific fashion during *C. albicans* infections (BARELLE *et al.*, 2006). We found that glucose catabolic enzymes (Glk1, Fba1, Pgi1, Cdc19/Pyk1, Pdc1, Tdh3 and Tkl1) and enzymes of the TCA cycle (Aco1, Lsc2 and Mdh1) were expressed at higher levels in *C. glabrata* cells growing at pH 4.0 (Tables 3 and 4). In *S. cerevisiae* gluconeogenic, glyoxylate cycle and TCA cycle genes are repressed by glucose, even at relatively low glucose concentrations (BOLES and ZIMMERMANN, 1993; GANCEDO, 1998; YIN *et al.*, 2003). Different species of the evolutionary *Saccharomyces* clade have been shown to display the Crabtree Effect (MERICO *et al.*, 2007), a phenomenon describing the production of ethanol also under aerobic conditions. Instead of oxidizing the produced pyruvate in the TCA cycle, as usually done under aerobic conditions, pyruvate is reduced to ethanol by fermentation when external glucose concentrations are high. Given that *C. glabrata* also displays this Crabtree Effect, one might have expected TCA cycle enzymes to have been repressed in our glucose-containing medium, which they are not. However, increased glucose catabolism might be required to generate energy for the maintenance of intracellular pH homeostasis when the ambient pH is acidic resulting in continued expression of TCA cycle genes.

There were two notable exceptions to the observation that glucose catabolic enzymes were induced in *C. glabrata* cells growing at pH 4.0 compared with cells growing at pH 7.4 or 8.0: Pfk1 and Pfk2 (Table 5). Significantly these are the *alpha* and *beta* subunits of phosphofructo-kinase 1, which is specific to and essential for glycolysis. At first view, this fact seems very surprising as it should be difficult to maintain glycolytic flux in *C. glabrata* cells during growth at low pH while phosphofructo-kinase 1 levels are decreased. However, in *S. cerevisiae*, phosphofructo-kinase 1 activities are maintained by increasing the levels of the positive allosteric effector fructose-2,6-*bis*phosphate (FRANCOIS *et al.*, 1988; FRANCOIS *et al.*, 1986; PEARCE *et al.*, 2001). Therefore, analogous mechanisms might exist in *C. glabrata* to maintain phosphofructo-kinase 1 activity, and hence glycolytic flux, during growth at acidic ambient pHs.

4.2.2 Alkaline ambient pH induces protein catabolism and reduces protein synthesis

An interesting difference between the pH responses of *C. glabrata* and *C. albicans* lies in the behaviour of their protein synthesis and degradation functions. In *C. albicans*, protein synthesis genes are generally up-regulated at pH 8, and protein degradation genes are down-regulated at this alkaline pH (BENSEN *et al.*, 2004). Fradin and co-workers found similar results when they

examined the transcript profiles of *C. albicans* when exposed to human blood (FRADIN *et al.*, 2003). Genes encoding for protein synthesis products represented 12% of the upregulated and only 1% of the downregulated genes. Strong induction of these genes was observed after only ten minutes of exposure to human blood but decreased markedly during the following 30 to 60 minutes. Thus, the observed growth initiation in this experiment might reflect the transfer from nutrient-poor to nutrient-rich medium. However, we found that *C. glabrata* proteins involved in protein synthesis were expressed at lower levels at pH 8.0, and protein catabolic enzymes were expressed at higher levels at alkaline pHs. These results suggest growth retention of *C. glabrata* at alkaline pH as protein synthesis is a prerequisite of cell propagation and the induction of protein catabolism is generally considered as a stress reaction. It is noteworthy, that the correct and solid attachment of cell wall proteins is essential for the fungus as the mannoproteins make up the outermost protective layer. If protein degradation diminishes the amount of mannoproteins present in the fungal cell wall, the fungus might be more exposed to environmental influences and stresses. Cell wall proteins might therefore function as a shield for inconvenient conditions such as alkaline pH. Secondly, we have shown that the incorporation of cell wall proteins, specifically the two very abundant cell wall proteins Cwp1.1p and Cwp1.2p, is strengthened at acidic pH as a second covalent and mild alkali sensitive bond is formed in addition to the GPI anchor. Thus, alkaline pH might not only reduce the amount of incorporated cell wall proteins but also weaken the anchorage of those proteins in the cell wall structure.

Additionally, we found that the generation time of the fungus is much longer when grown in YPD at pH 8 as compared to standard YPD (pH ~5) (Fig. 3-11) pointing to the difficulties *C. glabrata* seems to have when growing at alkaline pH. This is further supported by the fact that the functional category "response to stress" was significantly enriched in the subset of *C. glabrata* proteins that were up-regulated at alkaline pHs (Table 8). As it has been shown that *C. albicans* can survive the internalization by macrophages (IBATA-OMBETTA *et al.*, 2003) one might speculate that *C. glabrata* uses cells of these immunosystem (e.g. macrophages), which have an acidic intracellular pH to move within the human blood without being exposed to the alkaline pH.

Taken together, our data suggest that, in contrast to *C. albicans*, *C. glabrata* perceives acidic pHs as less stressful than the more basic pHs. This is consistent with the idea that *C. glabrata* is an opportunistic pathogen of humans that has evolved primarily in relatively acidic environmental niches such as rotting fruit and is more commonly found as the cause of vaginal candidiasis than in disseminated systemic infection.

5 SUMMARY AND CONCLUSIONS

C. glabrata causes a variety of infections ranging from superficial skin and vaginal infections to disseminated disease in immuno compromised patients. The encountered milieus at the different host sites vary considerably in respect to oxygen or nutrient availability as well as pH conditions. The first contact between pathogen and host as well as the recognition of environmental clues are of utmost importance for the establishment of an infection. We therefore examined two important aspects at the host-pathogen interface: the response of *C. glabrata* to changing ambient pH and the role of the mannoprotein family Cwp1 in the fungal cell wall structure.

Members of the Cwp1 protein family are major structural components of the fungal cell wall in *C. glabrata*. We could demonstrate that they can be anchored in the cell wall via two different covalent linkages, namely a GPI-anchor and a Pir like mild alkali sensitive linkage. Furthermore, we showed that deletion of the encoded genes results in an altered microscopic structure of the cell wall as well as a reduction of its protective potential. Thus, mutants were more sensitive to calcofluor white and β-1,3-glucanase treatment than wild type strains. The fungus' ability to adhere to epithelial cells was also diminished by *CWP1.1/1.2/1.3* deletion. We speculate that the deletion of *CWP1* genes might be compensated by alternate incorporation of other cell wall components counterbalancing induced weaknesses to some extend, for example β-1,3-glucan, which is supported by the increased β-1,3-glucanase sensitivity and enhanced expression of Bgl2, a β-glucan transglycosidase, in *CWP1* deletion strains. Strikingly, the growth of *Δcwp1.1/1.2/1.3* mutants was significantly slowed down at alkaline pH as compared to the wild type. Another speculative possibility could therefore be that incorporated proteins compensating for *CWP1* deletion are Pir proteins, which are sensitive to high pH. Although *C. glabrata* is able to cause bloodstream infections, at pH 7.4, the fungus seems to prefer an acidic environment. We performed a proteomic analysis of the fungus' pH response and found protein catabolism to be upregulated at alkaline pH whereas translational regulators, protein synthesis and protein complex assembly were downregulated, indicating reduced metabolic activity. Additionally, carbon metabolic enzymes were particularly highlighted in the analyses. Enzymes involved in glucose catabolism and enzymes of the TCA cycle were downregulated at alkaline pH as compared to acidic pH. At the same time also respiration is restrained, as indicated by the downregulation of participating enzymes. We observed constriction of cell redox homeostasis, chromosomal segregation and cellular polarization due to the downregulation of involved proteins as well as the concurrent induction of stress response proteins at alkaline pH.

All these observations indicate that growth of *C. glabrata* is favoured under acidic environmental conditions and the fungus perceives alkaline pH as more stressful. Nevertheless, *C. glabrata* is able to cause disseminated disease in the face of alkaline pH in the blood. The first front of protection from environmental impacts is the cell wall. In our analyses, we demonstrated that Cwp1 proteins mediate different aspects of cell protection as deletion mutants showed reduced growth at alkaline pH and were more susceptible to different kinds of cell wall stress. The protein also functions as a glucan cross linker connecting β-1,3- and β-1,6 glucan thus forming a resilient three dimensional network. Interruption of this network by *CWP1* deletion led to a decreased adherence to epithelial cells, which might be caused by insufficient incorporation of fungal adhesins due to the described restructuring events in the fungal cell wall.

Thus, we demonstrated that proteins of the Cwp1 family are important structural components of the fungal cell wall, which mediate protection from alkali induced and cell wall targeted stress conditions. The fact that *C. glabrata* perceives acidic environments as less stressful than alkaline conditions, contrasts with the human pathogen *C. albicans* but is consistent with the idea that *C. glabrata* is an opportunistic pathogen that has evolved primarily in relatively acidic environmental niches such as rotting fruit.

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APPENDIX

A) List of abbreviations

A	adenosine
A	alanine
A. fumigatus	*Apergillus fumigatus*
aa	amino acid
AIDS	acquired immuno deficiency syndrome
ALS	agglutinin like protein
Amp	ampicillin
Amp^R	ampicillin resistance
APS	ammonium persulfate
ARS	autonomously replicating sequence
Asn	asparagine
ATCC	American type cell culture
ATP	adenosine triphosphate
BCA	bicinchoninic acid
bp	base pairs
BSA	bovine serum albumin
°C	degree Celsius
C	cytosine
C	cystein
C. albicans	*Candida albicans*
C. glabrata	*Candida glabrata*
C. neoformans	*Cryptococcus neoformans*
Ca^{2+}	calcium ions
cDNA	copy-DNA
CEN	centromere
Cg	*Candida glabrata*
CW	cell wall
CWP	cell wall protein
2D	two dimensional
D	aspartic acid
Da	Dalton
dATP	deoxy-adenosine-triphosphate
dCTP	deoxy-cytosine-triphosphate
ddH_2O	bidestilled water
dGTP	deoxy-guanosine-triphosphate
DIG	digoxygenin
DMEM	Dulbecco's Minimal Essential Medium
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleosidtriphosphate
DSS	disuccinimidyl suberate
DTT	dithiothreitol
dTTP	deoxy-thymidine-triphosphate
dUTP	deoxy-uridine-triphosphate
E	glutamic acid

E. coli	*Escherichia coli*
EA926	human endothelial cells
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
et al.	and others (et alii)
EtN	ethanolamine
EtOH	ethanol
F	farad
F	phenylalanine
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
Fig	figure
FLO	lectin-like protein, involved in flocculation
FLP	flippase
FRT	FLP recombination target
FUO	fever of unknown origin
G	guanosine
G	glycine
g	gram
Gas	β-glucanosyltransferase
GN	glucosamine
GPI	glycosyl-phosphatidyl-inositol-phosphate
GST	glutathione S-transferase
6xHis	6x Histidine tag
h	hour
HaCaT	human keratinocytes
HeLa	human cervix carcinoma epithelial cells
HEp2	human laryngeal carcinoma
HIV	human immuno deficiency virus
HO	homothallism
HRP	horse radish peroxidase
HWP1	hyphae specific wall protein 1
I	isoleucine
I	myoinositol
IPTG	isopropyl-β-D-thiogalactopyranoside
k	kilo
K	lysine
K. lactis	*Kluyveromyces lactis*
Kan	kanamycin
kb	kilo bases
ko	knock out
L	litre
L	leucine
LB	Luria-Bertani
LHS	left hand side
m	milli
M	molar
M	mannose
m/z	mass to charge ratio
MAT	mating type

β-ME	β-mercaptoethanol
MetOH	methanol
min	minute
mRNA	messenger RNA
MS	mass spectrometry
MTLmating	type locus
MW	molecular weight
µ	micro
n	nano
N	asparagine
NCBI	National Centre of Biotechnology Information
NEB	New England Biolabs
o/n	overnight
OD	optical density
ORF	open reading frame
Ω	ohm
P	plasmid
PAGE	polyacrylamide gel-electrophoresis
PBS	phosphate buffered saline
PCA	principle component analysis
PCR	polymerase chain reaction
PFA	para formaldehyde
PFM	Pan Fungal Minimal
pH	preponderance of hydrogen ions
PHR	pH responsive protein
pI	isoelectric point
PI	propidium-iodid
Pir	proteins with internal repeats
PM	plasma membrane
PRR1	pheromone response regulator
RE	restriction enzyme
RHS	right hand side
RIM101	Regulator of IME2 (inducer of meiosis)
RNA	ribonucleic acid
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
RT	room temperature
S	serine
S. cerevisiae	*Saccharomyces cervisiae*
SAP2	secreted aspartyl protease 2
SB	sample buffer
SC	synthetic complete
SDS	sodium dodecyl sulfate
sec	seconds
Ser	serine
SLAD	synthetic, low ammonia, dextrose
spp.	species
T	thymidine
T	threonine
Taq	*Thermus aquaticus*

TBST	tris buffered saline – Tween
TE	Tris, EDTA
TEF3	transcription elongation factor 3
TEL	Tris, EDTA, lithium acetate
TEMED	N,N,N',N'-tetramethylenediamin
Tet	tetracycline
tetO	tetracycline responsive element
Thr	threonine
TOF	time of flight
TOP2	DNA topoisomerase 2
Tris	Tris-(hydroxymethyl)-aminoethan
Triton® X-100	octylphenol ethylene oxide condensate
UV	ultraviolet
V	valine
V	volt
v/v	volume per volume
Vero	kidney epithelial cells from African green monkey
vol	volume
w/v	weight per volume
wt	wild type
www	world wide web
X-Gal	5-Brom-4-chlor-3-indoxyl-β-D-galactopyranosid
Y	tyrosine
YCB	yeast carbon base
YNB	yeast nitrogen base
YPD	yeast extract, peptone, dextrose

B) List of primers

Oligonucleotide	5'-3' Sequence
ExCgcwp1.1 f	CGGCGGATCCGACTCCCAAGCCTTCGGTTTGTTGGC
ExCgcwp1.1 r	GCGCAAGCTTGTTGGTGGTTTGTTGGTGGATGGTGG
ApaI-F1f	CGACGTATGGGGGCCCCAAAGTGGTGTCGAACGGGTA
XhoI-F1r	GTATCCGCTCGAGGCGAGTGTAAATTGGTTACTG
NotI-F2f	CGTAAGGAAAAAAGCGGCCGCACTAGAGTTTTACTGGCGAG
SacII-F2r	ATGCATCCCCGCGGTATCCAGTTGTGTATCAG
NotI-F3 f	CGTAAGGTCAAGCGGCCGCACTTCAATATACAATTTTACGC
SacII-F3 r	AGTCATCTCCGCGGATGTATCGGTGACAAAGAGAATC
SacI-F4f	TAGAGCTCGGAGCAGCAACATTGACGGATAG
NotI-F4r	ATGGCGGCCGCGAGTTCCCCCTTCACATC
ApaI-F5 f	CGACGTATTCGGGCCCTAATAAGTTGTATATCAGCTCTTCTC GAA
XhoI-F5 r	GTATCCGCTCGAGGATTTCTTTCTAATCAAGTTTTATTGTTC
NotI-F6 f	CGTAAGGAATTAAGCGGCCGCAATGTATTTGAAAAAAAAAA AGGTAAT
SacII-F6 r	ATTGCATCCCGCGGTAAATGGAGGTTTTCAACTATTACTT
SOU His f	GGGAGAGCATACTGGGCAAGGAA
SOU His r	AGCGGTGGTGGTCGTTGGTG
SOU F1 f	AGTGGTGTCGAACGGGTAG
SOU F1 r	TGCCACTCACTAAGACGGG
SOU F2 f	TGTCCCTCAAGAATTTTTAGC
SOU F2 r	CATGACAAGCTTGACAAAACTG
SOU FLP f	AGCATTATGTGCTGCTGAACTA
SOU FLP r	GTATTTTCTCAGTGATCTCCCAG
RT Cgact f	CAGAGAAAAGATGACTCAAATCATGT
RT Cgact r	GAGTAACCACGTTCACTCAAGATC
RT Cgcwp1+2 f	CATCCCATCCTGGTTCTGAATACA
RT Cgcwp1.1 r	TTGGTGGATGGTGGAGGT
RT Cgcwp1.2 r	TGTTAAAATTAGATTAAAAAAGAGTTAAAGTTT
RT Cgcwp1.3 f	GGAAACTTGAAGGAAGTCAGCGA
RT Cgcwp1.3 r	GAACAGGTGGAACATGGGAAGTTG
RT Oligo dT	TTTTTTTTTTTTTTTT
ApaI-cwp1.1/1.2 f	TCGATACCGGCCCGTTTGCCCACGTATAAACCCA
ApaI-cwp1.1/1.2 r	TCGATACCGGGCCCCAGGTACTTCTCATCCAGCGTCTT
ApaI-cwp1.1 FLP f	TAGATCGGGCCCGGACTCACAGGTAAACCCTAAC
XhoI-cwp1.1 FLP r	TCGATGCTCGAGGAATGAGATTGTGATAGATTGAACT
EcoRI-cwp1.1 p f	CGGAATTCCCTAACGGCACTCTGTGATACATC
BamHI-cwp1.1 p r	GCGGATCCAGAAACAGAGGATTGTGCGAC
ApaI-cwp1.2 FLP f	CGACGTATGAGGGCCCCCGTGCCCAGCAATTATCCAAC
XhoI-cwp1.2 FLP r	TCGATGCTCGAGCAGGTACTTCTCATCCAGCGTCTT

ApaI-cwp1.3 FLP f	TGATCAGGGCCCCGATCCTACTATTTATATAGTTTTTCCC
XhoI-cwp1.3 FLP r	TAGTCGCTCGAGCCCAGTGTGTTTTTTATTTAGTATT
His ver	ACCACCACCGCTCCGAATCAG
His ver	ATTCGTCTCCTGCGTAACCCTCTT
FLP ver f	ATGTACTGGTACTGGTTCTCGGGA
FLP ver r	AATTTGTGTTTGTTGACGTTTCAAGT
F1 dFLP ver f	TTTTTACAGCACTGAAGTTGGAA
F2 dFLP ver r	ATGTTCAATTCCATGATAAAGGG
Cgcwp1.1 ver f	GTAAACCCTAACGGCACTCTGT
Cgcwp1.1 ver r	GCGCGGTGTTTTTTGTTTGCTC
Cgcwp1.2 ver f	ATAAATTTCCTCCTCCTAATCCGGTAAT
Cgcwp1.2 ver r	CAGGTACTTCTCATCCAGCGTCTT
Cgcwp1.3 ver f	CAGTAAAGCCCATTGAGAAGCACG
Cgcwp1.3 ver r	GTTGTAAATCAATTCCAAAACTAGAG

C) List of figures

D) List of tables

E) Publications

Published literature

Kaplanek, P., de Boer, A., Gross, U., de Groot, P., Hube, B. and Weig, M. (2006). *Candida* and Candidosis today: where are we, and where to go? The Interdisciplinary Forum on Candidosis (IFOCAN) 2005, Göttingen (Germany), 23-25 September 2005. *FEMS Yeast Res* **(6),** 1290-4

Manuscripts in preparation

Schmidt, P., Walker, J., Selway, L., Stead, D., Yin Z., Enjalbert, B., Weig, M. and Brown, A.J.P. Global proteomic analysis of the pH response in the fungal pathogen *Candida glabrata*. (Submitted 03.09.2007 to *Proteomics*)

Schmidt, P., Goretzki, A., Bader, O., de Boer, A., Groß, U. and Weig, M. The mannoprotein family Cwp1 of *Candida glabrata* mediates cell wall integrity. (in progress)

F) Curriculum vitae

PERSONAL INFORMATION

Name: Pia Schmidt, born Kaplanek
Date of birth: August 16th 1979
Place of birth: Halle/Saale, Germany
Marital status: married
Nationality: German

EDUCATION

since 04/2004 **Georg August University Göttingen**
PhD student in the group of OA Dr. med. Michael Weig, Head: Prof. Dr. med. Uwe Groß, Institute for Medical Microbiology

10/2002 - 03/2004 **Georg August University Göttingen**
MSc/PhD Program "Molecular Biology"
International Max Planck Research School
Degree: Master of Science

09/2001 - 05/2002 **University of Toronto, Canada**
Study: Biochemistry, at the Faculty of Arts and Sciences
Transatlantic Science Student Exchange Program (TASSEP)

10/1998 - 08/2001 **Friedrich Schiller University Jena**
Study: Biochemistry/Molecular Biology
Degree: Vordiplom

09/1986 - 08/1998 **Georg Cantor Gymnasium, Halle**
Degree: Abitur

PRACTICAL EDUCATION

09/2001 - 5/2002 **Research project at the University of Toronto**
Identification and confirmation of interacting partners of Yeast Hsp90 using a genome-wide Two Hybrid approach
Prof. Dr. Walid Houry, Dept. of Biochemistry, Toronto

10/2003-03/2004 **Master Thesis at the Georg August University Göttingen**
Functional characterization of the GPI-protein Cwp1p in Candida glabrata
Prof. Dr. med. Uwe Groß and OA Dr. med. Michael Weig, Institute for Medical Microbiology, Göttingen

04/2006-08/2006 **Collaborative research project at the University of Aberdeen**
Proteomic analysis of the pH response in Candida glabrata
Prof. Dr. Alistair Brown, Molecular and cell biology, Aberdeen

Since 04/2004 **PhD Thesis at the Georg August University Göttingen**
Molecular mechanisms of the human pathogen Candida glabrata involved in the interaction with the host
Prof. Dr. med. Uwe Groß and OA Dr. med. Michael Weig, Institute for Medical Microbiology, Göttingen

SCHOLARSHIPS/AWARDS

2002-2003	Scholarship of the International Max Planck Research School and Scholarship of the biotechnology company Develogen, Göttingen
2003-2006	Georg Christoph Lichtenberg PhD-Stipend of the State Lower Saxony
2004	MSD Award "Mycology" for scientific exchange of young investigators

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