Characterization of *Candida albicans* Genes Involved in Cell Wall Biogenesis and Infection

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I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person. Materials of work found by other researcher are mentioned by reference. This thesis, neither in whole nor in part, has been previously submitted for any degree.

Stuttgart, June 2010 Martin Zavrel

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1 SUMMARY

Candida albicans is a commensal organism living on skin and mucosal surfaces of humans and warm blooded animals. Its presence becomes a problem in immunocompromised patients where it may turn into an opportunistic pathogen. Candida colonizes various host niches including skin, gastrointestinal and the urogenital tract, which offer various environments in terms of pH and nutrient availability. The cell surface of the fungus is the site of direct interaction of Candida with the host, mediating environmental sensing, adhesion and also interaction with the host immune system. Since the cell wall is not present in humans its components are a prime target for drug development.

One part of this work is focused on characterization of cell wall changes induced by deletion of genes *efg1* and *cph1*. These genes encoding for transcription factors were previously described to be involved in many cellular functions including filamentation and pathogenicity. Deletion of *efg1* affects mostly the glucan polysaccharide part of the fungal cell wall. In contact with macrophages and dendritic cells, deletion of *efg1* itself or together with *cph1* alters significantly the immunogenicity of the strains, while deletion of *cph1* alone plays only a minor role.

In order to reveal whether *C. albicans* is able to specifically respond to different host niches, transcriptional profiling was used in order to identify *C. albicans* genes differentially regulated during adhesion on polystyrene, vaginal and intestinal tissue models. The second part of this work is following up on the results of the transcriptional profiling. It is focused on functional characterization of several genes differentially regulated under the tested conditions and encoding for putative cell surface proteins. Among the genes characterized in detail are two encoding for predicted GPI-proteins, Pga7 and Pga23 and one for a protein secreted to the medium, Pra1. Functional studies of *PGA7*, *PGA23* and *PRA1* with regard to adhesion and invasion as well as cell wall structure and stability were performed using deletion and overexpression strains. These studies qualify Pga7, Pga23 and Pra1 as structural elements of the cell wall rather than adhesins. The proper function of Pra1 seems to be also in interaction with host components.

Finally, a family of genes with one member induced during adhesion (*AUF8* Adhesion Upregulated Factor 8) was analyzed. These genes were predicted to be localized in the plasma membrane, carrying four transmembrane domains. There are totally seven homologues in the *C. albicans* genome including *AUF8*, and of which

six are located on chromosome 5 in a 10 kb gene-cluster. When heterologously expressed in *S. cerevisiae*, one of the proteins, Auf8GFP localizes to the plasma membrane. Deletion studies did not show any direct involvement of the *AUF* genes in the adhesion or invasion process. However, there are some indications about their importance during stationary phase of growth.

ZUSAMMENFASSUNG

Candida albicans ist ein kommensaler Mikroorganismus, der auf der Haut und Schleimhaut von Menschen und warmblütigen Tieren lebt. Problematisch wird seine Anwesenheit bei immunsupprimierten Patienten, bei denen sich *C. albicans* zu einem opportunistischen Pathogen wandeln kann. Im Wirt besiedelt *Candida* unterschiedliche Nischen, darunter die Haut, den Gastrointestinaltrakt sowie den Urogenitaltrakt. Diese bieten somit verschiedenartige Milieus bezüglich pH-Wert und die Verfügbarkeit von Nährstoffen. Die direkte Interaktion zwischen *Candida* und dem Wirt erfolgt über die Zelloberfläche des Pilzes, wodurch die Wahrnehmung der Umgebungsbedingung, die Adhäsion und auch die Interaktion mit dem Immunsystem des Wirts vermittelt werden. Da menschliche Zellen keine Zellwand aufweisen, sind deren Bausteine die wichtigsten Ziele für die Entwicklung von Medikamenten.

Der erste Teil der vorliegenden Arbeit konzentriert sich auf die Charakterisierung von Veränderungen der Zellwand, die durch die Deletion der Gene *efg1* und *cph1* induziert werden. Wie bereits beschrieben, sind diese Gene, die für Transkriptionsfaktoren kodieren, an vielen zellulären Funktionen beteiligt, einschließlich der Ausbildung von Filamenten und von Pathogenität. Eine Deletion von *efg1* beeinflusst hauptsächlich den Glukan-Polysaccharid-Anteil der Zellwand des Pilzes. Bei Kontakt mit Makrophagen und Dendritischen Zellen verändert eine Deletion von *efg1* allein oder in Kombination mit *cph1* die Immunogenität der Stämme signifikant, wohingegen die alleinige Deletion von *cph1* eine untergeordnete Rolle spielt.

Um herauszufinden, ob *C. albicans* in der Lage ist spezifisch auf die unterschiedlichen Nischen im Wirt zu reagieren, wurden Transkriptionsprofile erstellt zur Identifizierung von *C. albicans* Genen, die während der Adhäsion auf Polystyren sowie auf vaginalen und intestinalen Gewebemodellen differentiell reguliert werden. Der zweite Teil der Arbeit baut auf den Ergebnissen dieser Transkriptionsprofile auf. Der Fokus liegt dabei auf der funktionalen Charakterisierung mehrerer, unter den getesteten Bedingungen differentiell regulierter Gene, die für putative Zelloberflächenproteine kodieren. Unter den im Detail charakterisierten Genen befanden sich zwei codierende Gene für potentielle GPI-Proteine, Pga7 und Pga23 sowie eine für ein Protein, Pra1, das ins Medium sekretiert wird. Funktionale Studien von *PGA7*, *PGA23* und *PRA1* bezüglich Adhäsion und Invasion sowie

Zellwandstruktur und Stabilität wurden mittels Deletions- und Überexpressionsstämmen durchgeführt. Diese Studien qualifizieren Pga7, Pga23 und Pra1 eher als strukturelle Elemente der Zellwand als Adhäsine. Die eigentliche Funktion von Pra1 scheint auch in der Interaktion mit Wirtskomponenten zu liegen.

Abschließend wurde eine Genfamilie analysiert, von denen ein Mitglied (*AUF8* Adhesion Upregulated Factor 8) während der Adhäsion hochreguliert wird. Diese enthalten vier Transmembrandomänen und sind voraussichtlich in der Plasmamembran lokalisiert. Es konnten sieben homologe Gene im Genom von *C. albicans* identifiziert werden, von denen sich sechs auf dem Chromosom 5 in einem 10 kb-Gencluster befinden. Während der heterologen Genexpression als GFP-Fusionsprotein in *Saccharomyces cerevisiae* ist das Protein Auf8-GFP an der Plasmamembran lokalisiert. Deletionsstudien zeigten keinen direkten Einfluss der *AUF*-Gene auf den Adhäsions- oder Invasionsprozess. Allerdings gibt es Anzeichen für die Bedeutung dieser Gene während der stationären Wachstumsphase.

2 LIST OF ABBREVIATIONS

2.1 Standard Abbreviations

A	Adenine
aa	Amino acid
b	Base
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
cDNA	Cytosolic DNA, Complementary DNA
CFW	Calcofluor White
CFU	Colony forming units
CR	Congo Red
CWP	Cell wall protein
dH_2O	Deionized water
Da	Dalton
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
EtOH	Ethanol
FBS	Fetal bovine serum
FCS	Fetal calf serum
G	Guanine
GlcNAc	N-Acetyl-D-glucosamine
GPI	Glycosylphosphatidylinositol
HBSS	Hanks' Balanced Salt Solution
HPLC	High performance liquid chromatography
HPIC	High performance ion chromatography
HRP	Horseradish peroxidase
IFNβ	Interferon beta
Ig	Immunoglobulin
IL-x	Interleukin (x stands for number)
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride
kb	Kilobase
kDa	Kilodalton
KOAc	Potassium acetate
LB	Luria Bertani medium
LiAc	Lithium acetate
MAP kinase	Mitogen Activated Protein kinase
mDC	Myeloid dendritic cell
M	Molar concentration [mol/l]
MPH	Macrophage
mRNA	messenger RNA
NAD	Nicotinamide adenine dinucleotide
NaOAc	Sodium acetate
NP-40	Nonidet P-40, Nonyl phenoxylpolyethoxylethanol

OD_{600}	Optical Density at 600 nm
o/n	Overnight
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDNA	Plasmid DNA
pH	Inverse logarithmic representation of hydrogen proton concentration
Pir	Protein with internal repeats
PNS	Polymorphonuclear neutrophil
PMS	Phenazine Methosulphate
PMSF	Phenylmethanesulphonylfluoride
qRT-PCR	Quantitative Real Time PCR
RLU	Relative luminescence units
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room Temperature
RT-PCR	Reverse Transcription PCR
SC	Synthetic complete medium
SDS	Sodium dodecyl sulphate
SLAD	Synthetic low-ammonium dextrose
ssDNA	Single stranded DNA
T	Thymine
TAE	Tris-Acetate-EDTA Buffer
Taq	*Thermophillus aquaticus* DNA-polymerase
TNFα	Tumor Necrosis Factor alpha
Tris	T*ris*(hydroxymethyl)aminomethane
tRNA	transfer RNA
TWEEN 20	polyoxyethylene (20) sorbitan monolaurate
U	Unit
U, Ura	Uracyl
Uri	Uridine
UV	Ultraviolet
UTR	untranslated region
v/v	volume/volume
WT	Wild-type
w/v	weight/volume
XTT	2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide
YCB	Yeast carbon base
YPD	Yeast extract/peptone/dextrose
YNB	Yeast nitrogen base

2.2 Gene Abbreviations

In the following text the gene nomenclature is based on the nomenclature developed initially for *S. cerevisiae*. Gene symbols comprise three italic letters and a suffixed Arabic number. Wild type genes are designated with capital letters in italics (e.g. *GAL4*), while mutant/deleted alleles of the genes are indicated in lowercase italic (e.g. *gal4*). This is, in some cases, also used for description of the mutant strain genotypes. Encoded proteins are indicated by roman type, with the first letter capitalized (e.g. Gal4).

ACE2	Transcriptional factor involved in regulation of morphogenesis
ACT1	Actin
ALS1-9	Adhesin Like Sequence, adhesins
AUF1-8	Adhesion Upregulated Factor, unknown
BCR1	Biofilm Cell wall Regulator, transcriptional factor
BGL2	1,3-β-glucosyltransferase
CHS1-7	Chitine Synthase
CHT1-3	Chitinase
CPH1, 2	Candida Pseudohyphal Regulator, transcriptional factor
CRH11, 12	Glycosyl hydrolases
CSA1, 2	Candida Surface Antigen, unknown function
CSH1	Cell Surface Hydrophobicity, cell wall protein
CZF1	*C. albicans* Zinc Finger protein, transcriptional factor
ECE1	Extent of Cell Elongation, unknown
ECM33	Unknown cell wall protein
EFG1	Enhanced Filamentous Growth, transcriptional factor
ENG1	Endo-1,3-β-glucanase
EXG2	Predicted exo-1,3-β-glucosidase
GLC3	Predicted β-1,4-glucan branching enzyme
GSC1	β-1,3-Glucan Synthase Catalytic subunit
GSL1	Glucan Synthase Like, β-1,3-glucan synthase subunit
HWP1	Hyphal Wall Protein, adhesin
HYR1	Hyphally Regulatedr, cell wall protein
IFF4	IPF Family F, adhesin
KRE1, 9	Killer Resistant, β-1,6-glucan biosynthesis involved
KRE62	β-1,3-glucan synthase subunit
lacZ	β-galactosidase from *E. coli*
MSB1	Involved in ranscriptional regulation of adhesins
MP65	Mannoprotein of 65 kDa, involved in glucan metabolism
PGA1-115	Protein GPI Anchored, various functions
PHR1, 2	pH Responsive, glycosidases
PIR1	Protein with Internal Repeats, structural cell wall protein
PLB3-5	Phospholipase B
PRA1	pH Regulated Antigen, unknown cell surface protein
RBT1, 4, 5	Repressed by Tup1, cell wall proteins of various functions
RIM101	Transcriptional factor involved in pH response
SAP1-9	Secreted Aspartyl Proteinase

SCW4, 11	Unknown essential cell wall proteins
SKN1	Involved in β-1,6-glucan biosynthesis
SOD1-6	Superoxide Dismutase
SPR1	Predicted exo-1,3-β-glucanase
SUN41	Predicted cell wall glycosidase
SWI1	Transcriptional factor, subunit of chromatin remodeling complex
TDH3	Glyceraldehyde-3-phosphate dehydrogenase
TEC1	Transcriptional factor in pheromone response pathway
TEF2	Translational Elongation Factor
TUP1	Transcriptional corepressor
UTR2	Transglucosidase
WOR1	White-Opaque Regulator, transcriptional factor
XOG1	Exo-1,3-β-glucanase
YWP1	Yest Wall Protein, anti-adhesin

3 INTRODUCTION

3.1 Introduction to *Candida albicans*

The fungus *Candida albicans* phylogenetically belongs to the phylum of *Ascomycota* and the class of *Saccharomycetes*. *Candida* is a genus which comprises about 150 species, of which most are non-pathogenic and not able to grow at 37℃. Only a very small number of *Candida* species are facultative pathogens, among those are *C. albicans*, *C. tropicalis*, *C. dubliniensis* and *C. glabrata* (Calderone, 2002). *C. albicans* contains a diploid 2 x 16 Mb genome in eight chromosome pairs and reproduces by budding (Berman and Sudbery, 2002). It is a saprophytic facultative aerobe which can be found on the mucosa of the mouth, pharynx, genital and intestinal tract of warm-blooded animals and humans as well as on the skin especially between toes and fingers (Calderone, 2002).

C. albicans often lives as a harmless commensal on the skin and mucosa of healthy individuals. Actually, about 60% of the healthy population is estimated to carry *Candida* species (Calderone, 2002). Clearly, changes in the host immune system and often related changes in body-associated microflora (e.g. following treatment with broad-spectrum antibiotics) are the major determinants of deep seeded candidal pathogenesis. These symptomatic infections can range from surface infections, such as thrush and vaginal yeast infections, to more serious and life-threatening systemic infections, particularly in immunocompromised individuals. Chemotherapy, immunosuppression after tissue transplantation and HIV infections are generating a growing pool of individuals susceptible to this kind of systemic infections (Corner and Magee, 1997) which carry high mortality rates of approximately 40% (Krcmery and Barnes, 2002). The specific mechanisms by which the organism might sense disturbances in its normal commensal relationship with the host and transform itself into a pathogen, however, are not well understood.

One of the factors discussed as *C. albicans* virulence factors is dimorphism, the ability to grow in more than one morphological form (Figure 1). *C. albicans* grows either as budding yeast cells, as filamentous hyphae (chains of elongated, parallel-sided cells lacking constrictions at the septa) or pseudohyphae (chains of variably elongated cells with constrictions at the septa). The two filamentous morphologies, however, are fundamentally different and can be distinguished by a number of characteristics (Sudbery*, et al.*, 2004).

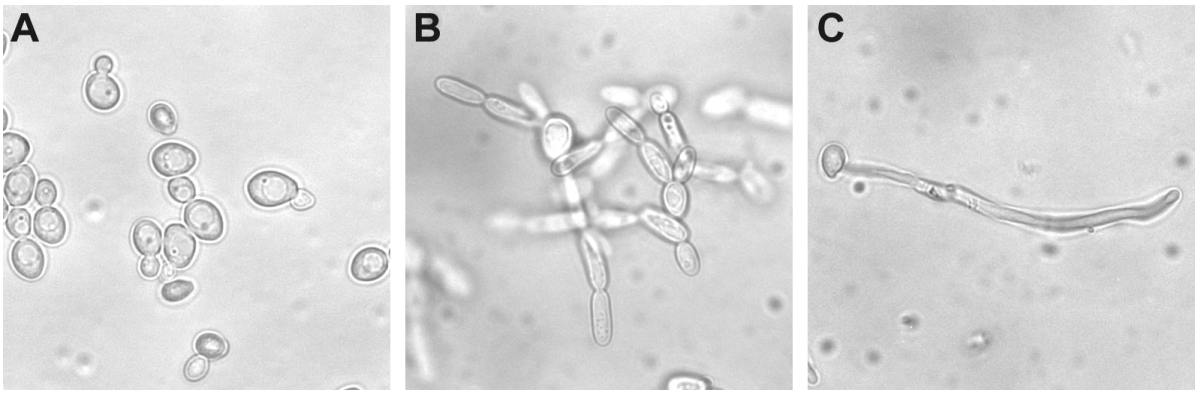


Figure 1. Morphological stages of *Candida albicans*.

Three morphological stages of *C. albicans* are presented: yeast stage - blastospores (A), pseudohyphal form (B) and the induction of filamentation (C).

3.2 Candidal Infections

In recent years several review articles focusing on host pathogen interactions were published (Hube, 2004; Kumamoto, 2008; Romani*, et al.*, 2003; Rupp, 2007). *Candida albicans* is an extremely successful pathogen of human beings. Unlike most other medically important fungi, such as *Histoplasma capsulatum*, *Cryptococcus neoformans* and *Aspergillus fumigatus*, *C. albicans* is rarely found in environmental niches such as soil. It has to be more considered as a part of the human microbial flora. This is indicated by the fact that *C. albicans* is highly adapted to and specialized for survival and proliferation on host surfaces. Most often, endogenous *C. albicans* infections are established by cells that normally colonize mucosal surfaces or skin as harmless commensals. Therefore a significant amount of infections caused by *C. albicans* is derived from a population of Candida cells acquired prior to infection (Taylor*, et al.*, 2003).

It is generally accepted that *C. albicans* can precisely sense its environment and run specific transcriptional programs allowing it to grow in various niches offering different osmolarity, pH, temperature, nutrient and oxygen availability. The human gut, for example, representing the natural reservoir of *C. albicans* in healthy individuals, significantly differs, especially with respect to pH, microflora, metabolism of the epithelium, to the vaginal environment where *C. albicans* colonizes in women suffering from vulvo-vaginal candidiasis. Even more, *C. albicans* can also grow on completely artificial surfaces like plastic devices and for example leading to biofilm

formation in catheters, which represents a significant problem especially in intensive care units (Douglas, 2003).

Many genes have been shown to be differentially expressed in response to conditions mimicking different host niches. The site specific transcriptional adaptation of *C. albicans* to different host niches during infection as well as interaction with immune cells is mostly involving metabolic pathways, as a response to different nutrient availability, and oxidative stress response in contact with immune cells (for review see (Kumamoto, 2008)).

In fact, adaptation to the different environments is accomplished by complex regulatory networks in *C. albicans* that are tightly controlled in response to environmental stimuli. This is ensured by regulation of many signal transduction pathways, including MAP kinase pathways or cyclic AMP/protein kinase A pathway (Biswas*, et al.*, 2007; Staib*, et al.*, 2000).

The effect of specific transcriptional profiles as a response to different environments can be demonstrated in case of two differentially pH regulated genes. The pH-regulated genes *PHR1* and *PHR2*, both encoding putative glycosidases with similar functions, are expressed differentially according to the pH of the environment (Muhlschlegel and Fonzi, 1997; Saporito-Irwin*, et al.*, 1995). The alkaline pH-upregulated gene *PHR1* was shown to be expressed and required for systemic (neutral pH) but not vaginal (low pH) infections. Conversely, mutants lacking *PHR2*, which is induced at acidic pH, are compromised in their ability to cause vaginal but not systemic infections (De Bernardis*, et al.*, 1998). Also the genes *RBT1* and *RBT4* have been shown to be required for *C. albicans* to cause lethal infection following intravenous inoculation and for invasive infection of the cornea, however, these genes are not required for colonization of the intestinal tract (Braun*, et al.*, 2000).

To the genes allowing colonization of multiple host sites families of differentially regulated isozymes/proteins belong (SAP proteases and ALS adhesins with different substrate specificity). The finding that distinct *SAP* genes are expressed preferentially during symptomatic disease (pathogenic stage) and not during asymptomatic carriage (commensal stage), even on the same mucosal surface, indicates that at least the expression of certain genes is modulated during the transition from a commensal to a pathogen (Naglik*, et al.*, 2003).

Although disruptions of *C. albicans* genes encoding for adhesive proteins, secreted hydrolytic enzymes, iron permeases and regulators of cell morphogenesis

attenuate mortality in animals with disseminated candidiasis, no individual gene encodes a dominant determinant of candidal virulence (Calderone and Fonzi, 2001).

3.3 Hypha Formation

Yeast to hypha transition in *C. albicans* appears as a response to environmental conditions like starvation, adherence, hypoxia, elevated CO_2 levels, 37℃ temperature, neutral pH, and presence of N-acetylglucosamine, L-proline or serum in cultivation media. Morphogenesis appears to be crucial for pathogenesis (reviewed by (Kumamoto and Vinces, 2005b)). Cells that are trapped in either the yeast (Lo*, et al.*, 1997; Rocha*, et al.*, 2001), filamentous (Bendel*, et al.*, 2003) or pseudohyphal (Braun and Johnson, 1997) states are less virulent in murine systemic infection models. Thus, the determinants of the morphological yeast-to-hyphal switch appear to be very important for virulence.

Hyphal cells have stronger adhesive properties (e.g. owing to the expression of the Als adhesins) (Hoyer, 2001) that may help the fungus to adhere to endothelial cells. Hyphal cells, but not yeast cells, have been also shown to further induce phagocytosis by endothelial cells, a mechanism that is discussed as a potential strategy of *C. albicans* to escape from the bloodstream (Phan*, et al.*, 2000; Zink*, et al.*, 1996). Furthermore, hyphae are thought to exert mechanical force, further damaging the endothelial cells and aiding the escape into the deeper tissue (Calderone and Fonzi, 2001).

C. albicans cells phagocytosed by macrophages induce hyphae and secrete hyphal-associated proteinases that together with the mechanical penetration of the surrounding membranes aid in killing of the macrophage (Borg-von Zepelin*, et al.*, 1998). Besides, hyphae express additional specific substances with potential to inhibit killing by neutrophils (Smail*, et al.*, 1992). Expression of superoxide dismutases for example aids in disrupting the oxidative burst of phagocytic cells (Nantel*, et al.*, 2002). Hyphal morphogenesis is thus an integral part of the overall virulence strategy of *C. albicans.* By several approaches, sets of genes that are expressed exclusively in the hyphal state have been identified. Signal transduction cascades, modulated by elements such as cAMP, mitogen-activated protein kinases, and pH-responsive modules appear to regulate this yeast-to-hyphal transition (Whiteway, 2000). Recent reviews have comprehensively described the molecular

details of such signaling pathways (Figure 2) as well as genes that are important for forming a hyphal structure (Biswas, *et al.*, 2007).

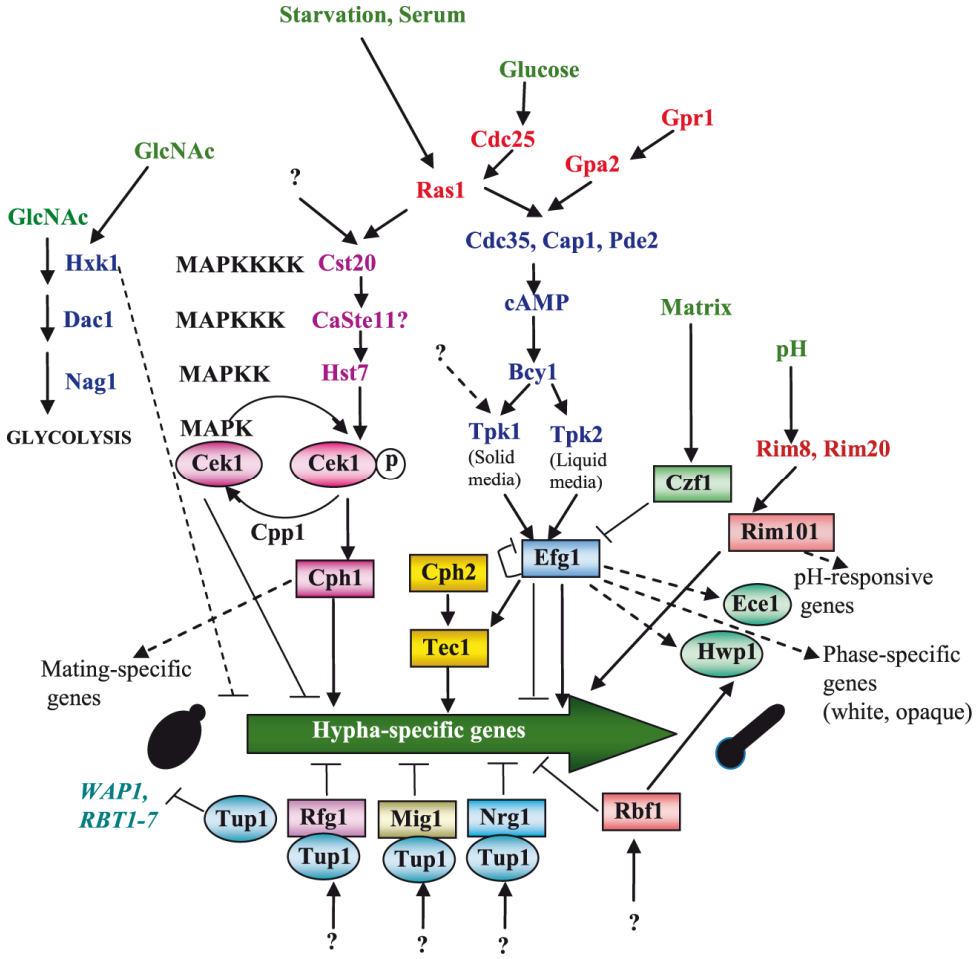


Figure 2. Regulation of dimorphism in *C. albicans* by multiple signaling pathways.
Stimuli and complex signaling cascades responsible for change of *C. albicans* morphology, with transcription factors presented in rectangular boxes (Biswas, *et al.*, 2007).

In vitro studies show that multiple transcription factors regulate overlapping sets of genes during growth under different conditions. In particular, several transcription factors regulate the expression of the hypha co-regulated genes. In addition to Efg1 and Cph1, those are for example Cph2, Tec1, Czf1, Rim101, and Tup1.

Expression of numerous genes is altered during hyphal morphogenesis (Kadosh and Johnson, 2005; Lane, *et al.*, 2001; Nantel, *et al.*, 2002). Actually, many genes found to be important for infection are also upregulated during yeast to hypha transition (Kumamoto and Vinces, 2005b). It is clear that genes which control hyphal morphology are co-regulated with genes that encode virulence factors without any effect on the morphogenesis itself. This co-regulation ensures that the morphological

conversion of yeast-form cells to hyphae occurs under conditions when proteolytic and lipolytic activities and adhesions, that enhance virulence, are also expressed. Thus, formation of hyphae is a part of the overall virulence strategy of *C. albicans.* Among genes induced during hypha formation belong the adhesins *ALS1*, *ALS3* and *HWP1*, proteases *SAP4*, *SAP5* and *SAP6*, and virulence factors *RBT1* and *RBT4*. Recently it has been also described that hypha-specific promoters (*ALS3*, *ECE1*, *HGC1*, *HWP1*, *HYR1*, *RBT1*, and *RBT4*) are unusually long compared to *C. albicans* gene promoters in general. The average length of the upstream intergenic regions for these seven hypha-specific genes is 4.5 kb (3-9 kb compared to 0.5-1 kb for other genes). This indicates high complexity of morphogenetically regulated promoters in *C. albicans* (Argimon*, et al.*, 2007). Similar long promoters of genes involved in morphogenesis have been described previously even in other yeasts like *S. cerevisiae* (Rupp*, et al.*, 1999).

3.3.1 Transcription Factors Efg1 and Cph1

Transcription factors Efg1 and Cph1 were originally identified by their ability to promote pseudohyphal growth in the non-pathogenic yeast *S. cerevisiae* (Liu*, et al.*, 1994; Stoldt*, et al.*, 1997). Efg1 (Stoldt*, et al.*, 1997), a member of the APSES family of basic helix–loop–helix transcriptional regulators, is one of the best-characterized regulators of hyphal morphogenesis in *C. albicans* and it is a key regulator of virulence. As its relatives, pseudohyphal activator *PHD1* and repressor *SOK2* of *S. cerevisiae*, *EFG1* has been reported to be downstream of the Ras-cAMP-PKA pathway (Sonneborn*, et al.*, 2000). The second transcription factor, Cph1, is a homeodomain protein and a homologue of *S. cerevisiae* Ste12. It is known to be downstream of a MAP kinase cascade in *C. albicans* and involved in hypha formation on solid media (Liu*, et al.*, 1994). When both genes *efg1* and *cph1* are deleted, *C. albicans* is practically unable to filament (Lo*, et al.*, 1997). Overexpression of *CPH1* showed that Efg1 and Cph1 regulate a largely overlapping set of genes (Lane*, et al.*, 2001).

As mentioned previously, many hypha induced genes are co-regulated together with genes encoding for known virulence factors including proteases and adhesins. Efg1 is involved in the regulation of many of those genes including *SAP4*, *SAP5*, *SAP6*, *ALS1*, *ALS3*, *HWP1*, *RBT1*, *RBT4*, *ECE1* (Doedt, et al., 2004; Fu, et al., 2002; Harcus, et al., 2004; Sharkey, et al., 1999; Schroppel, et al., 2000; Sohn, et

al., 2003). Besides these known pathogenesis related genes, Efg1 and Cph1 also regulate many proteins involved in the cell wall biogenesis like chitinases *CHT1*, *CHT2* and *CHT3*, chitin synthases *CHS1*, *CHS2*, *CHS4* and *CHS5*, β-1,3-glucan synthase subunits *GSL1*, *GSC1* and *KRE62*, β-1,4-glucan branching enzyme *GLC3*, β-1,6-glucan biosynthesis genes *KRE1* and *SKN1*, glycosyl hydrolase *CRH11*, glycosidases *PHR1* and predicted *SUN41* and glucosidase *EXG2* (Doedt*, et al.*, 2004; Harcus*, et al.*, 2004; Sohn*, et al.*, 2003) probably participating also in the cell wall changes during morphology switching. Thus Efg1 and Cph1 belong to the most important transcription factors regulating the cell wall biogenesis in *C. albicans*. Alterations in the cell walls are also followed by altered immunological properties of the strains deleted for *efg1* and *cph1* (Barker*, et al.*, 2008; Dongari-Bagtzoglou and Kashleva, 2003; Korting*, et al.*, 2003; Lu*, et al.*, 2006; Phan*, et al.*, 2000; Villar*, et al.*, 2004). Following all these changes, *C. albicans* deleted for both transcription factors *cph1* and *efg1*, in contrast to the *cph1* strain or the congenic clinical isolate SC5314, is neither able to adhere to, nor to penetrate *in vitro* adhesion and invasion models, while the strain deleted for *efg1* shows partial defects (Dieterich*, et al.*, 2002). Moreover, strain deleted for *efg1* shows reduced virulence in mouse systemic infection model, while strain deleted for both genes *efg1* and *cph1* is almost avirulent (Lo*, et al.*, 1997).

3.4 Cell Wall of *Candida albicans*

One of the key structures mediating host-pathogen interaction is the fungal cell surface defined by the polysaccharide cell wall and its integrated protein components. The cell wall is an essential structure surrounding the cell and is characteristic for all fungi. It has two essential roles, to maintain the integrity of the cell and to interact with the environment. The cell wall is also the contact point between the microbe and host surfaces including phagocytic cells. Since the cell wall is not present in mammals, it is a prime target for medical treatments.

The cell wall of *C. albicans* and its protein components were recently reviewed (Chaffin, 2008). Structural rigidity of the cell wall is conferred by polysaccharides which also maintain the cell shape. Candida must balance cell wall integrity with cell expansion in order to grow and allow the insertion of more constituents. Thus, synthesis, remodeling, and hydrolysis must be coordinated in time and space to provide integrity, flexibility and cell separation. The protein components of the cell

wall play also a role in maintaining of the structural integrity. Besides that they mediate adherence, whether to host or microbes, or may have additional enzymatic functions.

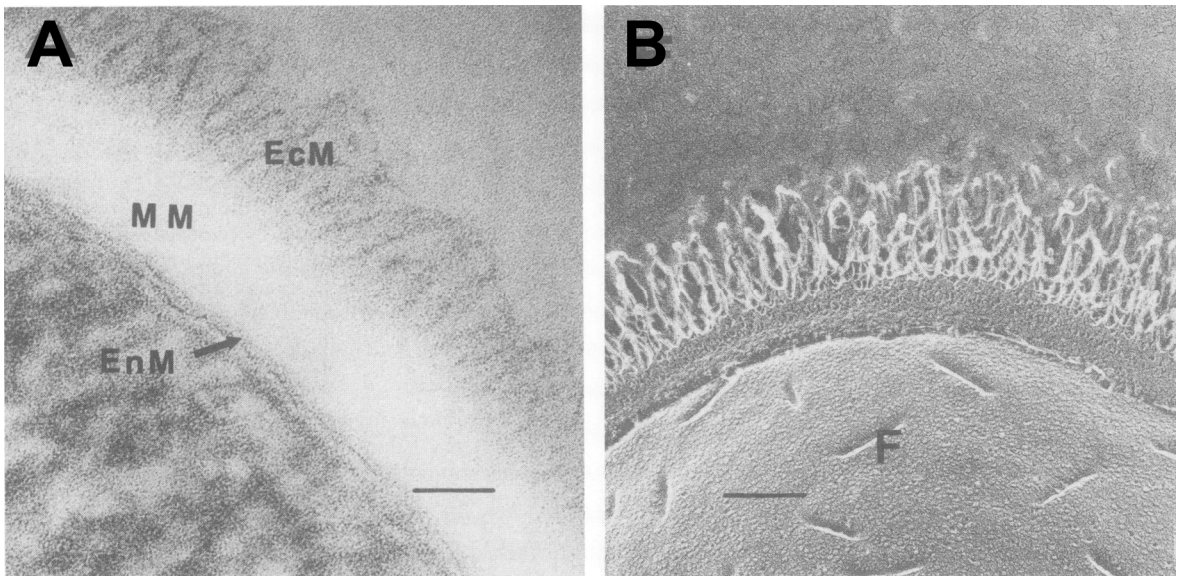


Figure 3. Electron microscopy images of *C. albicans* cell wall.

Images of *C. albicans* cell wall using transmission and scanning electron microscopy with cell freeze-fracturing (Hazen and Hazen, 1992). Thin section through the cell wall of freeze-substituted, uranyl acetate-stained *C. albicans* (A) shows three (endomural, EnM; mesomural, MM; and ectomural, EcM) general layers of the wall. The electron-dense ectomural basement region is the site of attachment of the mannoproteinaceous fibrils. Freeze fractured cell wall (B) presents its three dimensional fibrillar ectomural layer. The fibrillar structure changes with hydrophilicity/hydrophobicity of the cells. In general hydrophilic cell populations show highly fibrillar structures compared to the hydrophobic ones.

Electron microscopy of thin sections of the *C*. *albicans* cell wall shows layers which appear to be derived from differential abundances of cell wall constituents (Figure 3) (Klis*, et al.*, 2001; Ruiz-Herrera*, et al.*, 2006). The inner layer, enriched for chitin and glucans, is more electron translucent than outer layers, which are enriched for mannoproteins. Structurally, the outer portion of the cell wall appears to have perpendicularly aligned fibrils that differ in length correlated with surface hydrophobicity (Hazen and Hazen, 1992; Tokunaga*, et al.*, 1986). In the inner polysaccharide matrix there is a flexible, three-dimensional network of branched β-1,3-glucan to which β-1,6-glucan and chitin are attached through their reducing ends. Some chitin may also be attached to β-1,6-glucan. The inner part of the cell wall close to the plasma membrane is rich in chitin that forms chains of tight antiparallel hydrogen-bonded structures associated with high insolubility. The more distant layers

are dominated by glucans which can also have hydrogen-bond-mediated local alignments. The cell wall proteins (CWPs) covalently attached to this meshwork of structural fibrillar polysaccharides are in the most outer layer of the cell wall and can be distinguished into two classes. The first and most abundant class of CWPs is linked to β-1,6-glucan through a glycophosphatidylinositol (GPI) remnant (GPI-CWP). The second class of proteins, termed Pir (proteins with internal repeats), are linked directly to the β-1,3-glucan with alkali-labile ester linkage (Ecker*, et al.*, 2006; Kandasamy*, et al.*, 2000; Kapteyn*, et al.*, 2000). There is also a third class of proteins that lack the covalent attachment to the polysaccharide matrix. Some of these proteins may be heterogeneously distributed at the surface like in case of Pra1 (described later).

Many of the cell wall proteins have enzymatic functions and are involved in its processing and remodeling. A combination of GPI-CWPs and soluble noncovalently linked enzymes contribute to this vital activity (for example glucosyltransferases like Phr1, Phr2 and Bgl2; transglucosidases Pga4, Crh11, Crh12 and Utr2; glucanases Xog1 and Mp65; chitinases Cht1 and Cht2; chitin synthases Kre9 and Kre1; glucan synthases or glucanases Exg2, Spr1 and Eng1; structural protein Pir1; other cell wall remodeling enzymes Scw4, Scw11, Eng1 and Sun41 and many others) (Chaffin, 2008).

In the cell wall there are also many enzymes associated with pathogenesis and degradation of extracellular compounds (phospholipases Plb3, Plb4.5 and Plb5; proteases Sap9 and Sap10; superoxide dismutases Sod4, Sod5 and Sod6). Some of these proteins do not remain cell associated, but end in the extracellular environment. They have the capacity to hydrolyze large or complex substrates into small units that can be transported into the cell as a source of nutrition (SAP proteases, phospholipases, and lipases).

3.4.1 GPI Anchored Proteins - PGAs

In the *C. albicans* genome 115 putative GPI-anchored cell wall proteins were identified (66 % with unknown function) and sorted into 25 families (Richard and Plaine, 2007). Characterized GPI proteins have mostly a function in cell wall biogenesis and remodeling, cell adhesion and diverse enzymatic properties. The expression of some GPI-CWPs differs between yeast cell and hyphal morphology, and their expression responds to regulators of this process (Chaffin, 2008; Richard

and Plaine, 2007). Because of the putative localization of these proteins at the cell surface, they are in a very good position to be involved in interactions with the surrounding environment, including the host cells.

In terms of pH, oxidative stress, phagosomes, etc. *C. albicans* is highly adapted to its environment, compared to other opportunistic fungi. This suggests that during evolution it has developed several mechanisms to colonize its host. Specialization of *C. albicans* as a pathogen colonizing various environments could lead to the unusual amount of GPI-anchored proteins. The number of putative GPI proteins identified in *C. albicans* (115) is almost twice that identified in *S. cerevisiae* (58). However, the number of these proteins for both *C. albicans* and *S. cerevisiae* are the results of computerized predictions and may retain some annotation artifacts.

3.5 Adhesion

The ability to adhere to a surface is a widely distributed biological phenomenon that share highly diverse organisms to capture their respective habitats. A tight interaction of the organism with its environment contributes to its survival. Microorganisms use adhesion to different surfaces, including epithelia, in order to colonize them. Similarly, many different fungi, especially pathogens including *Candida albicans*, *Histoplasma capsulatum* or *Aspergillus fumigatus*, for example, adhere tightly to human epithelia to colonize their corresponding hosts (Mendes-Giannini*, et al.*, 2005).

Adherence of *C. albicans* to different cellular substrates requires the expression of adhesive structures on the surface of the fungus that are able to interact very tightly with the host tissues. Among those structural motifs are for example α-1,2 and β-1,2 linked oligomannosides present on glycolipids and proteins of the cell wall, that are crucial for the adhesion of *C. albicans* to Caco-2 cells (Dalle*, et al.*, 2003). But in addition to these common structural motifs the expression of specific adhesive proteins including Hwp1 (mentioned later) has evolved to mediate efficient adhesion (Staab*, et al.*, 1999).

Contact with a surface has also specific effects on transcriptional profile of *C. albicans*. Initiation of biofilm formation may occur as soon as a cell comes in contact with a surface. Fungal cells are surrounded by a rigid cell wall and lack appendages that may serve as sensory organelles. Nonetheless, by 30 min after contact of *C. albicans* yeast cells with a polystyrene surface, a gene expression program is initiated that is distinct from that of planktonic cells grown under otherwise similar

conditions (Murillo*, et al.*, 2005; Sohn*, et al.*, 2006). Actually, the transcriptional response to a surface was reported to be very fast, the promoter activity increases within 15-30 minutes after adherence (Mateus*, et al.*, 2004). The signaling pathways that mediate the gene expression response to surface contact has yet to be identified, but for example the MAP kinase Mkc1 was reported to be involved in this process (Kumamoto, 2005). This MAP kinase is the downstream target of the *C. albicans* cell wall integrity pathway (Monge*, et al.*, 2006), which responds to a variety of cell wall stresses. Thus, the interesting possibility is that the cell wall itself serves as a contact sensor, perhaps through a constraint of its elasticity by multiple surface contacts (Klis*, et al.*, 2006).

In general, yeast adhesins are of necessity mosaic proteins, because they need several domains with discrete functions. Their localization on the outer surfaces of thick cell walls determines the order of the domains and therefore determines their overall architecture. In the cell wall there are many adhesive proteins and deletion of one single gene reduces adhesion but does not completely abolish it (Zhao*, et al.*, 2004), showing that the cells express many adhesins with partially overlapping functions. The most prominent adhesins are described in the following section.

3.5.1 Proteins Involved in Adhesion Process

Adhesion of Candida cells to various substrates is a complex process affected by many factors. Basically there are three functionally distinct groups of proteins affecting the adhesion.

The first group involves transcription factors affecting the expression rate of adhesins and genes that are maintaining the proper cell wall structure. Among the transcription factors affecting the cell wall and the Candida adhesion belong Efg1 and Cph1. Strains deleted for *efg1* and *efg1*/*cph1* show a strong reduction of adhesive properties as well as a severely changed transcriptional profile of genes encoding for putative cell wall proteins (Dieterich*, et al.*, 2002; Sohn*, et al.*, 2003). For additional genes *WOR1/EAP2*, *SWI1*, *MSB1*, *ACE2* and *TEC1* similar roles were suggested, based again on expression experiments in *S. cerevisiae*, where they improve its adhesive properties (Li and Palecek, 2005).

The second group of proteins, with mostly indirect action in the adhesion process, acts through changes of the cell wall structure. One of such genes is *UTR2*/*CSF4*. The encoded GPI anchored cell wall glycosidase has a negative effect

on filamentation and also adhesion to mammalian cells when deleted (Alberti-Segui*, et al.*, 2004). Additionally, deletion of 1,3-beta-glucosyltransferase *bgl2* leads to aggregation of stationary cells (Goldman*, et al.*, 1995), what is often observed in strains with changes of adhesive properties. Also Sun41, a predicted glycosidase, has been shown to be involved in adhesion to mammalian cell line, when the null mutant strain displays lower adherence and defects in biofilm formation (Hiller*, et al.*, 2007). Ecm33 is another GPI anchored cell wall protein involved in adhesion. Mutant cells have a tendency to flocculate in a medium-dependent manner, have severe defects in the cell wall structure and have modestly reduced adherence to endothelial and oral epithelial cells (Martinez-Lopez*, et al.*, 2004; Martinez-Lopez*, et al.*, 2006). Another protein Mp65 is functionally a putative β-glucanase, with a sequence containing an RGD motif that has been implicated in a variety of adhesins (La Valle*, et al.*, 2000). Besides the inability of the mutant cells to form germ tubes, the cells adhere with a lower efficiency to polystyrene surfaces (Sandini*, et al.*, 2007). Recently we have published (as equal first author) a similar function for the cell surface glycosidase *PHR1* (Calderon*, et al.*, 2010). Its deletion negatively affects adhesion of *C. albicans* to abiotic polystyrene surface and *in vitro* tissue model. This is further supported by evidence of negative regulation of several genes encoding for cell wall proteins (including *HWP1* adhesin), probably as a feedback due to the cell wall defects.

The third group is consisting of the adhesive proteins themselves. The largest family of known adhesins in *C. albicans* is the *ALS* (agglutinin-like sequence) gene family. It has been already reviewed with all its members (Hoyer, et al., 2008; Chaffin, 2008). The Als family members are cell-wall GPI-anchored proteins known to interact with several different substrates including fibronectin, laminin, collagen and others (Hoyer, 2001; Kumamoto and Vinces, 2005a; Sheppard*, et al.*, 2004; Sundstrom, 2002). Als adhesins mediate adhesion to epithelia, yeast aggregation, biofilm formation, and *ALS* gene activity accompanies *C. albicans* pathogenesis. The family is encoded at least at eight loci, and each locus may be heterozygous (Zhao*, et al.*, 2003), thus creating great variation in sequence. Briefly, the encoded proteins have three domains (Hoyer, 2001; Sheppard*, et al.*, 2004). The N-terminal region is relatively conserved among Als proteins and contains a putative signal peptide followed by the substrate specific binding domain. The following globular Ig-like domain homologous to the *S. cerevisiae* sexual adhesin α-agglutinin is necessary

and sufficient for binding to the cell surfaces. It contains tandem repeats (~36 aa), which can vary in number between genes and alleles and can be used to classify three subgroups (Hoyer, 2001). The serine-threonine-rich C-terminal region, which contains also a glycosylphosphatidylinositol anchor sequence, has multiple glycosylation sites that are predicted to lead to a stretched conformation of the protein. To the most widely expressed members belong *ALS1* and *ALS3.* Als1 binds to endothelia and epithelia and its expression is maximal just after inoculation into fresh growth medium (Zhao*, et al.*, 2004), while *ALS3* expression is maximal when germ tubes are microscopically visible, and it may be the cause of the well-known autoaggregation of germ tubes (Nobile*, et al.*, 2006a; Zhao*, et al.*, 2004). It has also been reported that Als3 is involved in iron acquisition from the host by binding of ferritin, an iron binding protein in epithelia (Almeida*, et al.*, 2008).

The hypha-specific cell wall protein Hwp1/Ece2 is another GPI-modified cell wall protein involved in adhesion. There are structural and functional similarities to α-agglutinin and *Sc*Flo1 (Staab and Sundstrom, 1998). *HWP1* encodes an outer mannoprotein, with a cell surface-exposed N-terminal domain and C-terminal features conferring covalent integration into cell wall β-glucan (Staab*, et al.*, 1996; Staab and Sundstrom, 1998). Indeed, the majority of the Hwp1 molecules (about 75 %) is being covalently linked to the β-glucan as predicted (Staab*, et al.*, 2004). Although tandem repeated sequences were found, *HWP1* is not part of a family of *C. albicans* genes that vary in the copy number of tandemly repeated sequences as was reported for Als adhesins (Hoyer, 2001). Hwp1 plays an important role in adherence to epithelial cells (Staab*, et al.*, 1999), where it serves as a substrate for the epithelial enzyme transglutaminase and can be stably cross-linked to the surface of the epithelial cells. Therefore, Hwp1 mediates very tight binding of *C. albicans* cells to the host cells (Staab*, et al.*, 1999). Hwp1 shows additional involvement in *C. albicans* cell-cell interactions and adhesion independent of its transglutaminase substrate activity (Nobile*, et al.*, 2006a; Nobile*, et al.*, 2006b). *HWP1* has no general homologue in other yeast species besides closely related *Candida dubliniensis* (51.4/56.9), *Candida africana* (86.6/87.1, subspecies of *C. albicans*), *and Candida tropicalis* (41.8/42.7). Even Hwp1 from the closely related *C. dubliniensis* is significantly shorter (421 aa, compared to 634 aa for *C. albicans*), and may be non-functional.

There are several less studied proteins with possible adhesive functions. Eap1/Pga47 is an adhesive protein containing a GPI anchor. Its expression promotes binding to polystyrene and to a human embryonic kidney cell line (Li and Palecek, 2003). These properties are those required for stable adhesion to indwelling catheters and other devices. *EAP1* is expressed even in yeast cells and the transcript is only slightly increased during hypha formation (Nantel*, et al.*, 2002).

CSH1/IFD4 was identified as a protein responsible for cell hydrophobicity, which has been implicated as a contributor to adherence to host extracellular matrix as well as host cells and resistance to macrophage killing. The deletion of *CSH1* decreases cell surface hydrophobicity about 75% and decreases adherence to fibronectin (Singleton*, et al.*, 2001). Most of the 38-kDa protein is in the cytoplasm, however, Csh1 can be found in the cell wall extracts and the amount of protein on the cell surface is increasing in extracts of hydrophobic cells compared to what was seen for hydrophilic cells (Singleton and Hazen, 2004).

The GPI-anchored yeast-specific Ywp1/Pga24/Flo1/Tep1 is responsible for decrease in adherence of yeast cells (Granger*, et al.*, 2005). It has some structural similarity to the *S. cerevisiae* adhesin Flo1 (de Groot*, et al.*, 2004). In a biofilm model that contains only yeast cells, deletion of *ywp1* improves adherence, while the overexpression abolishes it (Granger*, et al.*, 2005). Ywp1 expression is greatest on yeast cell surface just after the end of the most rapid exponential growth and thereafter declines; it is also decreased during filamentation. When cells reach the stationary phase of growth, the fraction of soluble Ywp1 secreted to the medium increases, showing possible release from the cell walls (Granger*, et al.*, 2005). However, the direct action of Ywp1 is not clear yet.

ECE1 is a gene encoding for putative single-pass membrane protein of unknown function. Ece1 does not resemble an adhesin: it is comprised of novel 34-residue repeats that surround a possible transmembrane domain. Recently its contribution to biofilm formation has been shown and the idea that Ece1 functions in adhesion is suggested by observation that the overexpression of *ECE1* in *bcr1* mutant strain, with reduced biofilm formation, leads to increase in biofilm as in case of *ALS3*, *ALS1* or *HWP1* adhesin overexpression (Nobile*, et al.*, 2006a). However, its mechanism of action is uncertain.

Additionally, GPI anchored protein Iff4, has been recently reported to be directly involved in adhesion, when its deletion leads to decreased adhesion of the

respective strain to plastic and silicone catheters (Kempf*, et al.*, 2007; Kempf*, et al.*, 2009). Also integrin-like protein Int1 (Gale*, et al.*, 1996) seems to be directly involved in adhesive properties of *C. albicans* (Gale*, et al.*, 1998). GPI anchored Rbt5, involved primarily in hemoglobin utilization, may also have some adhesive functions. When overexpressed in *bcr1* mutant strain, it improves its biofilm formation (Nobile*, et al.*, 2006a). Secreted aspartic proteases (SAPs) were described to have a minor role in adhesion to various surfaces as well (Watts*, et al.*, 1998). However, the mechanism of action, else direct involvement in adhesion or change of the cell wall structure, is unclear.

3.5.2 Identification of Genes Relevant for Adhesion

Many adhesins like *EAP1* and *ALS3* have been identified and characterized by their expression in *S. cerevisiae* (Gaur and Klotz, 1997; Li and Palecek, 2003). In order to mimic more closely the host-pathogen interaction novel strategies were designed to identify additional proteins with possible adhesive functions. Sohn *et al.* (Sohn*, et al.*, 2006) identified several candidates by transcriptional profiling of *C. albicans* cells incubated on various substrates, both abiotic polystyrene and epithelial *in vitro* models. Expression of genes *PGA7*, *PGA23, PRA1* and *AUF8* was in all cases induced during adhesion to different substrates, but not expressed under identical conditions in suspension culture. The putative cell wall localization of Pga7, Pga23 and Pra1 as well as predicted plasma membrane localization for Auf8 puts them in a good position as proteins directly or indirectly involved in adhesion. These four proteins were further studied in this work.

3.5.2.1 PGA7

Using a bioinformatic approach, *PGA7* and *PGA23* were originally identified as genes encoding for putative GPI-anchored cell wall proteins in *C. albicans* (De Groot*, et al.*, 2003). Pga7 consists of 219 amino acids and has several homologues in other fungi, but none of them was functionally characterized so far. As reported in the literature, *PGA7* is transcriptionally active under several conditions including response to antimicrobial/ion chelating agents, pH, oxidative stress and during cell wall regeneration of protoplasts (Barelle*, et al.*, 2008; Bensen*, et al.*, 2004; Castillo*, et al.*, 2006; Hromatka*, et al.*, 2005; Lee*, et al.*, 2005; Liu*, et al.*, 2005; Sigle*, et al.*, 2005).

PGA7 is also differentially expressed (first down- and then upregulated) in planktonic cells during first 390 minutes of biofilm formation (Murillo*, et al.*, 2005). This short term cultivation is similar to the original experiments, where the *PGA7* was identified as gene induced during adhesion process (Sohn*, et al.*, 2006). Pga7 together with the proteins Pga7, Rbt5, Pga10, Csa2 and Csa1 share a cysteine-rich CFEM domain that has been identified as common in proteins involved in fungal pathogenesis (Kulkarni*, et al.*, 2003). The CFEM domain is the most conserved amino acid region in this group and is indicating its functional importance for these proteins. Moreover, Rbt5, Pga10 and Csa1 have all been identified to be implicated in biofilm adherence (Nobile*, et al.*, 2006a; Perez*, et al.*, 2006) indicating a possible function for Pga7.

Interestingly, overexpression of *PGA7* in a *rim101* strain from the *pTDH3* promoter restores partially interaction of this strain with oral epithelial cells, by enhancing endocytosis, but it does not enhance cell damage. When *PGA7* was overexpressed in a wild-type strain, decreased damage of oral epithelia was observed (Nobile*, et al.*, 2008b).

3.5.2.2 PGA23

The second GPI-anchored protein of interest, Pga23, is consisting of 271 amino acids. The function of Pga23 is completely unknown to date. It is only known as being induced during osmotic stress and heat stress through transcription factor Hsf1 (Nicholls*, et al.*, 2009) and regulated in response to pH (Ramon and Fonzi, 2003). *PGA23* is also upregulated by Cas5 (transcription factor involved in cell wall damage response) during exposure to caspofungin (Bruno*, et al.*, 2006) and is induced in *sur7* mutant strain with cell wall defects (Alvarez*, et al.*, 2008). In the *C. albicans* genome, there are no significantly homologous proteins to Pga23. No significantly homologous proteins (except *C. dubliniensis*) were identified in other organisms either.

3.5.2.3 PRA1

Pra1 was identified in early 90's as a 58 kDa fibrinogen binding protein localized on *C. albicans* cell surface (Casanova*, et al.*, 1992), but its fibrinogen binding activity was revised recently (Marcil*, et al.*, 2008). Immunogenic reactivity against Pra1 is commonly found in blood sera of patients suffering from candidiasis (Sepulveda*, et*

al., 1998; Thomas*, et al.*, 2006). *PRA1* encodes a protein of 299 aa in length, predominantly localized to hyphae (Lopez-Ribot*, et al.*, 1995; Sentandreu*, et al.*, 1998; Urban*, et al.*, 2003). *PRA1* is transcriptionally active in pH dependent manner with maximum expression occurring at neutral pH and with no expression detected below pH 6.0 (Bensen*, et al.*, 2004; Sentandreu*, et al.*, 1998). In hyphae strong expression on mRNA and mostly on protein level appears with some indications for additional posttranscriptional regulation (Choi*, et al.*, 2003). The protein is *in vivo* highly glycosylated of mass around 58 kDa, while its sole protein prediction shows only 31 kDa (Sentandreu*, et al.*, 1998). Pra1 does not seem to be covalently linked to the cell wall, since it is easily extractable with detergents like SDS (Sentandreu*, et al.*, 1998) and also is commonly found in the cell culture supernatant (Hiller*, et al.*, 2007; Soloviev*, et al.*, 2007). Due to successful construction of a deletion mutant strain, *PRA1* is not an essential gene. However, *pra1* deletion leads under heat stress conditions to inability of the respective strain to form hyphae and to altered chitin distribution in the cell wall (Sentandreu*, et al.*, 1998). Recently an interaction of Pra1 with $\alpha_M\beta_2$ (Mac-1, CD11b/CD18, CR3) integrin, a leukocyte receptor involved in *C. albicans* recognition and present on human polymorphonuclear neutrophils, was reported. A strain lacking *pra1* is also killed by neutrophils with much lower efficiency than the wild-type (Soloviev*, et al.*, 2007). However, this effect can not be observed in contact with macrophages (Marcil*, et al.*, 2008). Pra1 was also shown to interact with complement regulators Factor H and Factor H like protein-1 as well as plasminogen, which could possibly regulate host complement attack to the fungus (Luo*, et al.*, 2009). Interestingly, besides closely related and pathogenic *Candida dubliniensis* and *Candida tropicalis*, there are many predicted homologous proteins to Pra1 in other fungal species (*Debaryomyces hansenii*, *Pichia stipitis*, *Pichia pastoris*, *Pichia guilliermondii*, *Magnaporthe grisea*, *Penicillium chrysogenum*, *Aspergillus nidulans*, *Aspergillus fumigatus* and others), often with identities over 50%. In fact, Pra1 is closely related to the *Aspergillus fumigatus* antigen Aspf2 (43.3/59.6), induced under low zinc conditions (Segurado*, et al.*, 1999), as well as to the *S. cerevisiae* homologue Zps1 (25.7/44.1), a GPI-anchored protein induced under low-zinc conditions and alkaline pH (Lyons*, et al.*, 2000). Thus it seems that Pra1 homologues with similar pH and zinc regulation are widely spread even in non-pathogenic fungi, and often contain a GPI anchor.

3.5.2.4 AUF8

ORF19.3908 (*AUF8* – Adhesion Upregulated Factor 8) was identified besides *PGA7, PGA23 and PRA1* to be upregulated during adhesion process (Sohn*, et al.*, 2006). *AUF8* encodes a protein consisting of 204 aa and is predicted to carry 4 transmembrane domains with the highest probability of localization to the plasma membrane. 6 additional ORFs encoding for proteins with significant similarity to Auf8 were identified in *C. albicans* genome. However, none of the seven ORFs was functionally characterized so far.

3.5.3 Aims of Work

The aim of this work is to contribute to the understanding of cell wall biogenesis in *C. albicans*. For this purpose structural changes in the cell wall, induced by deletion of two key regulators of cell wall biogenesis Efg1 and Cph1 were analyzed, focusing on polysaccharide composition, structure and immunogenicity of the respective strains. Furthermore, a set of four genes *PGA7*, *PGA23, PRA1* and ORF19.3908 (*AUF8*) identified previously as upregulated during adhesion and encoding for predicted cell surface proteins, were functionally characterized with respect to adhesion and invasion behavior of *C. albicans in vitro*, in order to understand their contribution to virulence mechanisms in this organism.

4 MATERIAL

4.1 Lab Equipment

Table 1. Laboratory equipment.

Equipment	Company
Agarose gel electrophoresis	Amersham Pharmacia Biotech EPS 301 power supply Amersham Pharmacia Biotech HE 99X gel system
Autoclave	SX-700E, Tomy Digital Biology
Base Molds for paraffin blocking	Labonord
Centrifuges	Heraeus Megafuge 1.0, rotor number 2704, Thermo Scientific Heraeus Biofuge pico, tabletop centrifuge, Thermo Scientific Heraeus Biofuge fresco, tabletop cooling centrifuge, Thermo Scientific neoLab micro centrifuge
Colony counting pen	Carl Roth
Electronic cell counter	Schärfe System CASY COUNTER 1
Freezer, -20℃ and -80℃	Liebherr Comfort (-20℃) Heraeus Instruments (-80℃)
Gel documentation system	Raytest Camilla
Heating blocks	Digi-Block® -JR, Laboratory Devices
Heating plates	Thermal stirrer 34533, Snijders
Laminar flow hoods for cell culture	Gelaire BSB 4A, Gelaire PTY
Incubators for *Candida albicans*	Heraeus FunctionLine Memmert BF40
Incubators for cell culture, invasion and adhesion assays	Heraeus FunctionLine B12 Heraeus Hera Cell CO_2-Inkubator (B 5060 EK-CO_2)
Incubators for histological processing (60℃)	Heraeus Instruments
Improved Neubauer cell counting chamber	Carl Roth
Incubator with shaker	HAT Infors AG
Micropipettes	Gilson
Microscopes	Axiovert 25 (for black and white documentation), Zeiss Axiovert 200M (for colored documentation), Zeiss
Microtome	Leica RM2145
Microwave	Moulinex Optimo Duo Optiquick, Samou
pH meter	Greisinger Electronic Mess- und Regeltechnik
Paraffin heating bath	Medax
Pipetting aids	Brand

Real Time PCR cycler	LightCycler 480, Roche Mastercycler ep realplex, Eppendorf
Retsch mill	Retsch MM200, Retsch
Scales	Sartorius BP 410, Sartorius Sartorius BP 121S, Sartorius
Spectrophotometers	Thermo Spectronic Genesys10uv, Thermo Electron
Software for processing of biological images	Aida Image Analyzer Version 4.15, Raytest
Tissue processor for embedding	Shandon, Citadell 1000, Thermo Electron
Stretching table for microscopy slides	Medax
Thermocycler	Hybaid TouchDown PTC200 MJ Research, Thermo Electron
Water baths	Medingen W12, MS-L Julabo UC, Julabo GFL Type 1008, Hilab

4.2 Lab Consumables

Table 2. Laboratory consumables.

Product	**Company**
Cell culture flask 75 cm^2	Greiner
Cell culture 24well polystyrene plate	Greiner
Cell culture inserts for 24 well plates: ThinCert™ 12 mm ∅, 0.4 µm pore size	Greiner
Cell scraper	Sarstedt
Coverslips for histological samples (18 x 18mm and 18 x 36mm)	Carl Roth
Cuvettes	Carl Roth
Embedding Cassettes	Labonord
Glass beads (5 mm and 0.25 mm)	Carl Roth
Glass slides (76 x 26 mm)	Carl Roth
Sterile filter units	Carl Roth
Tubes 1.5ml and 2.0ml	Eppendorf, Carl Roth
PCR tubes 0.2 ml	Biozym Scientific
Petri dishes, 90 mm	Sarstedt
Pipette tips	Steinbrenner Laborsysteme
15 ml and 50 ml tubes with screw caps	Greiner

4.3 Chemicals

All the used chemicals were ordered in the highest purity from the following companies: Carl Roth GmbH, Fluka and Sigma Aldrich. For culture media and buffer preparation the deionized water was used, using Ionic-exchange filtering device from the Millipore company.

Table 3. List of chemicals.

Company	Chemicals
Carl Roth	Acetic acid, Acetone, Ammonium persulphate, Ammonium sulphate, Ampicillin sodium salt, Bacto agar, β-mercaptoethanol, BSA, Bromphenolblue (sodium salt), Caffeine, Chloramphenicol, dCTPs, Di-sodiumhydrogenphosphate, ethanol, D-Mannit, EDTA, Ethidiumbromide, Formaldehyde, Formamide, Glutaraldehyde, Glycine, Glucose monohydrate, Glycerol, , HEPES, Hydrochloric acid, L-Lactate, Lithium chloride, Methanol, Methylene blue, Meyers hematoxilin,MES, MOPS, Nitric acid, Periodic acid, Phenol, Phenol-chloroform-isoamylalcohol (25:24:1), Phosphoric acid, Potassium acetate, Potassium chloride, Potassium di-hydrogen phosphate, Potassium hydroxide, Roticlear, SDS, Sodium acetate, Sodium bisulphite solution, Sodium chloride, Sodium Hydroxide, Sodium hypochlorite, Sulphuric acid, Tris, Trichloracetic acid, Tri-sodium citrate dihydrate, Triton X, Urea, Xylene cyanol, Xylol
Bio-Rad	Acrylamide-/(N,N'-Methylenebisacrylamide) solution: 40% solution, 37.5:1, TEMED
Invitrogen Gibco	Amphotericin B, Desoxyribonucleotides (100mM), DMEM, DMEM powder, FCS, Gentamicin, Sodium pyruvate, Tryphan blue, Trypsin/EDTA (0.25% Trypsin, 1 mM EDTA)
Becton Dickinson	Bacto peptone, ChromAgar, Difco Nutrient Broth, Difco YNB w/o AA and NH_4SO_4, Difco Yeast Carbon Base
Biozym Scientific	Biozym LE agarose
Sigma-Aldrich	Amino acids, 4-aminoantipyrine, Bouin's solution, Calcofluor White, Coomassie brilliant blue, Congo Red, DEPC, Diamide, DMSO, DTT, Ferrozine, INT, IPTG, Lithium acetate, Menadione, NAD, Osmium tetraoxide, Paraquat, Phenazine methosulphate, Ponceau s, Polychromic staining solution EA50, Rnase Zap, Schiff's reagent, Tween-80, X-gal, Uranyl acetate, XTT sodium salt
Schärfe-System	Casyton
Merck	Yeast extract
Labonord	Isomount, Paraffin wax (t_m 54-56℃)
Hartmann analytic	[α-^{32}P]-dCTP

4.4 Enzymes

All the enzymes were used according to the instructions of the producer in combination with the recommended buffers.

Table 4. Enzymes and Antibodies.

Enzyme	Supplier/Producer
Restriction Enzymes	New England Biolabs
Pfu-DNA-polymerase	Stratagene
RNase A	Sigma Aldrich
Shrimp Alkaline Phosphatase	New England Biolabs
T4-DNA Ligase	New England Biolabs
Zymolyase 100T	Seikagaku
Glucose oxidase	Sigma Aldrich
Peroxidase	Sigma Aldrich
β-1,3-glucan antibody	Gentaur

4.5 Other Material (Reaction Kits)

Table 5. List of kits and other material.

Kit/Product	Supplier/Producer
QIAprep Spin Miniprep Kit	Qiagen
QIAEX Gel Extraction Kit	Qiagen
RNeasy Mini Kit	Qiagen
RNeasy Midi Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
Prime-It® II Random Primer Labeling Kit	Stratagene
ECL Detection Kit	Amersham
RC DC Protein Assay	Biorad
QuantiTect SYBR Green PCR Kit	Qiagen
QuantiTect Reverse Transcription Kit	Qiagen
Light Cycler 480 Hydrolysis Probes UPL	Roche
Light Cycler 480 Probes Master	Roche
1 kb DNA Ladder	New England Biolabs

4.6 Tissue Cell-lines, Bacterial and Yeast Strains

Table 6. Tissue cell lines used in the work.

Cell line number	Type of cell	Reference
A-431 (ATCC CRL-1555)	Vaginal carcinoma	(Giard*, et al.*, 1973)
Caco-2 (ATCC HTB-37)	Colorectal adenocarcinoma cell line	(Fogh*, et al.*, 1977)
RAW264.7 (ATCC TIB-71)	Murine macrophage cell line	(Raschke*, et al.*, 1978)

Table 7. *E. coli* strains used in the work.

Strain	Genotype
DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–
Sure (Stratagene)	endA1 glnV44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC::Tn5 uvrC e14- Δ(mcrCB-hsdSMR-mrr)171 F'[$proAB^+$ $lacI^q$ lacZΔM15 Tn10]

Table 8. Strains of *C. albicans* used in the work.

Strain	Original Name	Genotype	Parent	Reference
SC5314	SC5314	Wild-type	-	(Gillum*, et al.*, 1984)
CAI4	CAI4	*ura3::imm434/ura3::imm434*	SC5314	(Fonzi and Irwin, 1993)
efg1	HLC52	*ura3::imm434/ura3::imm434 efg1::hisG/efg1::hisG-URA3-hisG*	CAI4	(Lo*, et al.*, 1997)
efg1/cph1	HLC54	*ura3::imm434/ura3::imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG-URA3-hisG*	JKC18*	(Lo*, et al.*, 1997)
HLC69	HLC69	*ura3::imm434/ura3::imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG*	HLC54	(Lo*, et al.*, 1997)
efg1::*EFG1*	HLC74	*ura3::imm434/ura3::imm434 efg1::hisG/efg1::hisG (EFG1)*	HLC67	(Lo*, et al.*, 1997)
HLC17	HLC17	*ura3::imm434/ura3::imm434 efg1::hisG-URA3-hisG /EFG1*	CAI4	(Lo*, et al.*, 1997)
HLC46	HLC47	*ura3::imm434/ura3::imm434 efg1::hisG /EFG1*	CAI4	(Lo*, et al.*, 1997)
HLC17Rev	HLC17Rev	*ura3::imm434/ura3::imm434 EFG1/ efg1*::[*EFG1p-HA-EFG1-URA3*]	HLC46	This work
HLCEEFG1	HLCEEFG1	*ura3::imm434/ura3::imm434 efg1*::*hisG/efg1*::[*EFG1p-HA-EFG1-URA3*]	HLC67	(Noffz*, et al.*, 2008)
efg1::*EFG1*/*cph1*	HLC84	*ura3::imm434/ura3::imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG (EFG1)*	HLC69	(Lo*, et al.*, 1997)
cph1	JKC19	*ura3::imm434/ura3::imm434 cph1::hisG/cph1::hisG-URA3-hisG*	CAI4	(Liu*, et al.*, 1994)
pga7	*pga7*	*ura3::imm434/ura3::imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*	BWP17	(Plaine*, et al.*, 2008)

pga23	*pga23*	*pga7::ARG4/pga7::URA3 ura3::imm434/ura3::imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG pga23::ARG4/pga23::URA3*	BWP17	(Plaine*, et al.*, 2008)
BWP17	BWP17	*ura3::imm434/ura3::imm434 his1::hisG/his1::hisG arg4::hisG/arg4::hisG*	RM1000 *	(Wilson*, et al.*, 1999)
pra1	CAMB43	*ura3::imm434/ura3::imm434 pra1::hisG/pra1::hisG-URA3-hisG*	CAI4	(Sentandreu*, et al.*, 1998)
pra1::PRA1	CAMB9	*pra1::hisG/PRA1-pUC18-URA3-pra1 ura3::imm434/ura3::imm434*	CAMB435	(Sentandreu*, et al.*, 1998)
auf8		*auf8::FRT/auf8::FRT*	SC5314	This work
auf8::AUF8		*auf8::FRT/auf8::AUF8-FRT*	*auf8*	This work
auf1		*auf1::FRT/auf1::FRT*	SC5314	This work
auf1::AUF1		*auf1::FRT/auf1::AUF1*	*auf1*	This work
auf2-8		*auf2-8::FRT/auf2-8::FRT*	SC5314	This work
auf2-8::AUF8		*auf2-8::FRT/auf2-8::AUF8*	*auf2-8*	This work
auf1-8		*auf1::FRT/auf1::FRT auf2-8::FRT/auf2-8::FRT*	*auf1*	This work
auf1-8::AUF8		*auf1::FRT/auf1::FRT auf2-8::FRT/auf2-8::AUF8-FRT*	*auf1-8*	This work
auf1-8::AUF1		*auf1::FRT/auf1::AUF1-FRT auf2-8::FRT/auf2-8::FRT*	*auf1-8*	This work
pTDH3-AUF8		*AUF8/pTDH3-AUF8*	SC5314	This work
AUF8GFP		*AUF8/AUF8GFP*	SC5314	This work
pTHDH3-AUF8GFP		*AUF8/pTDH3-AUF8GFP*	*pTDH3-AUF8*	This work
CAI4 *URA3*		*RP10::URA3/RP10 ura3::imm434/ura3::imm434*	CAI4	This work
CAI4 *pACT1_PGA7*		*RP10::URA3-pACT1-PGA7/RP10 ura3::imm434/ura3::imm434*	CAI4	This work
CAI4 *pACT1_PGA23*		*RP10:: URA3-pACT1-PGA23/RP10 ura3::imm434/ura3::imm434*	CAI4	This work
CAI4 *pACT1_PRA1*		*RP10:: URA3-pACT1-PRA1/RP10 ura3::imm434/ura3::imm434*	CAI4	This work
CAI4 *pACT1_HWP1*		*RP10:: URA3-pACT1-HWP1/RP10 ura3::imm434/ura3::imm434*	CAI4	This work
CAI4 *pACT1_lacZ*		*RP10:: URA3-pACT1-lacZ/RP10 ura3::imm434/ura3::imm434*	CAI4	This work
HLC69 *URA3*		*RP10:: URA3-URA3/RP10 ura3::imm434/ura3::imm434 cph1::hisG/cph1::hisG*	HLC69	This work

	efg1::hisG/efg1::hisG		
HLC69 *pACT1-PGA7*	*RP10:: URA3-pACT1-PGA7/RP10 ura3::imm434/ura3::imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG*	HLC69	This work
HLC69 *pACT1-PGA23*	*RP10:: URA3-pACT1-PGA23/RP10 ura3::imm434/ura3::imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG*	HLC69	This work
HLC69 *pACT1-PRA1*	*RP10:: URA3-pACT1-PRA1/RP10 ura3::imm434/ura3::imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG*	HLC69	This work
HLC69 *pACT1-HWP1*	*RP10:: URA3-pACT1-HWP1/RP10 ura3::imm434/ura3::imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG*	HLC69	This work
HLC69 *pACT1-lacZ*	*RP10:: URA3-pACT1-lacZ/RP10 ura3::imm434/ura3::imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG*	HLC69	This work

* Originally derived from CAI4.

Table 9. Strains of *S. cerevisiae* used in the work.

Strain	Genotype	Parental strain	Reference
10560-14A	MATa**,** leu2::hisG	Σ1278b	(Guo*, et al.*, 2000)

4.7 Plasmids

Name	Description	Reference
pAU36RP10_KKf	Integrative *C. albicans* plasmid derived from pCaEXP (Care*, et al.*, 1999); *URA3*, Amp^R, pUC-ori, *RP10*, *pACT1* promoter, *lacZ*, *tACT1* terminator	M. Roehm
pAU36-PGA7	Integrative *C. albicans* plasmid derived from pAU36RP10_KKf; *PGA7* gene under actin promoter for overexpression	This study
pAU36-PGA23	Integrative *C. albicans* plasmid derived from pAU36RP10_KKf; *PGA23* gene under actin promoter for overexpression	This study
pAU36-PRA1	Integrative *C. albicans* plasmid derived from pAU36RP10_KKf; *PRA1* gene under actin promoter for overexpression	This study

pAU36-HWP1	Integrative *C. albicans* plasmid derived from pAU36RP10_KKf; *HWP1* gene under actin promoter for overexpression	This study
pAU36-empty	Integrative *C. albicans* plasmid derived from pAU36RP10_KKf; for *URA3* complementation	This study
pRS425TEF	Plasmid for *S. cerevisiae* overexpression; *lacZ*, *LEU2*, 2µ, Amp^R, pMB1-ori, *pTEF1* promoter, polylinker, *tCYC1* terminator	(Mumberg*, et al.*, 1995)
pRS425A8	*S. cerevisiae* overexpression plasmid derived from pRS425TEF with *AUF8* ORF under *pTEF1* promoter	This study
pRS425A8G	*S. cerevisiae* overexpression plasmid derived from pRS425TEF with *GFP* tagged *AUF8* ORF under *pTEF1* promoter	This study
pSFS1A	*E. coli* plasmid with cassette for gene deletions in *C. albicans*; flipase under *pSAP2* inducible promoter and *tACT1* terminator, *SAT1* dominant marker under *pACT1* promoter	(Reuss*, et al.*, 2004)
pSFS1A_d1	Plasmid derived from pSFS1A with cassette for deletion of *AUF1* gene	This study
pSFS1A_d8	Plasmid derived from pSFS1A with cassette for deletion of *AUF8* gene	This study
pSFS1A_d28	Plasmid derived from pSFS1A with cassette for deletion of *AUF2-8* genes	This study
pSFS1A_r1	Plasmid derived from pSFS1A with cassette for reintroduction of *AUF1* gene into the original locus	This study
pSFS1A_r8	Plasmid derived from pSFS1A with cassette for reintroduction of *AUF8* gene into the original locus	This study
pSFS1A_r8b	Plasmid derived from pSFS1A with cassette for reintroduction of *AUF8* gene into *auf2-8* deleted region	This study
pSFS1A_o8	Plasmid derived from pSFS1A with cassette for overexpression of *AUF8* gene under *pTDH3* promoter in its locus	This study
pUC119	*E. coli* cloning vector; M13-ori, Amp^R, pMB-ori, *lacZ* including polylinker	(Vieira and Messing, 1987)
pGFP-SAT1	Plasmid derived from pUC119 containing cassette for PCR amplification and *GFP* tagging; GFP, *tADH1* terminator, SAT1 dominant marker under pACT1 promoter an followed by *tURA3* terminator	This study
pYFP-SAT1	Plasmid like pGFP-SAT1 but for YFP tagging	This study
pCFP-SAT1	Plasmid like pGFP-SAT1 but for CFP tagging	This study
pTD38-HA	Plasmid for reintegration of HA-tagged *EFG1* into its own locus, *URA3* marker	(Noffz*, et al.*, 2008)

4.8 Buffers and Solutions

Table 10. List of buffers and solutions used in the work.

Buffer or solution	Composition *
DEPC water	0.1% (v/v) DEPC in dH2O Stirring for 6 h at RT, autoclaved
DNA loading buffer (10x)	0.25% (w/v) Bromophenol blue 1 mM EDTA pH 8.0 50% (w/v) Glycerol
Gelificant solution	103 ml DMEM (5.4 g DMEM powder dissolved in 103 ml distilled water) 15 ml 3 M HEPES 20 ml 7.5% sodium bicarbonate 40 ml FCS 4 ml 10 M NaOH 2 ml Gentamicin Storage at 4℃
PBS buffer	140 mM NaCl 3 mM KCl 8 mM Na_2HPO_4 1 mM KH_2PO_4 Adjusted to pH 7.4
PBS^- buffer	140 mM NaCl 3 mM KCl 1 mM KH_2PO_4 8 mM Na_2HPO_4 1 mM EDTA Adjusted to pH 7.4
RNA-loading buffer	1 x RNA MOPS buffer (from 10 x stock solution) 2.25 M Formaldehyde 50% formamide 16.5% DNA loading buffer 10x Storage at -80℃
RNA MOPS Buffer 10x	0.2 M MOPS pH 7.0 80 mM NaOAc 10 mM DEPC-EDTA DEPC H_20 – stored in dark
Sodium bisulphite solution	1100 ml distilled water 66 ml 10% sodium bisulphite stock solution 55 ml 1 N HCl
20 x SSC Buffer	3 M NaCl 0.3 M tri-Sodium Citrate 2-hydrate Adjusted to pH 7.0
10 x TAE Buffer	0.4 M Tris 0.2 M Acetic acid 22 mM EDTA
10 x TE Buffer	100 mM Tris-HCl, pH 7.5 10 mM EDTA
Yeast Lysis Buffer	2 % (w/v) Triton X-100 1% (w/v) SDS

	100 mM NaCl
	10 mM Tris HCl, pH 8.0
	1 mM EDTA
TFB1	30 mM KOAc
	50 mM $MnCl_2$
	10 mM KCl
	10 mM $CaCl_2$
	15 % glycerol
TFB2	10 mM Na-MOPS, pH 7.0
	75 mM $CaCl_2$
	10 mM KCl
	15 % glycerol
Church Buffer	7% SDS
	1% BSA
	1 mM EDTA
	250 mM Na-PO_4 buffer pH 7.2 (70 mM NaH_2PO_4 and 180 mM NA_2HPO_4)
Depurination Buffer	0.25 M HCl in dH_2O
Denaturation Buffer	2% (w/v) NaOH
	8.77% (w/v) NaCl in dH_2O
Neutralization Buffer	8.77% (w/v) NaCl
	6.05% (w/v) Tris pH 7.0 in dH_2O
Protein isolation buffer	8 M Urea
	50 mM Tris-HCl pH 6.8
	5% SDS
	0.1 mM EDTA
	1.5% DTT
Protein Electrophoresis	25 mM Tris
Buffer 10 x	192 mM glycine
	0.1% (w/v) SDS
Resolving gel buffer	1.5 M Tris-HCl pH 8.8
	0.4% (w/v) SDS in water
Stacking gel buffer	0.5 M Tris-HCl pH 6.8
	0.4% (w/v) SDS in water
	0.01% bromophenol blue
Protein loading buffer 4 x	40 mM Tris-HCl pH 8.0
	4 mM EDTA
	4% (w/v) SDS
	40% glycerol
	20% β-Mercaptoethanol
	0.02% bromophenol blue
Ponceau s solution	0.2% Ponceau s in 3% TCA
PBST	1 x PBS
	0.05% Tween 20
Towbin Buffer	25 mM Tris-HCl pH 8.0
	192 mM glycine
	20% methanol in water
Buffer A	50 mM Tris-HCl pH 7.9
	50 mM glucose
	1 mM EDTA
Storage Buffer	50 mM Tris-HCl pH 7.9

	50 mM KCl 0.1 mM EDTA 1 mM DTT 0.5 mM PMSF (100 mM stock in isopropanol) 50% glycerin
Lysis buffer	10 mM Tris-HCl pH 7.9 50 mM KCl 1 mM EDTA 0.5% NP-40 0.5% Tween-20
LDH buffer	0.2 M Tris pH 8.2 0.5% lactic acid 0.1% Triton X 12 ml of buffer prior to experiment was mixed with 16 mg of chemical mixture (167 mg INT, 43 mg PMS, 431 mg NAD)
Binding buffer	100 mM $NaHCO_3$ 0.5 M NaCl Adjusted pH 8.3
Elution Buffer	0.1 M Glycine Adjusted pH 2.8
ROS detection Solution	5.5 ml HBSS buffer (Invitrogen) 11 µl luminol (0.1 M stock) 220 µl HRP (2 kU/ml)
HEPES-buffered BSA	10 mM HEPES pH 7.3 5.5 mM glucose 1 mM $MgCl_2$ 5 mM KCl 145 mM NaCl 4 mM $NaHCO_3$ 1 mM $CaCl_2$ 0.1% BSA

* All buffers were stored at room temperature unless otherwise noted.

4.9 Media

Table 11. List of media used in the work.

Medium	Composition
LB	1% Trypton peptone 0.5% Yeast extract 0.5% NaCl pH adjusted to 7.0
YPE	1% Yeast extract 2% Bacto Peptone
YPD	1% Yeast extract 2% Bacto Peptone 2% glucose
SC	0.17% YNB w/o AA and NH_4SO_4

	0.5% NH_4SO_4
	0.2% AA-mix minus 5 (Ade, His, Leu, Trp, Ura)
YNB	0.17% YNB w/o AA and NH_4SO_4
	0.5% NH_4SO_4
Cornmeal agar	1.7% Cornmeal agar
	1% Tween-80
YCB-BSA	2.34% YCB
	0.1% BSA
Spider agar	1% DIFCO Nutrient Broth
	1% D-Mannit
	0.2% K_2HPO_4
	2% agar
SLAD agar	0.17% YNB
	50 µM NH_4SO_4
	2% glucose
	2% agar
Supplemented DMEM	DMEM with 0.45% glucose (Gibco)
	10% FCS
	1% Gentamicin from a 10 mg/ml stock solution
	1 mM sodium pyruvate
α-MEM agar	1.02% α-MEM
	0.22% $NaHCO_3$
	2% glucose
	adjusted to pH 7.4

Solid media were having the same composition like liquid media containing additionally 2% agar. All the media containing additional amino acids and bases were supplemented after autoclaving with filter sterilized, water stock solutions to the final concentration of 50 µg/ml Ade (5 mg/ml stock ~ 30 mM), 60 µg/ml His (20 mg/ml stock ~ 100 mM), 200 µg/ml Leu (20 mg/ml stock ~ 100 mM), 80 µg/ml Trp (8 mg/ml stock ~ 40 mM), 50 µg/ml Uri (5 mg/ml stock ~ 20 mM) and 22 µg/ml Ura (2.2 mg/ml stock ~ 20 mM). LB was supplemented with ampicillin to final concentration 100 µg/ml (filter sterilized, water stock solution 100 mg/ml) or chloramphenicol to 10 µg/ml (filter sterilized, methanol stock solution 25 mg/ml). Buffered media were containing additional 100 mM buffers (Tris, MOPS, MES, HEPES) and pH was adjusted by hydrochloric acid and sodium hydroxide.

4.10 Oligonucleotides

Table 12. Oligonucleotide sequenced used in the work.

Oligonucleotide	**Sequence 5'→3'** (restriction sites underlined)
4691dU_F	AAGAGGGCCCCCTTTTCATATTCGCTCATC
4691dU_R	AGTCCTCGAGCTTATTTTACAAGCACAGCC

4691dD_F	AATACCGCGGATAGTATGTTGTGGTGTTTG
4691dD_R	TAGAGAGCTCTGTCTCCTTGATACACTTTG
3908dU_F	GATAGGGCCCAATCGCACATAAAATGAGAAG
3908dU_R	TACTCTCGAGATTGCGTTCAGAAATGCTTG
3902-08dD_F	AATTCCGCGGTTTTGTAGCTGGGCTAATTG
3902-08dD_R	CTATGAGCTCATTTCTGATACTCTCTAGGTGTG
3902-08dU_F	GAGTGGGCCCATAGTGGATAATACTTAC
3902-08dU_R	CAGGCTCGAGCAGTCCTTTAAATATAAGAGGAG
4691revU_F	GGCTCCGCGGGTAAAATAAGTATCTCGACT
3908revU_F	GCATCCGCGGACGCAATGACACAAGTATTC
pTDH3_F_SacII	TACCGCGGTAGTAGTAGTAGTGGTATGATGC
pTDH3_R_StuI	CTAGGCCTATTGTTAATTAATTTGATTGTAAAG
AUF8_F_StuI	ACTTAGGCCTTAATGAGTACCGCTAGAGTTTATATTG
pGFP-SAT1_F1	TAAGCGCTCAGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAT GGGCCCCGAGCGTCAAAACTAGAGAATAATAAAG
pGFP-SAT1_R1	GTTGATCAATTTTGAATGATTATATTTTTTTAATA
pGFP-SAT1_F2	TATGATCACTCATATGAAAATTTCGGTGATCC
pGFP-SAT1_R2	GTGAATTCATGAGCTCCACCGCGGTGGCGGCCGCTC
3908GFP_F	ATGCAGCATAGCACAAGTAATAATACTGGAACCAAACAAAATGAAG TTGAAAAACAAGAATCAATTGTGTTCGGTGGTGGTTCTAAAGGTGA AGAATTATT
3908GFP_R2	ACAATTAGCCCAGCTACAAAAATTCACAATTAATTATATATATTAT CCACATTACATCAAAGTCAATATTTAGGCCGCTCTAGAACTAGTGG ATC
GFP_Ca_R	TGGACCATCACCAATTGGAG
3908_F_B	ACTTGGATCCATGAGTACCGCTAGAGTTTATATTG
3908GFP_R_X	GTTGCTCGAGCTGTAAAAGATGCAGCTGCAG
3908_R_X	AAGTCTCGAGTTAGAACACAATTGATTCTTGTTTTTC
PGA7_F_XhoI	CGCGCTCGAGATGCATTTCATATTCTACTTGATTC
PGA7_R_BamHI	CGCGGGATCCTTATACAAGAAGGTACATCATGAAT
PGA23_F_XhoI	CGCGCTCGAGATGAGAGTTTCCACATTAGTTTTAT
PGA23_R_BamHI	CGCGGGATCCTTAAATCAAAACCAATGCTGCTAAG
PRA1_F_XhoI	CGCGCTCGAGATGAATTATTTATTGTTTTGTTTAT
PRA1_R_BamHI	CGCGGGATCCTTAACAGTGGACTTCACCATCTGCA
HWP1_F_XhoI	TTTCCTCGAGATGAGATTATCAACTGCTCAAC
HWP1_R_BamHI	GTTAGGATCCTTAGATCAAGAATGCAGCAATAC
pACT1_pAU36_F	TTAATATTAAAAAAATATAATCATTCAAACTCG
PGA7_F	CAGGATGTTTCTGCGTGATG
PGA23_F	GGTGGTCTGGGCTCATCTAA
PRA1_F	TGTCGGTGCTGACAAATCAT
HWP1_500b_F	CCAGAAAAGCCAACAACTCC
HWP1_1000b_F	ATTTACACCACTTACTGTCCATTG
HWP1_1500b_F	CAACCATCTATTCCTGCCGGTG
RP10_F	GGTTTAAAGAATGCCGCTGA
RP10_R	ATTTCACGGCCAATGACTTC
URA3_F	GACCTATAGTGAGAGAGCAG
URA3_R	TCTTGTCCACCCATATCACG
AUF8_F1_B	ACTTGGATCCATGAGTACCGCTAGAGTTTATATTG
AUF8_R1	CCAAAATCATCTTCAGTCCAATCG
EFG1_F1	CCCCCATACCTTCCAATTCT
EFG1_R1	GAGGAGCCGAAGCAGAAGT

Table 13. qRT-PCR oligonucleotide and probe list.

Gene	Primers 5'→3'	Probe / Sybr Green *
GAPDH (mouse)	CATGGCCTTCCGTGTTCCTA GCGGCACGTCAGATCCA	SG
IL1β (mouse)	CAACCAACAAGTGATATTCTCCATG GATCCACACTCTCCAGCTGCA	SG
IL4 (mouse)	CATCGGCATTTTGAACGAGGTCA CTTATCGATGAATCCAGGCATCG	SG
IL10 (mouse)	GGAAGACAATAACTGCACCCA CCCAAGTAACCCTTAAAGTCCTG	SG
IL12 (mouse)	GGAAGCACGGCAGCAGAATA AACTTGAGGGAGAAGTAGGAATGG	SG
IL23 (mouse)	GCAGATTCCAAGCCTCAGTC TTCAACATATGCAGGTCCCA	SG
TNFα (mouse)	CAAAATTCGAGTGACAAGCCTG GAGATCCATGCCGTTGGC	SG
IFNβ (mouse)	TCAGAATGAGTGGTGGTTGC GACCTTTCAAATGCAGTAGATTCA	SG
AUF1 (*C. albicans*)	TTACGTTCATAATGTATGTGTGTTGG CCAAATCTGAATGGTTGTCTTTC	#142
AUF2 (*C. albicans*)	CTGTTGGTTTGGCTTCATCC TTGCAGGAGCTTCATGGATA	#21
AUF3 (*C. albicans*)	GGCTGTATGGTTTTTGATACCG CCTCTTCACTGTAAGAAAACTCTTCAA	#102
AUF4 (*C. albicans*)	TGCTACACTCTATTGTTGACTCCATT TGTGTATAGAATGAACTCACTTGCAG	#55
AUF5 (*C. albicans*)	CCTTGTTTGAGGTCTACGTGTTT TCGAGGTCTTTCAGGTTTCG	#76
AUF8 (*C. albicans*)	CAATTATTGGGGGCTTGAAA CCAATGTACAAAAAGAAAAACGAG	#39
AUF8 (*C. albicans*)	ATTTGGTTTGGTGTGCCAAT TCAGTCCAATCGTCGGTGTA	SG
PGA7 (*C. albicans*)	GCTCGTCATCTACAGGCTCA GCAGAAGATGAAGGAGATGAGG	#35
PGA23 (*C. albicans*)	TCTGGTCCAGCAGGTTTAGG CAGAAGTCTGCAATTGTCTAGTGG	#35
PRA1 (*C. albicans*)	CTCTGATTCAGGGGCCAGTA GGGTTGCTATCGGTATGTTGA	#90
HWP1 (*C. albicans*)	ATTGCTCCAGGTGCTGAAAC TTCCGGAATAGTAATAGCACCAC	#78
TDH3 (*C. albicans*)	GCCGTCAACGATCCATTC AGAATCGTATTTGAACATGTAAGCA	#50
rRNA (*C. albicans*)	CCTTAACGAGGAACAATTGGA TACGCTTTTGGAGCTGGAAT	#66

* Number stands for probe from Roche Universal Probe Library, SG for Sybr Green measurement method.

4.11 Computational Analysis and Software

For protein and nucleotide blast searches Candida genome database (www.candidagenome.org) and National Center for Biotechnology Information (blast.ncbi.nlm.nih.gov) were used. For quantification of DNA and RNA bands from gels Aida Image Analyzer software v4.15 was used. Transmembrane spanning domains prediction was performed with TMHMM v2.0 (www.cbs.dtu.dk/services/TMHMM/). Microscopic images were processed using Zeiss Axiovision software. All the figures were adjusted with use of Adobe Photoshop CS2.

5 METHODS

5.1 Sterilization of Media and Instruments

Media components, buffers and laboratory equipment were sterilized by hot steam autoclaving (121℃, 120 kPa, and 20 min). Media and buffers that are not recommended to be autoclaved were sterilized by sterile filtration through Millipore filters with pore size 0.22 µm.

5.2 Cultivation and Storage of *C. albicans* and *S. cerevisiae* Strains

All the strains of *C. albicans* and *S. cerevisiae* were cultivated in the lab at 30℃. For the sufficient agitation and aeration of liquid cultures, the volume of the cultivation medium did not exceed 1/5 of the total volume of cultivation flask. Strains on the agar plates were stored at 4℃ and were not used for inoculation for longer than two weeks. Strains carrying auxotrophy were cultivated in minimal media (YNB, SC) containing the essential supplements. Strains were stored long-term in 30% glycerol at -80℃. The freshly prepared cell culture from the agar plate was resuspended in 30% glycerol, vortexed and stored at -80℃.

5.3 Cultivation and Storage of *E. coli* Strains

Bacterial strains were cultivated at 37℃ in LB medium supplemented for ampicillin or chloramphenicol if necessary (strains carrying plasmids). Strains were stored in 20% glycerol at -80℃. To the fresh liquid culture 1/5 of the volume of 100% glycerol was added, vortexed and placed at -80℃. On the agar plates, strains were stored at 4℃ no longer than for 7 days.

5.4 Measuring of the Cell Optical Density

Optical density (OD_{600}) of the cell suspension was measured at wavelength 600 nm. Since the optical density is equal to cell concentration only in a certain range, the cell suspension was always diluted by water to be measured in the linear range between 0 and 1.

5.5 Isolation of Plasmid DNA from *E. coli*

For purification of pDNA from *E. coli* strains the Spin Miniprep Kit (Qiagen) was used. The protocol of the manufacturer was followed and the plasmid was usually eluted into 30 µl of sterile dH_2O resulting usually in yield 0.2-0.5 µg/µl.

5.6 Isolation of Genomic DNA from *C. albicans*

Genomic DNA was isolated from Candida strains by the Phenol/Chloroform method. Up to 10 ml of stationary phase culture grown in YPD for 14 to 18 h at 30℃ was spun down (2 min, 6,000 x g). The cell pellet was transferred to 2 ml Eppendorf tube, resuspended in 300 µl lysis buffer. Equal volume (300 µl) of 0.25 mm glass beads and a Phenol-Chloroform-Isoamylalcohol (25:24:1) mixture was added. The suspension was vortexed for 20 min at 4℃ in order to lyse the yeast cells. Afterwards the mixture was spun down (10 min, 16,000 x g). The water-phase supernatant (containing nucleic acids) was transferred to a 1.5 ml Eppendorf tube and the gDNA was precipitated by adding 1 ml of 100% ethanol. The mixture was vortexed and incubated at -20℃ for at least 15 min. The precipitated nucleic acids were spun down (10 min, 4℃, and 16,000 x g). The pellet was resuspended in 300 µl 1 x TE buffer pH 8.0 with 3 µl of RNase A (10 mg/ml stock) in order to get rid of any RNA contaminants. The nucleic acids were precipitated again at -20℃ for at least 15 min after adding 1 ml of 100% ethanol. The nucleic acids were pelleted again by centrifugation (4℃, 16,000 x g, 10 min). The pellet was washed once with 1 ml of ice cold 70% ethanol, spun down (1 min, 16,000 x g, 4℃), supernatant was discarded and the pellet was air dried. Finally, the pellet was resuspended in 50 µl of dH_2O and incubated for 10 min at 65℃. Samples were stored at -20℃.

5.7 DNA Digestion Using Restriction Endonucleases

Restriction was performed at 37℃ (if no other temperature optimum for the enzyme was required) for 1 hour using buffers recommended by the producer. The amount of enzyme was 2-5 U per 1 µg of digested DNA. When DNA was cleaved by two enzymes at the same time, the reaction buffer was chosen to allow the optimal enzymatic reaction for both the enzymes. In case that the enzymes did not share the

same buffer, the DNA was cleaved with one of the enzymes and after purification (PCR Purification Kit, Qiagen) with the second one.

In case of restriction of vectors used for cloning purposes, the amount of enzyme was usually 5 U/ µg and the incubation time was prolonged to 4 hours. After restriction the dephosphorylation usually followed.

5.8 Purification DNA Fragments after Enzymatic Reactions

For the purification of DNA fragments free of salts and enzymes the PCR Purification Kit (Qiagen) was used. The protocol of the manufacturer was followed. The DNA was usually eluted into 30 µl of sterile dH_2O ready for further enzymatic reactions.

5.9 Dephosphorylation

Since the shrimp alkaline phosphatase does not require necessarily its own buffer, the enzyme was added at the end of restriction reaction in concentration 10 U per 1 µg of DNA and incubated at 37℃ for additional 2 hours. In other cases the buffer recommended by producer was used. If necessary, the phosphatase was heat inactivated by incubation of the mixture at 65℃ for 10 minutes.

5.10 Ligation

Ligation of the DNA fragments was catalyzed by T4 DNA ligase (1 µl ~ 400 U) in buffer supplied with the enzyme in final volume of 20 µl, using 0.1 µg of the vector and 0.3-0.5 µg of the insert to be ligated. Linearized vector was dephosphorylated in case of complementary cohesive ends. Linearized vector and insert were purified from the enzymatic reaction or isolated from the agarose gel. The ligation was usually performed overnight at 4℃. If necessary (higher *E. coli* transformation efficiency), the ligase was inactivated by incubation at 65℃ for 10 minutes.

5.11 Electrophoretic Analysis of DNA

DNA fragments after PCR or restriction cleavage were separated by horizontal gel electrophoresis in 1% agarose gel in 1 x TAE buffer. The gel was prepared by

dissolving of the agarose in 1 x TAE buffer in microwave oven. After cooling down the EtBr (10 mg/ml stock solution) was added to final concentration 0.5 µg/ml. After covering the gel with 1 x TAE buffer, the samples mixed with 10 x concentrated loading buffer (0.25% bromophenol blue, 50% glycerol, 1 mM EDTA) were applied into the wells. As a standard for fragment size the commercial 1kb DNA ladder (NEB) was used. Electrophoresis was run at constant voltage 5 V/cm and 400 mA. Gels were documented under UV light using Camilla software.

5.12 Isolation of DNA Fragments from Agarose Gel

In case of obtaining the multiple fragments after enzymatic reactions, the mixture was separated in the agarose gel, bands excised and DNA purified by Gel Extraction Kit (Qiagen).

5.13 Verification of DNA Constructs

After creation of new constructs, it was preceded as follows. All the new plasmid constructs were transformed into *E. coli*, where the pDNA was amplified and further isolated. The constructs were always examined by restriction using proper restriction endonucleases and the size of expected bands was verified in agarose-gel electrophoresis. In case of verification of the correct sequence containing no mutations, the respective part of the vector was sequenced with use of suitable oligonucleotides (Biolux GmbH, Stuttgart).

5.14 RNA Isolation

The lab equipment used for the mechanical disruption of the frozen cell pellets (Teflon vials, wolfram carbide beads, scoops) was pre-treated for 10 min with 0.5% (v/v) sodium hypochlorite, rinsed with distilled water, 70% (v/v) ethanol and dried on air. An adequate amount of *C. albicans* culture was used for mechanical disruption by the Retsch mill MM200 (Retsch GmbH, Haan, Germany). Teflon vials with wolfram carbide beads inside were cooled in liquid nitrogen and about half filled with the frozen cell pellet obtained from the cell culture frozen in liquid nitrogen. The vials were fixed in the Retsch mill and shaken for 2 min with a frequency of 30 min^{-1}. The following steps were proceeded according to the protocol enclosed in the Qiagen

RNeasy Mini/Midi Kit. The resulting powder containing fractioned cells was resuspended into RLT buffer (included in the kit) supplemented with β-mercaptoethanol. RNA was purified using centrifugation columns of the RNeasy Mini/Midi Kit and followed the instruction manual of the producer. In case of higher RNA concentrations, the isolated RNA was further precipitated for enrichment with 2 M lithium chloride at -20℃ for 16 to 18 hrs. The p recipitated RNA was centrifuged (30 min, 4℃, and 16,000 x g), washed twice with 70 % EtOH (v/v) with DEPC treated water and centrifuged (4℃, 1 min, 16,000 x g). Pel let was air dried and resuspended in 50 - 100 µl of molecular biology grade water pre-warmed to 37℃ in order to facilitate the resuspension of the pellet. The quality of the RNA was checked on a 1% TAE agarose gel. 2 to 4 µg of RNA were dissolved in 15 µl of RNA loading buffer, incubated 10 min at 65℃ and subsequently loaded on the gel. RNA was considered as suitable for further experiments if the 18 S and the 26 S rRNA were visible on the gel and no degradation was detected. The RNA samples were stored at -80℃.

5.15 Determination of Nucleic Acid Concentration

The concentration of the DNA and RNA solution was determined with spectrophotometer in UV-cuvettes. The DNA/RNA solution was diluted 1:50 and the OD_{260} and OD_{280} was read. The amount of DNA/RNA in the solution was determined with the formula:

c [µg/ml] = OD_{260} x AF x DF

AF: absorption factor (OD_{260} = 1.0 in 1 cm cuvette for concentrations of dsDNA 50 µg/ml, ssRNA 40 µg/ml and ssDNA = 37 µg/ml)

DF: dilution factor

For pure dsDNA free of proteins the ratio OD_{260}/OD_{280} should be 1.7 - 1.9 and for ssRNA 1.8 - 2.0.

5.16 Northern Blot

10 µl of samples containing about 15 µg RNA were mixed with 30 µl of RNA-loading buffer and denatured at 65℃ for 5 minutes. The RNA was run in denaturing formaldehyde agarose gel (1% agarose, 1 x RNA MOPS buffer, 10% formaldehyde). Gel was run in 1 x RNA MOPS buffer at constant 5 V/cm until the bromophenol blue reached 2/3 of the gel. Gel was further stained for 45 minutes in 200 ml TAE buffer

containing 20 µl of SYBR Gold (10,000 x in DMSO) and documented under UV light. Gel was blotted to nylon membrane by performing the blot on Whatman paper column soaked by 20 x SSC buffer, 3 fresh Whatman papers soaked in 20 x SSC buffer, gel upside down, nylon membrane soaked in water (edges between gel and membrane covered with parafilm to avoid the buffer flow around the gel), 3 fresh Whatman papers soaked in 20 x SSC buffer and thick pile of paper towels to soak the buffer from the bottom of the blot. The blot was rid of all air bubbles between all the parts and blotting was performed overnight. Next day the membrane was air dried on bench and cross-linked in UV Stratalinker (twice 125 mJ/cm^2). The membrane was stored at -20℃ freezer sealed in plastic foil.

5.17 Southern Blot

25 µg of isolated genomic DNA was digested overnight with desired restriction enzyme and was load into 1% agarose TAE gel containing ethidium bromide. Gel was run at 5 V/cm until the bromophenol blue reached 2/3 of the gel. Following documentation under UV light, the gel was incubated for 15 min in Depurination buffer, 30 min in Denaturation buffer and 30 min in Neutralization buffer. The blotting procedure was performed exactly the same way like in case of Northern blotting.

5.18 Radioactive Probe Labeling and Blot Hybridization

5.18.1 Probe Labelling

For the radioactive labeling, Prime-It II Random Primer Labeling Kit performing nick translation in template DNA and incorporating radioactively labeled oligonucleotides was used. Up to 1 µg of purified PCR probe (200 – 500 bp) in 12.5 µl dH_2O was mixed with 5 µl of random 9-mer primer and denatured for 5 minutes at 95℃. After cooling down, 5 µl of 5 x dCTP reaction buffer, 2.5 µl of α^{32}P-dCTP and 1 µl of Exo (-) Klenow enzyme was added. The reaction took place at 37℃ for 10 minutes. The labeled probe was purified by gel filtration in ProbeQuant G-50 Micro Column in the table centrifuge. The probe was applied to the resin in the column and spun down in Eppendorf tube for 2 minutes at 735 x g. Purified sample was collected in the Eppendorf tube.

5.18.2 Blot Hybridization

Blotted membrane was placed into the hybridization bottle (side with bounded nucleic acids accessible for medium) with 15 ml of Church buffer and pre-hybridized at 65℃ with rotation for 1 hour. Church buffer was changed for 10 ml of fresh Church buffer containing the radioactive probe and the blot was hybridized overnight at 65℃ with rotation. Church buffer was discarded and the membrane was washed twice for 15 minutes with 15 ml of 1 x SSC buffer containing 1% SDS. Northern blot membrane was washed additionally for 15 minutes with 15 ml of 0.1 x SSC buffer containing 1% SDS. After wash was the membrane sealed in plastic foil and incubated with Fuji Phosphoimager screen. The screen was scanned usually after 24 hours. If necessary the membrane was stripped off by incubation twice for 15 min with boiling dH_2O at 65℃ and re-probed.

5.19 Isolation of *Taq* Polymerase

E. coli strain carrying vector pTrc99A for expression of Taq polymerase was inoculated overnight into LB ampicillin medium. Next day 400 ml of fresh LB ampicillin medium was inoculated into OD_{600} = 0.002. After reaching OD_{600} = 0.65 the Taq polymerase expression was induced by adding of IPTG to final concentration 0.5 mM. The culture was incubated at 37℃ for additional 16 h. Cell culture was spun down (6,000 x g, 10 min, 4℃) and washed with 40 ml of buffer A. Suspension was spun down once again (6,000 x g, 10 min, 4℃), the pellet was resuspended in 20 ml of buffer A containing 4 mg/ml lysozyme and incubated 15 min at room temperature. 20 ml of lysis buffer was added and the mixture was incubated for additional 1 hour at 75℃. The lysate was centrifuged (6,000 x g, 10 min , and 4℃) and the supernatant was recovered. For 10 ml of lysate 5.2 g of $NH_4(SO_4)_2$ was added and stirred at 4℃ for 30 minutes. Mixture was centrifuged for 10 min at 16,000 x g and 4℃ and additionally for 10 min at 22,000 x g and 4℃. The precipitate was resuspended in 8 ml of buffer A and dialyzed (dialysis membrane was once cooked in 2% $NaHCO_3$ + 1 mM EDTA and once in 1 mM EDTA and additionally washed in dH_2O) overnight at 4℃ against 400 ml storage buffer. Taq polymerase was aliquoted and frozen at -80℃.

5.20 PCR

PCR was usually performed with Taq polymerase (1 µl in 50 µl reaction) in NEB ThermoPol Buffer. Oligonucleotides were in final concentration 0.4 µM, dNTPs 0.2 mM and amount of gDNA in 50 µl of reaction at least 200 ng (plasmids 10 ng). Initial 5 min denaturation at 95℃ (prolonged to 10 min for colony PCR) was followed by thirty cycles of: denaturing 1 min at 95℃, 1 min annealing according to primer pair usually at 55℃ and amplification for 1 min/kb at 72℃. At the end 72℃ was kept for additional 10 minutes to complete the reaction. PCR reaction was load on the agarose gel or purified by PCR purification kit.

Colony PCR was performed from intact cells inoculated from the colony to the PCR tube without gDNA isolation step. Incubation of the reaction tubes with the cells at 65℃ for 30 min prior to PCR mixture addition was performed in order to improve the reaction.

In case of amplification of DNA fragments for further cloning, Stratagene Pfu low error rate polymerase was used according to the manual distributed with the enzyme.

5.21 Quantitative Real Time PCR

To quantify the cDNA the detection system with Taq-man probes from a universal probe library (Roche) was used. Oligonucleotides were designed on Roche web-based application in order to be used in the common program for the probe library. cDNA was synthesized from isolated RNA (up to 1 µg) with the Qiagen QuantiTect Reverse Transcription Kit as recommended in the handbook. Following mixture was used for the amplification in the Roche LightCycler 480: 2 µl of diluted cDNA (1:50), 10 µl LightCycler 480 Probes Master Mix, 40 pmol TaqMan probe from universal probe library and 40 pmol of each real time PCR primer (from 20 µM stock solution). The mixture was filled up to 20 µl with molecular biology grade water.

Following temperature protocol was used for the real time PCR reaction:

1	First denaturation	5 min, 95℃	ramp rate: 4.4℃/s ec
2	Denaturation	10 sec, 95℃	ramp rate: 4.4℃/sec
3	Annealing	5 sec, 60℃	ramp rate: 2.2℃/sec
4	Elongation	1 sec, 72℃	ramp rate: 4.4℃/sec
5	Repeat from step 2. 45 times		
6	Cooling	30 sec, 40℃	ramp rate: 1.5℃/sec

As an internal control the gene *TDH3* encoding for glyceraldehyde-3-phosphate dehydrogenase (glycolytic enzyme) or 18 S rRNA was used.

The first cycle at the beginning of the exponential phase, where the fluorescence is higher for the first time than the background fluorescence, is called Ct-Value (threshold cycle). The lower the Ct value is the higher is the original amount of template in the PCR reaction. Delta Ct method was used for the transcriptional analysis (Livak and Schmittgen, 2001).

In case of Candida-mDC interaction Mastercycler ep realplex (Eppendorf) was used with SYBR Green as a detection probe. 20 µl Real-time PCR mix contained 1 x MESA GREEN qRT-PCR MasterMix Plus for SYBR assay – dTTP (Eurogentec), 10 pmol of each real time PCR primer (from 20 µM stock solution) and 5 µl of template cDNA diluted 1:5. Abgene PCR plates were used and sealed with BioRad optical tape. Before the run the plates were vortexed and shortly spun down.
Following temperature protocol was used for the real time PCR reaction:

1	First denaturation	5 min, 95℃
2	Denaturation	10 sec, 95℃
3	Annealing	15 sec, 60℃
4	Elongation	15 sec, 72℃
5	Measurement*	10 sec, 80/79/78.5/72℃
6	Repeat from step 2. 45 times	
7	Denaturation curve	20 sec, 95℃
		20 sec, 60℃
		30 min, 60℃ to 96℃
		15 sec, 95℃
		infinite, 25℃

* Measurement at 80℃ (*IL-10*, *IL-4*, *IL-12*), 79℃ (*GAPDH*), 78.5℃ (*IFNß*) and 72℃ (*IL-23*, *IL-1β*).

Delta Ct method was used for the transcriptional analysis also in this case.

5.22 Transformation of *E. coli* Cells

5.22.1 Preparation of Competent Cells

All the steps were performed in the sterile flow-hood and on ice; all the centrifugations were performed at 4℃. Twice 500 ml of LB medium was inoculated with 2.5 ml of the overnight culture of DH5α strain and incubated at 37℃ until OD_{600} = 0.6. The culture was then cooled on ice and spun down (3,350 x g, 15 min, and 4℃). Resulting pellet was washed in 200 ml of ice cold TFB1 buffer and spun down (3,350 x g, 10 min, and 4℃). The pellet was then resuspended in 40 ml of ice cold TFB2 buffer. Aliquots in the Eppendorf tubes were frozen in -80℃.

5.22.2 Heat Shock Transformation

Frozen competent cells were thawed on ice for 20 minutes. Aliquot of 100 µl of cells was add to the ligation mixture or 0.1 – 10 ng pDNA respectively. After vortexing was the tube kept on ice for additional 20 minutes. The heat shock at 42℃ was performed for 1 minute and the tube was placed immediately on ice for next 5 minutes. Later 900 µl of LB-medium was added and the mixture was incubated with shaking at 37℃ for 45 minutes.

The cells were spun down (16,000 x g, 5 seconds), 800 µl of supernatant was removed and the pellet was resuspended in the resting volume. Cells were plated on LB agar medium containing ampicillin or chloramphenicol.

5.23 Transformation of *C. albicans* Cells by Electroporation

Usually 50 ml of YPD (+ 0.1 mM uridine for *ura3* strains) was inoculated by *C. albicans* strain and incubated overnight at 30℃ with shakin g until OD_{600} = 1.6 – 2.2. Cell suspension was centrifuged in 50 ml Falcon tube (4,000 x g, 5 minutes) and the pellet resuspended in 9 ml of ice-cold sterile double distilled water. 1 ml of 10 x TE-buffer and 50 µl of 1 M LiAc, pH 7.5 was added. This mixture was further incubated for 1 hour with shaking (30℃, 150 rpm). Later, 250 µl of 1 M DTT was added and incubated for additional 30 minutes with shaking (30℃, 150 rpm). Finally, the mixture was filled up with sterile dH_2O (room temperature) to 40 ml and the cells were centrifuged (4,000 x g, 5 minutes). The final pellet was washed with 25 ml of ice-cold dH_2O and centrifuged (4,000 x g, 5 minutes, and 4℃). Pellet was resuspended in 10 ml of ice-cold 1 M sorbitol and centrifuged (4,000 x g, 5 minutes, and 4℃) and the cell pellet was resuspended in 400 µl of ice-cold 1 M sorbitol. Aliquots of 40 µl of cells were placed into Eppendorf tube (kept on ice) containing the transformed DNA (up to 1 µg) and then incubated on ice for 5 minutes. Later, the suspension was transferred into 1 mm cuvette (pre-cooled on ice). Pulse was initiated (Biorad MicroPulser – program SC2 for fungi; equal to 3 – 5 ms, 1.5 kV). In case of transformation of *SAT1*-flipper cassette (containing dominant selection marker) 900 µl of ice cold YPD was added, transferred into new Eppendorf tube and incubated at 30℃ for 4 hours. In case of *URA3* marker on the transformed cassette, 100 µl of YPD was added and the suspension was kept in the room temperature for 30 minutes. After the incubation the cells were spun down, supernatant removed, 200 µl of SC medium or water added and the cells were plated.

5.24 Transformation of *S. cerevisiae* Cells by LiAc Method

50 ml of YPD fresh culture of *S. cerevisiae* strain in liquid YPD medium was prepared by inoculation from overnight culture or from fresh YPD plate to OD_{600} = 0.25 and

incubated at 30℃ until reaching OD_{600} = 1.0. Cells were centrifuged (4 000 x g, 3 minutes), resuspended in 10 ml of sterile double distilled water and aliquots 10 x 1 ml in Eppendorf tubes were prepared. Cells were spun down (4 000 x g, 3 minutes) and the supernatant removed. Resulting pellet was resuspended in 1 ml sterile 100 mM LiAc and incubated for 10 minutes at 30℃. After ce ntrifugation (4 000 x g, 3 minutes), the supernatant was removed and 240 µl of PEG4000 (50 % w/v), 36 µl of 1 M LiAc, 5 µl of salmon sperm ssDNA (10 mg/ml, incubated 5 min in 100℃) and aliquot of transformed DNA (0.1 – 0.5 µg) were added. The mixture was vortexed and incubated for 30 minutes at 30℃. Tubes were placed to 42℃ for 20 minutes. After the heat shock, cells were spun down (4 000 x g, 3 minutes), supernatant removed and pellet was resuspended in 1 ml of sterile water. This mixture was incubated at room temperature for 5 minutes, vortexed thoughtfully and plated in 200 µl aliquots on minimal medium agar plates lacking amino acids complemented in auxotrophic strain by the transformed vector.

5.25 Flipping Out of the *SAT1* Cassette

Using of *pSAP2* promoter for flipase expression from pSFS1A vector, the expression was activated by cultivation of the respective strain in YCB-BSA medium from low optical density to stationary phase for 2-3 days. Strains were diluted in order to plate out 200 – 300 cells on YPD plate containing 15 - 25 µg/ml nourseothricine. Smaller colonies formed on the plate were streaked out for further analysis. Using of *pMAL2* for flipase expression from pSFS2A vector the cells were inoculated into YPD and after reaching stationary phase the cultures were plated on YPD plate containing 15 - 25 µg/ml nourseothricine.

5.26 Preparation of Plasmids

5.26.1 Plasmids Used for *AUF* Gene Studies

For deletion of *AUF* genes new set of plasmids was prepared from original pSFS1A (Reuss*, et al.*, 2004), carrying a dominant selection marker *SAT1* and offering cells resistance to the antibiotic compound nourseothricine (Figure 4). Integration of the deletion cassette, present on this plasmid, is dependent on two site-specific flanking

regions upstream and downstream of the deletion cassette, which both have to be cloned prior to deletion.

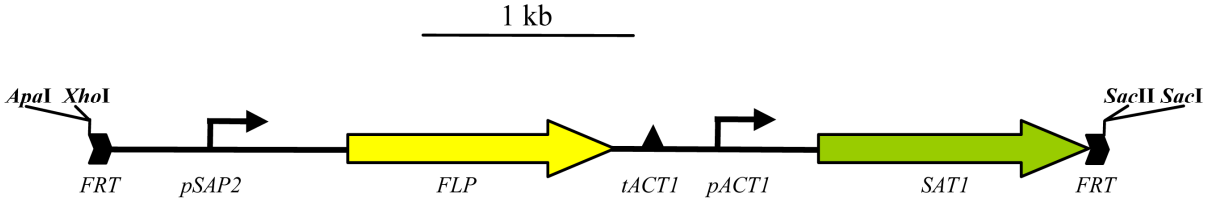


Figure 4. Scheme of deletion cassette from the plasmid pSFS1A.

The entire *SAT1* flipper cassette adapted from (Reuss*, et al.*, 2004) including polylinker sites at the ends is 4758 bp in size. *FRT* – flipase recombinant target, *pSAP2* – *SAP2* promoter inducible by presence of proteins in minimal medium, *FLP* – flipase, *tACT1* – *ACT1* terminator and *SAT1* – nourseothricine resistance marker gene under *pACT1* promoter encoding for nourseothricine acetyltransferase. About 500 bp DNA fragments upstream and downstream from the regions for deletion were amplified by PCR and ligated between *Apa*I/*Xho*I and *Sac*II/*Sac*I sites. The final cassette was restricted with enzymes *Apa*I and *Sac*I and transformed into desired Candida strain in order to perform the deletion.

Following oligonucleotides were used to amplify desired regions: *AUF1* upstream region (4691dU_F and 4691dU_R), *AUF1* downstream region (4691dD_F and 4691dD_R), *AUF8* upstream region (3908dU_F and 3908dU_R), *AUF8* downstream region (3902-08dD_F and 3902-08dD_R) and *AUF2* upstream region (3902-08dU_F and 3902-08dU_R). Oligonucleotides used for upstream regions were carrying *Apa*I and *Xho*I restriction sites, while those used for downstream regions *Sac*II and *Sac*I sites. After restriction of the PCR products, these regions were ligated into the pSFS1A plasmid linearized with identical enzymes. Following plasmids were constructed: pSFS1A_d1 (cloned *AUF1* upstream and downstream regions), pSFS1A_d8 (cloned *AUF8* upstream and downstream regions) and pSFS1A_d28 (cloned *AUF2* upstream and *AUF8* downstream regions). Plasmid pSFS1A_d1 was used for deletion of *auf1* gene, pSFS1A_d8 for *auf8* and pSFS1A_d28 for the whole region containing genes *auf2* to *auf8*. Strain containing deletions of all the *auf* genes was prepared from strain deleted first for *auf1* and then deleted for genes *auf2-8*.

Plasmids used for the deletions were further altered for reintroducing of a functional allele to the mutant strains. The downstream regions (cloned *Sac*II, *Sac*I) were replaced for regions containing the functional ORFs with the following downstream region. For reintroduction of the *AUF1* (*auf1* and *auf1-8* strains) the pSFS1A_r1 plasmid was constructed from pSFS1A_d1 by replacing of the downstream region (*Sac*II, *Sac*I) by PCR product (4691revU_F and 4691dD_R). For

reintroduction of *AUF8* (*auf8* strain), the pSFS1A_r8 plasmid was constructed from pSFS1A_d8 by replacing of the downstream region (oligonucleotides 3908revU_F and 3902-08dD_R). Finally for reintegration of *AUF8* into *auf1-8* or *auf2-8* strains, plasmid pSFS1A_r8b was constructed from pSFS1A_d28 by replacing of the downstream region by PCR product same as for pSFS1A_r8 (3908revU_F and 3902-08dD_R).

For *AUF8* overexpression plasmid pSFS1A_o8 was prepared. The downstream region of pSFS1A_d8 (*Sac*II, *Sac*I) was replaced by two PCR products with cloning sites *Sac*II/*Stu*I (pTDH3_F_SacII and pTDH3_R_StuI) and *Stu*I/*Sac*I (AUF8_F_StuI and 3902-08dD_R) constructing *AUF8* gene under 1 kb of *pTDH3* promoter.

The cassettes were restricted from the plasmids using restriction enzymes *Apa*I and *Sac*I, transformed into desired strains and selected on YPD agar plates containing 200 µg/ml nourseothricine.

5.26.2 Plasmids for *S. cerevisiae AUF8* Heterologous Expression

To create plasmid pRS425A8G, *GFP* tagged *AUF8* ORF was amplified from the genome of *C. albicans AUF8GFP* strain using oligonucleotides 3908_F_B and 3908GFP_R_X with restriction sites *Bam*HI and *Xho*I. PCR product was restricted with *Bam*HI and *Xho*I enzymes and ligated into plasmid pRS425TEF (Mumberg*, et al.*, 1995) linearized with the identical enzymes. Created plasmid pRS425A8G was after cloning containing strong promoter *pTEF1* of translational elongation factor from *S. cerevisiae*, *AUF8GFP* construct and *tCYC1* terminator.

To create plasmid pRS425A8, the *AUF8* ORF was amplified from genomic DNA of SC5314 strain using oligonucleotides 3908_F_B and 3908_R_X and cloned into linearized vector pRS425TEF using the same restriction sites like in previous case. This led to creation of plasmid pRS425A8.

5.26.3 pXFP-SAT1 Plasmids

The X stands for fluorescent color variants of the tag (GFP, YFP and CFP). In order to construct the pGFP-SAT1 plasmid, the whole GFP-tagging cassette from the

vector pGFP-HIS1 (Gerami-Nejad*, et al.*, 2001) was restricted by *Hind*III and *Eco*RI and ligated into vector pUC119 (Vieira and Messing, 1987). From this vector the *HIS1* marker was excised with enzymes *Eco*47III and *Eco*RI (leaving in vector *eGFP* ORF and *tADH* terminator). Into the linearized vector *FRT* site (on oligonucleotide pGFP-SAT1_F1) with 500 bp of *pACT1* promoter was ligated (PCR product from SC5314 genomic DNA using oligonucleotides pGFP-SAT1_F1 and pGFP-SAT1_R1, restricted *Eco*47II and *Kpn*I) together with *SAT1* gene, *tURA3* terminator and *FRT* recombination site (PCR product from pSFS1A using oligonucleotides pGFP-SAT1_F2 and pGFP-SAT1_R2, restricted *Kpn*I and *Eco*RI).

In order to produce different fluorescent protein variants, pGFP-SAT1 was restricted using enzymes *Hind*III and *Xho*I (*GFP* excision) and *Hind*III and *Xho*I fragments (*CFP* and *YFP* ORFs) from pCFP-URA3 and pYFP-URA3 were ligated instead. Positive transformants were selected according to the color of the *E. coli* colonies expressing the fluorescent proteins from the vector.

5.26.4 Plasmids Used for Overexpression Studies

Gene *PGA7* was from SC5314 genomic DNA amplified by PCR using primers PGA7_F_XhoI and PGA7_R_BamHI, *PGA23* by PGA23_F_XhoI and PGA23_R_BamHI, *PRA1* by PRA1_F_XhoI and PRA1_R_BamHI, and *HWP1* by HWP1_F_XhoI and HWP1_R_BamHI. All the PCR products were restricted by enzymes *Bam*HI (*HWP1* only partially due to internal *Bam*HI restriction site) and *Xho*I and ligated into *Bam*HI and *Xho*I linearized (excision of *lacZ* gene) vector pAU36RP10_KKf downstream from constitutive *pACT1* actin promoter (resulting in plasmids pAU36-PGA7, pAU36-PGA23, pAU36-PRA1 and pAU36-HWP1). As a negative control for *URA3* complementation of the background strains served *Hind*III restricted (excision of *lacZ* gene) and self-ligated vector (pAU36-empty). Verified plasmids were linearized by enzyme *Stu*I inside the *RPS1*/*RP10* gene (Care*, et al.*, 1999) and transformed into the desired strain. The positive transformants were further selected on agar plates without uridine.

5.27 Drop Tests

To test the growth defects and sensitivity of different Candida strains to various compounds the drop tests were used. Into wells of 96 well plate 90 µl of sterile water

was added. Into the first well column the suspensions of different strains of exact OD_{600} = 1.0 were transferred. In the following well columns dilution series of 1:10 were made by transferring always 10 µl of the suspension into the next well column. Usually 6 dilutions were made. The dilution series of strains were transferred from 96-well plate to the agar plates by replica plater (Sigma Aldrich), transferring about 4 µl of the suspension. Pure YPD or YNB agar plates were used as a control to exclude any growth defects of strains from those observed on the selective media. Agar plates were incubated for several days at 30 and 37℃ and documented by photography.

5.28 Wash Assay

To determine the ability of several strains to adhere to the agar plate the wash assay was performed. Strains were diluted into OD_{600} = 1.0 and 3 µl of this suspension were centrically spotted on YPD agar plate as three separate spots. Wild-type strain as positive control was included. After 2 days of cultivation at 30℃ plate was documented by photography, about 10 ml dH_2O was added to the center of the plate and the plate was incubated with rotatory shaking at 100 rpm. Less adhesive strains were washed off the agar prior to the more adhesive/filamentous ones. The process was continuously documented by photography.

5.29 Biofilm Formation Assay

2 ml of an overnight culture in YNB containing 0.9% glucose was centrifuged for 5 min at 4,000 x g and washed twice with 0.5 ml PBS. Cells were resuspended in 1 ml YNB containing 0.9% glucose and the OD_{600} was adjusted to exactly 1.0 with the same medium. For each strain 100 µl of suspension in triplicates was inoculated into individual wells of polystyrene 96 well plate. Medium without inoculum was used as negative control. Plates were incubated for 90 min at 37℃ to let the cells adhere. After this step, supernatant was discarded, well bottoms were twice carefully washed with 150 µl PBS to remove any non-adherent cells and 100 µl of fresh YNB containing 0.9% glucose was added. The 96 well plates were covered with lid and incubated at 37℃ for 48 hours. Before measuring of the cell mass, all planktonic cells were discarded during two washes with 200 µl of PBS. To each washed well with biofilm 100 µl PBS containing 1 mg/ml XTT with 1 µM menadione (fresh 1 M

stock in acetone) was added and developed at 37℃ in dark for 3 – 5 h. The intensity of colorimetric change (yellow tetrazolium salt to red formazan) was measured at 490 nm. Wells without inoculum were used as a blank.

5.30 Competition Assays

In order to compare fitness of two strains (usually wild-type and respective mutant) the co-cultivation competition assay performed. Two strains were inoculated in the same amount to the desired cultivation medium. During the experiment the culture was re-inoculated each day or the strains were kept in stationary phase (depending on the experimental conditions). Each day samples of the culture were frozen for further gDNA isolation. After collecting of all the samples, gDNA was isolated, cleaved by specific restriction endonuclease and a Southern blot was performed. Experiment was designed usually the way that the probe was specific for both, wild-type and mutant, but for different size of gDNA fragments on the blot. Probe on each band was quantified using Aida software and relative quantification of both strains in the culture was plotted in the graph.

5.31 Cell Wall Stability Assay

Cell wall Zymolyase treatment was performed in order to investigate changes in cell wall stability among various strains. Experiment was performed in 96 well plate. Strains grown in YPD were spun down, washed in dH_2O and resuspended in dH_2O to OD_{600} = 3.0. 33 µl of each strain in triplicates was added to the wells together with 33 µl of 150 mM Tris-HCl pH 7.5 containing 180 mM β-ME and incubated 30 min at 30℃. Prior to measurement 33 µl of Zymolyase (usual concentration 30 µg/ml ~ 3 U/ml or lower if necessary) in dH_2O was added and plates were red at OD_{600} every 2 min with 10 second shaking before each measurement. Experiment took 1 h in total.
In case of measurements in the cuvettes, 400 µl of cell suspension, buffer and Zymolyase was added. OD_{600} was measured every 5 min with cuvette vortexing prior to measurement.

5.32 Fungal Cell Wall Composition Analysis

5.32.1 Relative Concentrations of Polysaccharides

200 ml of exponentially grown cells in YPD medium around OD_{600} = 2.0 were spun down (6,000 x g, 4℃, 5 min) and washed in 20 ml of dH_2O. Cells were washed once with 20 ml of 10 mM Tris-HCl, pH 7.5 at 4℃, resuspended in 25 ml of the same buffer and kept on ice. The cell suspension was processed three times in French Press under pressure 1,500-2,000 PSI (10-14 MPa). Suspension was spun down (6,000 x g, 4℃, and 5 min) and washed with 20 ml of 1M NaCl. After three washes with dH_2O, cell walls were extracted three times at 100℃ with 5 ml of extraction buffer (50 mM tris-HCl pH 7.8, 2% SDS, 10 mM EDTA, 40 mM β-mercaptoethanol). The pellet was additionally washed three times with dH_2O, transferred to the 2 ml Eppendorf tubes, spun down, frozen and lyophilized.

Approximately 10 mg specimens of the lyophilized cell walls were incubated in glass reaction tubes with 75 µl of 72% H_2SO_4 and incubated at room temperature for 3 h. 950 µl of dH_2O containing 1 mg/ml galactose, as an internal control, was further added. Reaction tubes were transferred to a sand bath set at 100℃. After 4 h additional 2.5 ml of dH_2O was added to the mixture and transferred to 50 ml Falcon tubes. Reaction tubes were rinsed with dH_2O and transferred to the rest. Acid solution was neutralized with about 4 ml of saturated $Ba(OH)_2$ (approximately 40 g/l). The precipitate was left to form overnight at 4℃. The next day the pH was set up with the pH meter between 6.0 and 8.0 ($Ba(OH)_2/H_2SO_4$). The final volume was adjusted to 20 ml with dH_2O and the formed pellet was spun down (6,000 x g, 4℃, and 10 min). The sugar content was set up by HPIC (high performance ion chromatography) measurement (GlcNAc for chitin, glucose for β-1,3- and β-1,6-glucans, mannose for mannans and galactose as an internal control) using standard dilutions for the calibration.

5.32.2 Absolute Concentration of Glucans Related to Biomass

50 ml of exponentially grown cells in YPD medium of OD_{600} = 2.0 were spun down (6,000 x g, 4℃, 5 min) and washed twice with 20 ml of PBS. The cell pellet was

transferred to 2 ml Eppendorf tube, spun down in table centrifuge, frozen and lyophilized.

Approximately 10 mg specimens (the exact weight was recorded) of the lyophilized cells were incubated in 1.5 ml Eppendorf tube with 150 µl of 36% H_2SO_4 at 60℃. After 3 h 850 µl of dH_2O was added and reaction tubes were transferred for 4 h to heating block set at 100℃. After the hydrolysis procedure, the solution was measured for glucose concentration by enzyme coupled reaction. 10 µl of the sample was mixed with 1 ml of 0.1 M potassium phosphate buffer pH 7.4 containing 10 mM phenol, 0.3 mM 4-aminoantipyrine, 10 kU/l glucose oxidase and 700 U/l peroxidase. Samples were incubated at room temperature for at least 30 minutes and then OD_{505} was measured. Glucose concentration was set from calibration curve made of standard glucose samples of concentration up to 20 mM. The total amount of glucose was calculated and related to the biomass used for the hydrolysis. This enzymatic reaction has high specificity to glucose and is not affected by other monosaccharides in the hydrolysis mixture.

In case of measurement of the glucose units inside of the cell, about 10 mg specimens of dry cell biomass were disrupted in 1 ml of dH_2O with 300 ml glass beads by vortexing at 4℃ for 30 minutes. In order to hydrate all the polysaccharides, the Eppendorf tubes were incubated at 95℃ for 15 minutes and then shortly vortexed. The glass beads and the solid parts of the cells were centrifuged at 16,000 x g for 15 minutes. 500 µl of the suspension was transferred into a fresh Eppendorf tube and 50 µl of 72 % H_2SO_4 was added. The mixture was incubated at 100℃ for 6 hours. After the hydrolysis, glucose concentration was determined like in previous case and the concentration was related to the cell biomass entering the glass bead disruption.

5.33 Fluorescent β-1,3-glucan Staining

Approximately 1 x 10^8 Candida cells were fixed for 5 minutes in 3.7% formaldehyde, washed with PBS, and blocked for 1 hour with 1 ml PBS + 2% BSA at room temperature with rotation. Cells were stained with mouse anti-ß-1, 3-glucan primary antibody (1:100 in PBS + 2% BSA; 100 µl; original Ab concentration 1 mg/ml) (Gentaur) for 1 hour with rotation at room temperature. Cells were washed 4 x with 1 ml of PBS without any incubation time. Secondary antibody staining was performed for 1 hour with rotation at room temperature with Alexa488 goat anti-mouse antibody

(Molecular Probes) 1:100 in 100 µl PBS + 2% BSA. During this step, samples were protected from light. Cells were washed 4 times with 1 ml PBS. Cells stained just with the secondary antibody were used as negative controls. Cells were microscopically examined using microscope Axiovert 200M (Zeiss) with appropriate fluorescence filter and constant exposition time for all the samples. The rough intensity of the fluorescent signal was measured with Aida software, using 1D densitometry.

5.34 Cell Culture Techniques

5.34.1 Cell Culture

A-431 and Caco-2 cells thawed from cryostocks were grown in 75 cm^2 cell culture flasks in 15 ml of high glucose DMEM medium supplemented with 10 % FCS, 1 mM sodium pyruvate and 1 x gentamicin. When cells reached about 80% of confluency they were passaged. Cells were washed with 5 ml pre-warmed PBS^- in order to remove traces of medium, divalent ions and dead cells. Cells were detached from the cell culture flasks by incubation of the cells with 2.5 ml pre-warmed 0.25% Trypsin, 1 mM EDTA solution at 37℃ for 3 – 5 minutes. The Try psin was saturated by adding of 10 ml pre-warmed supplemented DMEM medium and the cells were spun down (3 min, 300 g). Afterwards, cells were resuspended in 10 ml pre-warmed supplemented DMEM medium and counted either in an electronic cell counter (CASY COUNTER 1) or in an improved Neubauer counting chamber (1:1 mixture of cell suspension with tryphan blue solution). After determination of the cell count 1 - 2 x 10^6 cells were seeded into a fresh cell culture flask in 15 ml warm supplemented DMEM medium. The medium was changed for fresh one every two to three days.

5.34.2 Preparation of Cryocultures

Passaged cells were resuspended to the concentration of 2 x 10^6 cells/ml in the freezing medium (culture medium containing 20% FCS and 5% DMSO). The cell suspension was aliquoted into 1 ml cryotubes, placed in the polystyrene container for gradual freezing about 1℃/min and placed into -80° C freezer overnight. When the samples reached the final temperature, they were removed and transferred to the liquid N_2 freezer.

5.34.3 Thawing of Stored Cryotubes

The cryotube thawing was done as rapidly as possible at 37℃ in water bath in order to minimize intracellular ice crystal growth during the warming process. The cell suspension was diluted slowly into 10 ml fresh supplemented DMEM medium to avoid the rapid dilution inducing osmotic shock and reducing viability.

5.35 Protein Coating of 24-Well Plates

For coating of 24-well plates with fibrinogen 800 µl of PBS pH 7.4 containing fibrinogen in concentration 5 µg/ml was used. Plate was incubated at 4℃ overnight, washed twice with 1 ml PBS pH 7.4 and used for adhesion experiments.

5.36 Adhesion Assays

After passaging 1-3 x 10^5 Caco-2/A-431 cells in 1 ml supplemented DMEM medium were seeded in each well of the 24 well plate. The cells were cultivated for two to three days at 37℃ and 5% CO_2 until they reached confluency. Prior to infection with Candida the medium was removed, the tissue cells were washed with 1 ml PBS^- and 250 µl new pre-warmed DMEM without gentamicin was added. For adhesion assays performed on inert polystyrene surface only 250 µl of fresh DMEM without gentamicin was added to the 24 well plates. Exponentially grown *C. albicans* cells in YPD of OD_{600} about 1.0 were diluted 1:5000 in sterile dH_20 in order to obtain about 6000 cells/ml. Ten wells per strain (five time points in duplicates) were infected with about 300 *C. albicans* cells in 50 µl of the diluted culture. Adherent and non-adherent *C. albicans* cells were plated out at 0, 0.5, 1, 2 and 4 h time points. After incubation of the plate for 2 min at 200 rpm on rotator shaker detaching the sedimented non-adherent cells, the non-adhering *C. albicans* cells were plated by removing the 300 µl medium from the wells and spreading it on YPD agar plate. After plating the non-adherent cells the wells were washed with 300 µl of PBS^- and again 300 µl of PBS^- was added to the well. Eventually, the tissue cell line with the adhering *C. albicans* cells were scratched off the bottom of the well with a pipette tip and spread on an YPD agar plate. The agar plates were incubated for 24-48 h at 30℃ and finally the colonies on each plate were counted. The number of adherent and non-adherent

cells for each strain, time point and duplicate sample was recorded and used for further calculations.

5.37 Invasion Assays

5.37.1 Isolation of Rat Tail Tendon Collagen

Frozen rat tails were collected from -80℃ freezer and thawed at the room temperature. Thawed tails were washed with 95% ethanol and the skin was peeled off with a sterile forceps. Starting from the tip, the separate tale spondyls were broken off and the tail tendons attached to the spondyl were pulled out of the tail. The tendons were then cut free and collected in a Petri dish with sterile PBS. After collecting all the tendons, they were incubated in 70% ethanol for 15 minutes. Tendons were weighted and after cutting into smaller pieces placed into 0.1 M acetic acid (1 ml per 10 mg of wet tendons). Tendons were dissolved at 4℃ with continuous stirring for 24 hours. Solution was clarified by centrifugation at 6,000 x g for 20 minutes. The supernatant was collected and stored at 4℃ until being used. The amount of gelificant necessary to neutralize each batch of collagen stock was tested by adding 300 - 700 µl of gelificant to 1 ml of collagen solution.

5.37.2 Construction of Human Reconstituted Epithelia

Collagen solution was mixed with gelificant in an appropriate ratio and 300 µl aliquots of this mixture were poured into inserts placed in cell culture 24 well plate. The inserts with the collagen mixture were incubated for at least 10 min at 37℃ to solidify. After the collagen turned solid, 500 µl of supplemented DMEM was added into each well outside of the insert. 100 µl of cell suspension of Caco-2/A-431 in supplemented DMEM containing 1 x 10^5 cells was added on top of each insert and incubated for 72 h at 37℃ and 5% CO_2, until the cells formed a confluent monolayer on the top of collagen matrix. The medium from the outer part of the insert was removed and replaced with 500 µl fresh DMEM without gentamicin. Exponentially grown *C. albicans* strains from YPD of OD_{600} about 1.0 were used for the infection of the reconstituted human epithelia. The cultures were diluted in sterile dH_2O to an OD_{600} = 0.1 and 15 µl of these diluted cultures were used for infection of inserts. The infected models were incubated usually for 24 hours, additional time points were

included when necessary. In each experiment the positive control represented by wild-type strain was added and only strains from one experiment were compared.

5.37.3 Histological Processing of Invasion Assays

After reaching the desired time point of infection of the epithelial models with the *C. albicans* isolates, the inserts were fixed by replacing of the culture medium with Bouin's solution and incubation at room temperature for 1 hour. After fixation the Bouin's solution was aspirated and the fixed samples were placed into tissue embedding cassettes with the filter paper. The cassettes were placed into tap water for at least 2 hours in order to remove the excess of the Bouin's solution. Afterwards, the cassettes were transferred into the tissue processor for replacing the water in the samples by paraffin. The program was as follows: tap water 1 h, tap water 1h, 70% (v/v) ethanol 1 h, 90% (v/v) ethanol 1 h, 96% (v/v) ethanol 1 h, 100% isopropanol 1 h, 100% isopropanol 1 h, xylol:isopropanol (1:1) 1 h, 100% xylol 1 h, 100% xylol 1 h, paraffin 2 h and paraffin 2 h. The fixed samples were cut into two pieces and embedded in hot paraffin into base molds with the cut side facing the bottom. The base molds with the samples were placed in the fridge for several hours to harden the paraffin. The paraffin blocks with the samples were cooled and cut on microtome to obtain 3 µm thick vertical cuts. The slices of the paraffin block were stretched on surface of a 42℃ water bath and mounted on microsc opy slide. The microscopy slides with the samples were dried for 2 hours on a 42℃ heated stretching plate to remove excess of water.

5.37.4 Staining

Glass slides were incubated for 30 min at 60℃ in o rder to fix the samples on the slides. Samples were then stained according to the PAS method (periodic acid-Schiff reaction stain). The samples were first deparaffinized and rehydrated as follows: Roticlear I (10 min), Roticlear II (3 min), 96% (v/v) ethanol I (2 min), 96% (v/v) ethanol II (2 min), 70% (v/v) ethanol (2 min), 50% (v/v) ethanol (2 min) and rinse in distilled water. Following steps were performed to stain the samples: 1% (w/v) periodic acid (5 min), short rinse in distilled water, Schiff's reagent (15 min), Sodium bisulphite solution (3 x 2 min - fresh solution each time), running tap water (10 min), Mayer's haematoxylin (15 sec), 0.03 M HCl (short rinse), warm running tap water (5 min),

50% (v/v) ethanol (2 min), 70% (v/v) ethanol (2 min), 96% (v/v) ethanol (2 min), Polychrome staining solution EA50 (1 min), 96% (v/v) ethanol (3 x for 2 min - three solutions), 100% ethanol (30 sec) and Isopropanol (2 x 5 min – two solutions). Finally, the stained samples were coated with Isomount mounting medium and covered with a cover slip for protection of the samples. The slides were viewed with a Zeiss Axiovert M200 Microscope and colored pictures were taken from the samples at 400 x magnification.

Cellulose, mucopolysaccharides, muco- and glycoproteins, glycolipids, unsaturated fatty acids and phospholipids which occur in the fungal cell wall are stained magenta-red by the periodic acid and the Schiff's reagent. The nuclei of the epithelial cells appear blue after staining with Mayer's haematoxylin and after dehydration steps the collagen matrix is counter-stained light-blue with Polychrome staining solution.

5.38 LDH Assay

LDH assay was performed to quantify the damage of tissue cell lined in presence of various Candida strains. 96 well plate was inoculated with 100 µl of fresh supplemented DMEM containing 10,000 passaged Caco-2 cells and incubated for two days until the tissue cell line reached confluency. Medium was aspirated and replaced with 100 µl of fresh supplemented DMEM medium without phenol red and gentamicin. Wells were infected at least in triplicates with 10 µl Candida cell suspension (OD_{600} = 0.14 ~ 80,000 cells) including wild-type as a control. The 96 well plate was incubated at 37℃ and 5% CO_2. After 20, 24 and 28 h the plate was incubated for 2 min at 160 rpm in order to resuspend the medium. Supernatant was transferred to a fresh 96 well plate. Ten minutes prior to each time point, 10 µl of 1% Triton X was added to several wells serving as a positive control of 100% Caco-2 cell lysis. Negative control, wells without Candida infection, was also included. 96 well plate was frozen at -80℃ and analyzed later.

To each well of 96 well plate 100 µl of LDH Buffer was added and the reaction was developed for five minutes. If necessary, the reaction was stopped by addition of 20 µl of 1 M HCl. The optical density was red at 490 nm. Triton X lysed Caco-2 cells served as positive control (100% lysis), uninfected wells as a negative control (0% lysis) and culture medium as a blank.

5.39 End-Point Dilution Survival Assays

Logarithmic phase growing murine bone marrow derived macrophage cells (starting from cryoculture, 7- to 9-week-old C57BL/6 wild-type mice, obtained as described (Frohner*, et al.*, 2009)) at day 12 were resuspended in culture medium (-L-conditioned supplemented DMEM) and at least 100 µl aliquots containing 1 x 10^5 cells were distributed into the wells of 96 well plate (every second column only). Next day the medium was aspirated, macrophages were gently washed twice with 100 µl of PBS and 100 µl of fresh supplemented DMEM w/o phenol red was added including the columns without macrophages. Overnight YPD grown cultures (5 ml) of *C. albicans* were spun down at 4,000 x g for 5 min and washed twice with 10 ml of PBS. The cultures were diluted in 1 ml of the supplemented DMEM w/o phenol red and after estimating the cell concentration the strains were diluted to concentration 2 x 10^6 cells/ml. Starting from this culture six serial dilutions were made (1:4, 1:16, 1:64, 1:256, 1:1024 and 1:2048). 50 µl of Candida serial dilutions were added to the wells – both with and without macrophages. Dilutions 1:256, 1:1024 and 1:2048 were made at least in triplicates. Plates were spun at 500 g for 1 min, followed by incubation at 37℃ and 5% CO_2 for 48 h. After this period the wells were washed with 100 µl PBS and 40 µl of 0.2 % crystal violet 20 % MeOH aqueous solution was added into each well. After 15 minutes crystal violet stain was aspirated, wells were washed twice with 100 µl PBS and the 96 well plate was scanned. The amount of colony forming units was counted for both wells with and without macrophages and the killing rate was determined. For non-filamentous strains (in this case derived from *efg1*/*cph1* strain background) the crystal violet staining was not possible, thus after the 48 h of interaction the content of the 96 well plate was resuspended and the OD_{600} was measured.

5.40 ROS Assay

5.40.1 Using Bone Marrow Derived Macrophages

For the detection of extracellular ROS, chemiluminescence assays are used exploiting horse radish peroxidase dependent electron acceptor luminol, which reacts weakly with O^{2-} and strongly with other ROS like H_2O_2. Overnight Candida cultures in YPD were re-inoculated into 5 ml fresh YPD to OD_{600} ~ 0.2 and grown for additional 4

– 5 h. Cultures were spun down for 5 min at 4,000 x g washed in 5 ml PBS and resuspended in 1 ml of HBSS buffer (Invitrogen). After estimation of the cell concentration all the cultures were diluted to 4 x 10^6 cells/ml in HBSS (interaction 5 Candida cells : 1 macrophage). Log phase murine (C57BL/6) bone marrow derived macrophages were harvested in HBSS with a cell scraper 45 min prior to measurement and diluted to concentration 4 x 10^5 cells/ml. 100 µl of macrophage suspension was transferred into each well of 96 well plate (Nunclon TM plate, Nunc). 50 µl of ROS detection solution was added. Right before the measurement 50 µl of different Candida strain suspensions were added. Chemiluminescence was measured at 2.5 min intervals for total 120 minutes of experiment in Victor3V multilabel plate reader at 37℃. Luminescence was e xpressed as relative luciferase units/minute cells. Positive control was included as 400 µg/ml zymosan. Negative control was represented by HBSS.

5.40.2 Using RAW264.7 Cell Line

Murine macrophage RAW264.7 cell line was grown in supplemented DMEM at 37℃ with 5% CO_2. When grown to confluence, the macrophages were detached from the 75 cm^2 cell culture flask by using trypsin-EDTA. For chemiluminescence assay the RAW264.7 cells were harvested using a cell scraper, further resuspended in HEPES-buffered BSA and diluted to 2 × 10^6 cells/ml. From this suspension 50 µl (1 × 10^5 cells) were added per well of a white 96-well plate (Nunc). After incubation at 37℃ and 5% CO_2 in the dark for about 30 min, 100 µl of HEPES buffered BSA containing 0.2 mM luminol and 40 U/ml HRP type VI was added. Prior to the measurement 50 µl of HEPES-buffered BSA containing 100 µg of the unopsonized zymosan (100 µg/ml) or 5 x 10^5 Candida cells (1 x 10^7 cells/ml) were added to the wells. As a negative control served HEPES buffered BSA without stimulus, zymosan or with Candida. The well content was mixed thoroughly. Chemiluminescence emitted from each of the wells was immediately measured in dark at 37℃ for 120 min at 2.5 min intervals using a 96 well plate reader (Synergy 2, BioTek). The luminescence was expressed as relative luminescence units (RLU) sec^{-1}.

5.41 Interaction of DCs with Candida

Murine myeloid dendritic cells (originally from cryostock, 7- to 9-week-old C57BL/6 wild-type mice) were harvested from a culture and plated to 60 mm square plates (2.5×10^6 mDC/plate, 3 ml). Next day YPD exponentially grown Candida cultures were spun down and resuspended in PBS. After estimation of the cell count the dendritic cells were infected with 100 µl of Candida suspension (5×10^7 cells/ml) in ratio 2 C. a. : 1 mDC. After 4 h incubation the plates were placed on ice and medium was removed (except negative control, where mDCs are in media non-adhered). Media of negative controls were centrifuged (750 x g, 5 min, and 4℃) and to pellet 350 µl of RNA lysis buffer (Promega) containing β-ME was added. To the infected plates 350 µl of the same buffer was added and the mDCs were scrapped off the plate. Samples were added on Filter column and DNA was sheared by spinning at 11,000 x g for 1 min at RT. Supernatants of the flow-throughs were collected and RNA was extracted with SV Total RNA Isolation System (Promega). 1 µg of RNA was transcribed to cDNA using Reverse Transcription System (Promega) and following the technical bulletin of the producer.

5.42 Microscopy and Fluorescent Microscopy

Candida cultures used for microscopical studies were fixed for 10 minutes at room temperature with 3.7% formaldehyde. Afterwards, the cells were washed once with PBS and resuspended in desired amount of fresh PBS. About 4 µl of cell suspension were transferred to polylysine glass slide and overlaid with cover slip. The edges were sealed with transparent nail polish. Microscopy was performed using microscope Zeiss Axiovert 200M with respective excitation filter for GFP.

For ordinary microscopy the cells were not fixed and they were examined at Zeiss Axiovert 25, where black and white pictures were taken. This microscope was also used for pictures of colony agar invasion.

5.43 Transmission Electron Microscopy

2 ml of exponentially grown Candida cells in YPD (OD_{600} ~ 1.0) were fixed for 1 hour with 2.5% glutaraldehyde. After fixation, cells were washed three times for 15 min with 1 ml of fresh YPD. After the last wash 0.5 ml YPD containing 2% $KMnO_4$ was

added to the cells and incubated at room temperature for 1 hour. Cells were washed three times for 15 min with 1 ml YPD and then slowly dehydrated by incubation for 15 min each using 1 ml of 30%, 50%, 70%, 90% and 100% acetone with centrifugation after each step (1 min, 2,000 x g). Finally, cells were washed twice in absolute acetone and incubated 1 hour in a mixture of 1/3 Spurr's medium hard resin (Polysciences) and 2/3 acetone in closed Eppendorf tube. Tubes were then opened and the acetone was evaporated overnight at room temperature. The cells were incubated twice for 1 h with fresh Spurr's resin, overlaid by the resin in a 5.6 mm Beem capsules and baked at 65℃ for 48 hours. After trimming with razor blade, samples were sectioned with glass knives (Ultramicrotom, Leica Ultracut) to about 60 nm sections and collected on a water surface. Sections were transferred to the surface of mesh grids covered with polyvinyl formal support film and stained for 5 minutes in 1 % uranyl acetate and after washing with dH_2O for additional 4 minutes in 1% lead citrate. Washed and dried samples were examined under electron microscope (Zeiss EM10 and FEI Tecnai G2). Multiple cells of each strain were imaged. The average cell wall thickness, from the membrane to the middle of electron dense outer mannoprotein layer, was measured manually for at least 20 individual yeast cells.

6 RESULTS

6.1 Effect of Efg1 and Cph1 Transcriptional Factors on *C. albicans* Cell Wall

Many different factors control cell wall biogenesis in *C. albicans*. Transcription factor Efg1 is a central regulator of metabolism, dimorphism and virulence in this organism, but it has also been shown to determine cell wall biogenesis to a large extent. Deletion of a second transcription factor, Cph1 further increases the effects observed for strains deleted in Efg1, indicating a synergistic effect of both transcription factors (Dieterich*, et al.*, 2002; Doedt*, et al.*, 2004; Harcus*, et al.*, 2004; Sohn*, et al.*, 2003). In order to reveal changes in cell wall polysaccharide composition, and understand the differences observed in their immunogenic behavior (Barker*, et al.*, 2008; Dongari-Bagtzoglou and Kashleva, 2003; Korting*, et al.*, 2003; Lu*, et al.*, 2006; Phan*, et al.*, 2000; Villar*, et al.*, 2004; Yang*, et al.*, 2009), the cell wall of *cph1*, *efg1* and *efg1*/*cph1* mutant strains was analyzed.

6.1.1 Determination of Cell Wall Changes

Deletion of the transcription factors Efg1 and Cph1 results in changes in expression levels of many cell wall proteins. This includes changes in expression of cell wall synthesizing and remodeling enzymes like the chitinases *CHT1*, *CHT2* and *CHT3*, chitin synthases *CHS1*, *CHS2*, *CHS4* and *CHS5*, β-1,3-glucan synthase subunits *GSL1*, *GSC1* and *KRE62*, β-1,4-glucan branching enzyme *GLC3*, β-1,6-glucan biosynthesis genes *KRE1* and *SKN1*, glycosyl hydrolase *CRH11*, glycosidases *PHR1* and *EXG2* and *SUN41*. In general, most of these genes are downregulated in *efg1* and *efg1/cph1* strains compared to the wild-type, with the strongest evidence in hypha inducing media, like α-MEM at 37℃. On the other side, different regulati on of many of these genes can be observed in YPD medium as well (Doedt*, et al.*, 2004; Harcus*, et al.*, 2004; Sohn*, et al.*, 2003). Until now the influence of these changes, on the transcriptional level, were not validated on a structural level, focusing on the polysaccharide composition of the cell wall in these strains.

In order to verify the expected cell wall defects, growth of the strains was investigated in presence of the cell wall disturbing agents Congo Red and Calcofluor White. They were both originally described as inhibiting glucan synthesis (Roncero

and Duran, 1985) and chitin microfibril assembly (Herth, 1980). Calcofluor White is considered to be preferentially associated with chitin, supported by the fact that it is generally used in microscopy to visualize chitin components of the cell walls of various microorganisms (Pringle, 1991). Moreover, Calcofluor White does not affect for example *Schizosaccharomyces pombe* that does not contain chitin in its cell wall in detectable levels (Roncero and Duran, 1985). Calcofluor White and Congo Red seem to have partially overlapping effects and in general they are indicators for cell wall stress. Analysis of strains defective in *cph1*, *efg1* or *efg1*/*cph1* on agar-plates containing Congo Red or Calcofluor White showed stronger sensitivity of the *efg1* deleted strains to Congo Red, whereas the strain defective in *cph1* showed no altered sensitivity to any of the two agents, if compared to the wild-type. Reversion of the *efg1* deletion resulted in restored resistance to Congo Red (Figure 5A). The increased sensitivity to the cell wall disturbing agent Calcofluor White in strain deleted for *efg1* observed previously (Harcus*, et al.*, 2004) was confirmed. However, reintroduction of one functional copy of the *EFG1* gene into *efg1* strain did not restore the observed phenotype. Moreover, this phenotype was not observed in *efg1*/*cph1* mutant (Figure 5A).

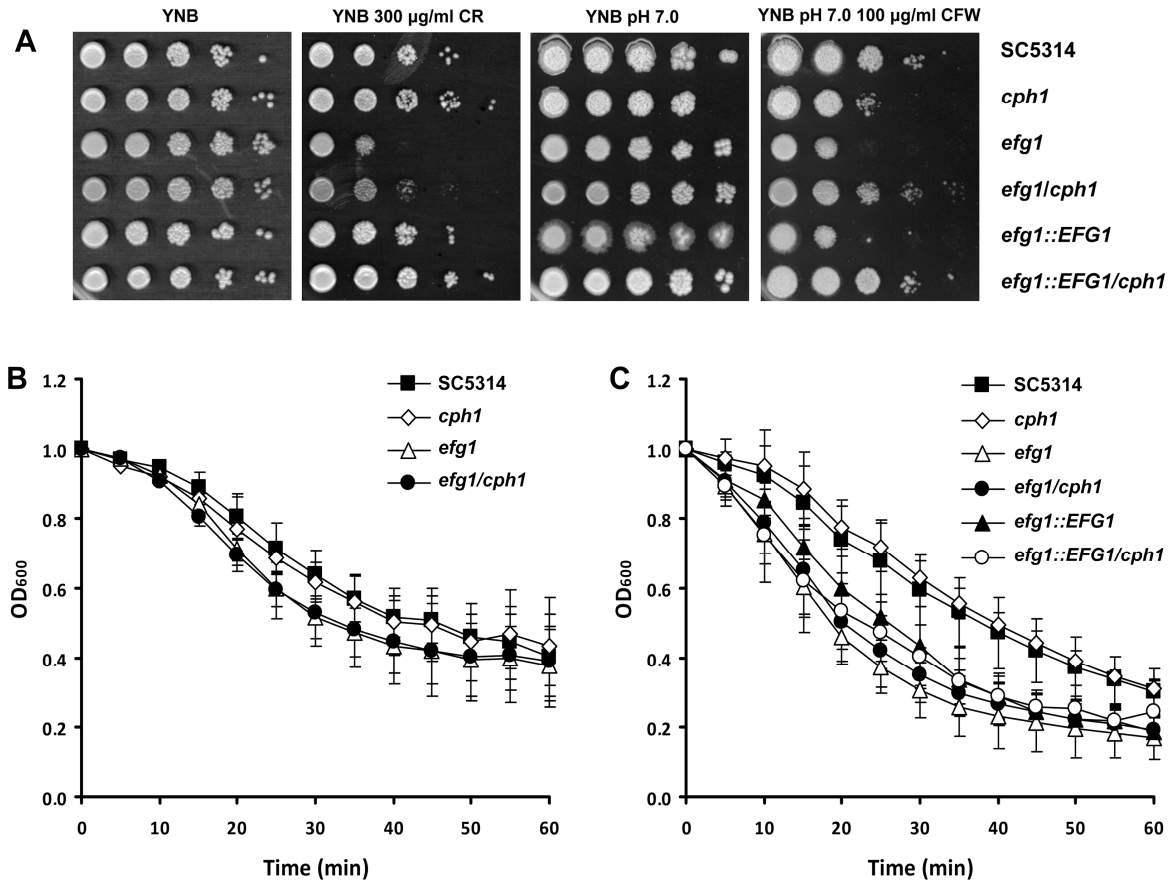


Figure 5. Effect of *efg1* and *cph1* deletion on cell wall stability.

Drop tests testing sensitivity to the cell wall disturbing agents Congo Red (CR) and Calcofluor White (CFW) (A). Strains were spotted on agar plates in 1:10 dilution series, cultivated at 30℃ and documented after 2 days (Congo Red) and 3 days (Calcofluor White). Sensitivity of exponentially (B) and post-exponentially (C) grown cells to the Zymolyase 100T. Cells were cultivated in presence of the cell wall degrading enzyme Zymolyase and changes in optical density of the culture starting from OD_{600} = 1 was monitored for 1 hour. Data represent average from three independent experiments.

To further confirm the cell wall defects observed, cells of SC5314, *cph1*, *efg1* and *efg1/cph1* strains were incubated with Zymolyase 100T. This enzyme is a β-1,3-glucan laminarinpentaohydrolase from *Arthrobacter luteus*, which hydrolyzes linear glucose polymers with β-1,3-linkages and releases protoplasts of the yeast cells. In hypotonic conditions the degradation of the cell wall can be measured spectrophotometrically, by cell lysis resulting in a decrease in OD_{600}. By analyzing the exponentially grown cells no statistically significant differences among the strains were observed (Figure 5B). Strains *efg1* and *efg1*/*cph1* tended to show higher sensitivity to the Zymolyase, however, this was within the error rate of the assay. By using cells from post-exponentially grown cultures (OD_{600} ~ 10) the *cph1* strain

showed the same kinetics of cell wall degradation as the wild-type, whereas the *efg1* and *efg1/cph1* strains showed significantly increased sensitivities (Figure 5C). When *EFG1* revertant strains *efg1*::*EFG1* and *efg1*::*EFG1*/*cph1* were included to the assay, only partial reversion of the phenotype was observed, indicative of a gene dosis effect. This indicates that deletion of *efg1* gene has prominent effect not only on the cell wall protein composition as reported previously (Sohn*, et al.*, 2003), but most likely also on the structural composition of the polysaccharide matrix. Interestingly, the stronger effect in post-exponential cells indicates a more prominent role of Efg1 in cell wall biogenesis during post-exponential growth phases.

6.1.2 Determination of Cell Wall Polysaccharide Composition

To investigate the structural changes of the cell wall more in detail, the polysaccharide composition in the different strains was analyzed (Figure 6). First, the total glucose amount released by hydrolysis of dry cell biomass was determined for the exponentially/post-exponentially grown strains SC5314, *cph1*, *efg1*, *efg1/cph1*, *efg1*::*EFG1* and *efg1*::*EFG1*/*cph1*. The most abundant polysaccharides in the cell wall are β-1,3 and β-1,6-glucans, which after acidic hydrolysis are converted to glucose. In order to account for the carbohydrates present in the cytosol (mainly trehalose and glycogen), the cells were additionally lysed by disruption with glass beads. After separation of the cell wall fragments by centrifugation, the concentration of glucose, released by hydrolysis of the extracted cell content, was determined (Figure 6A, C). Interestingly, deletion of *efg1* has a significant effect, both on the total amount of glucose units inside the cell, as well as the amount of glucans in the cell wall. The total amount of glucose released by hydrolysis of the exponentially/post-exponentially grown *efg1* and *efg1*/*cph1* strains was determined to be only about 77%/83% (*efg1*) or 67%/72% (*efg1*/*cph1*) respectively of the wild-type strain SC5314, while in case of *cph1* strain the level remained unaltered. After substraction of the cytosolic glucose, the amount of cell wall related glucose in exponentially/post-exponentially grown cells was determined to be 84%/90% for the *efg1* and 78%/82% for the *efg1*/*cph1* strain if compared to the wild-type. The amount of glucose released by hydrolysis of the cytosolic content was also significantly reduced in strains carrying a deletion in *efg1*. This effect seems to be even more prominent in case of

efg1/*cph1* double mutant indicating a contribution of *cph1* deletion as well. However, in the case of the revertant strains *efg1*::*EFG1* and *efg1*::*EFG1*/*cph1* no significant reversion of the reduced cell wall glucan levels was observed. Only a partial reversion of internal glucose levels occurred in these strains.

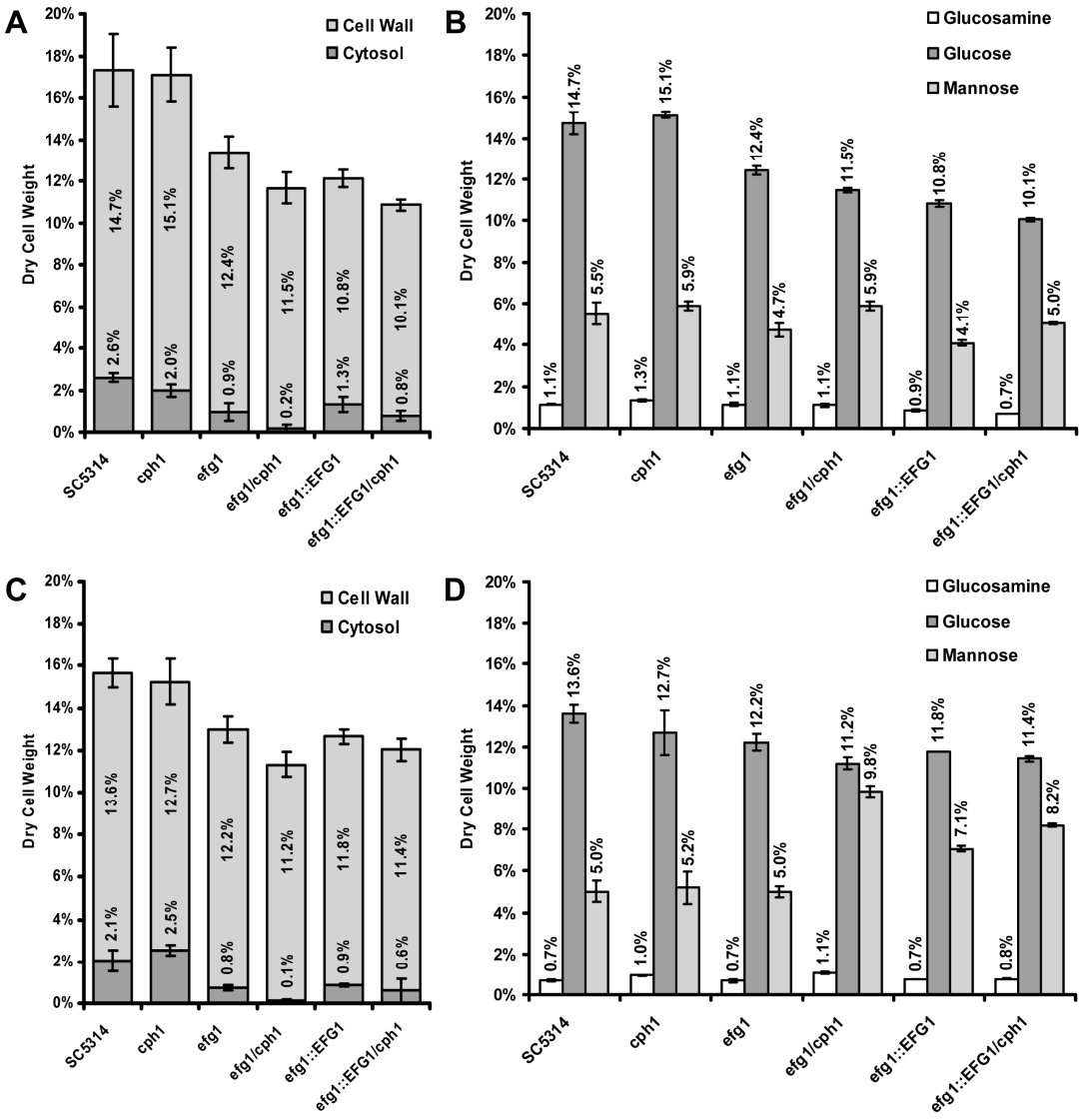


Figure 6. Polysaccharide composition of the cell wall following *efg1* and *cph1* deletion.

Absolute amount of glucose released by hydrolysis of total cell dry biomass and cytosolic content of exponentially grown (A) and post-exponentially grown (C) strains SC5314, *cph1*, *efg1, efg1*/*cph1, efg1*::*EFG1 and efg1*::*EFG1*/*CPH1*. Additionally cell walls were purified from Candida cells, hydrolyzed with sulphuric acid and relative cell wall composition was determined. From both these values absolute polysaccharide cell wall compositions related to the dry cell biomass of exponentially (B) and post-exponentially (D) grown strains were determined. All the data were based on three independent experiments and the average values with standard deviations are presented.

In addition to the determination of glucan amount related to the dry cell biomass, the relative concentration of glucose (glucans), glucosamine (chitin) and mannose (mannosylated proteins) in purified cell walls was determined. The respective relative amounts of glucans, mannans and chitin in the isolated cell walls of the exponentially and post-exponentially grown strains are listed in Table 14. By correlating these proportions with the absolute amount of glucose in the cell wall, it was possible to determine the absolute amount of the analyzed saccharides related to the dry cell biomass. Thereby it can be determined which component in the cell wall remains constant and which component changes (Figure 6B, D).

Table 14. Relative cell wall polysaccharide composition

Strain	Glucans (%)	Mannans (%)	Chitin (%)
Exponentially grown cells			
SC5314	68.9 ± 2.4	25.8 ± 2.6	5.3 ± 0.1
cph1	67.6 ± 0.5	26.4 ± 0.9	6.0 ± 0.2
efg1	68.0 ± 1.2	25.8 ± 1.7	6.2 ± 0.4
efg1/*cph1*	62.1 ± 0.6	31.9 ± 1.1	6.0 ± 0.4
efg1::*EFG1*	68.6 ± 1.0	26.0 ± 0.8	5.4 ± 0.2
efg1::*EFG1*/*cph1*	63.7 ± 0.6	31.9 ± 0.4	4.4 ± 0.1
Post-exponentially grown cells			
SC5314	70.4 ± 2.2	26.0 ± 2.6	3.6 ± 0.3
cph1	67.3 ± 5.7	27.6 ± 4.1	5.1 ± 0.1
efg1	68.3 ± 2.2	27.9 ± 1.6	3.8 ± 0.4
efg1/*cph1*	50.7 ± 1.3	44.5 ± 1.2	4.8 ± 0.2
efg1::*EFG1*	60.1 ± 0.1	36.1 ± 0.8	3.8 ± 0.0
efg1::*EFG1*/*cph1*	56.1 ± 0.6	40.1 ± 0.5	3.8 ± 0.2

In cells grown exponentially no significant changes in chitin and mannan levels related to the cell biomass were observed. The most dramatic change was observed in *efg1* and *efg1*/*cph1* strains, where a decrease of the glucan levels of about 20% occurred. More dramatic change appeared in cells grown post-exponentially. The deletion of both *efg1* and *cph1* genes led in this case to significant increase in mannans in the isolated cell walls, reaching almost the glucan level. An intermediate increase of mannans in post-exponentially grown cells was observed also in *efg1*::*EFG1* and *efg1*::*EFG1*/*cph1* revertant strains, although the *efg1* mutant was not affected. This effects could have been caused by alterations in *EFG1* expression from only one copy placed in the *leu2* locus in the revertant strains (Lo*, et al.*, 1997).

For wild-type strain SC5314 the total amount of all the cell wall polysaccharide components represented about 21.4%/19.3% (exponentially/post-exponentially grown) of total dry cell biomass, which is in agreement with the general knowledge that the cell wall of *C. albicans* contains about one fifth of the cell biomass (Chaffin, 2008).

For exponentially grown strains deleted in *efg1* or *efg1*/*cph1* a significantly lower level of cell wall polysaccharides was detected (decreased for 15% and 14%), indicating a significantly reduced cell wall biomass. In case of post-exponentially grown cells, this decrease is less dramatic and reaching only 2% and 7% difference from the wild-type for strains deleted in *efg1* or *efg1*/*cph1* respectively.

Transmission electron microscopy images revealed that the relative thickness of the cell wall of the exponentially grown *efg1*/*cph1* strain was reduced to about 50% of the wild-type (Figure 7). The cell wall thickness was measured between the plasma membrane and high electron density outer layer of the cell wall consisting predominantly of mannoproteins, as described in literature previously (Martinez-Lopez*, et al.*, 2006). The average thickness of the SC5314 cell wall in electron microscopy images was 62.6 ± 6.0 nm, while the thickness of *efg1*/*cph1* cell wall was 32.6 ± 5.3 nm. This finding is in agreement with the reduced amount of carbohydrates detected in the strains deleted for *efg1*. It can be expected that similar cell wall thickness reduction occurs in the *efg1* strain as well. Apart of the thickness of the β-glucan layer, both of strains seem to have normal cell wall architecture, consisting of an electron dense outer layer of mannoproteins, and an electron lucent inner layer composed mainly of β-1,3-glucan and chitin.

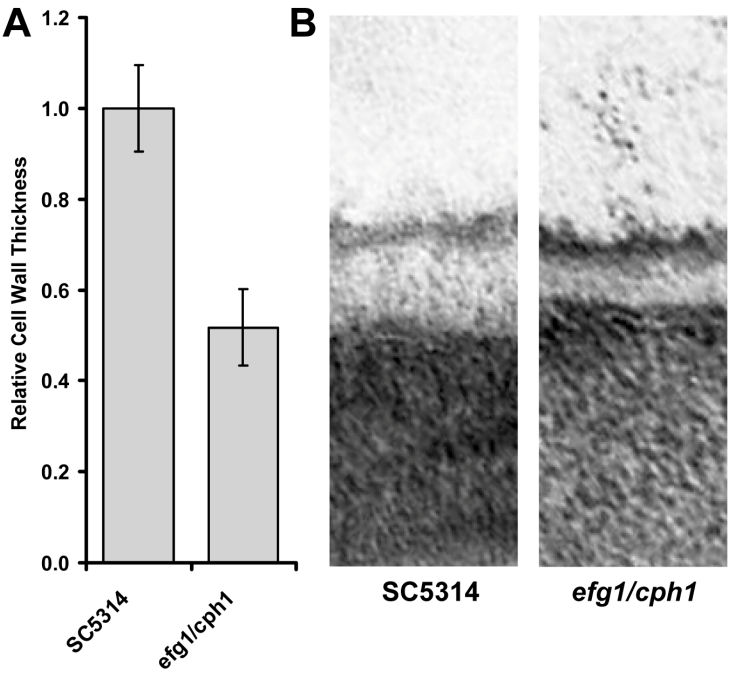


Figure 7. Cell wall thickness
The relative cell wall thickness (A) of wild-type strain SC5314 and *efg1*/*cph1* mutant was determined from over 20 independent cell measurements obtained from different microscopic images. Representative TEM images (B) of the cell walls presenting differences in the thickness of the electron-translucent polysaccharide layer. Images were taken at 16,000 x magnification.

6.1.3 β-1,3-Glucan Exposure

In order to check if the structural changes in the cell wall observed also have consequences on β-glucan exposure, a critical virulence factor in *C. albicans* (Wheeler and Fink, 2006), β-glucan of exponentially and post-exponentially grown cells of the respective strains (SC5314, *cph1*, *efg1, efg1*/*cph1, efg1*::*EFG1* and *efg1*::*EFG1*/*cph1*) was visualized using a β-1,3-glucan specific antibody (Figure 8). Interestingly, there was a clear difference for the *efg1* and *efg1/cph1* strains if compared to the WT. Deletion of both *efg1* and *cph1* results in significantly lower signal (reduced to about 50%), while deletion of *efg1* alone has an opposite effect (about double increase in intensity to the wild-type). When the revertant strains *efg1*::*EFG1* and *efg1*::*EFG1*/*cph1* were examined, at least partial restoration of the phenotypes of the respective mutants has been observed. In strain *efg1*::*EFG1* the signal intensity was reduced back to wild-type levels and in case of *efg1*::*EFG1*/*cph1* the signal intensity increased as well, however, it did not reach the wild-type level. In this revertant strain the shape of the cell wall is clearly visible, compared to the *efg1*/*cph1* mutant, where the fluorescent signal is more dispersed over the cell. In general the post-exponentially grown cells bound significantly lower amounts of β-1,3-glucan antibody. However, a stronger signal from the *efg1* strain remained even under this condition (Figure 8).

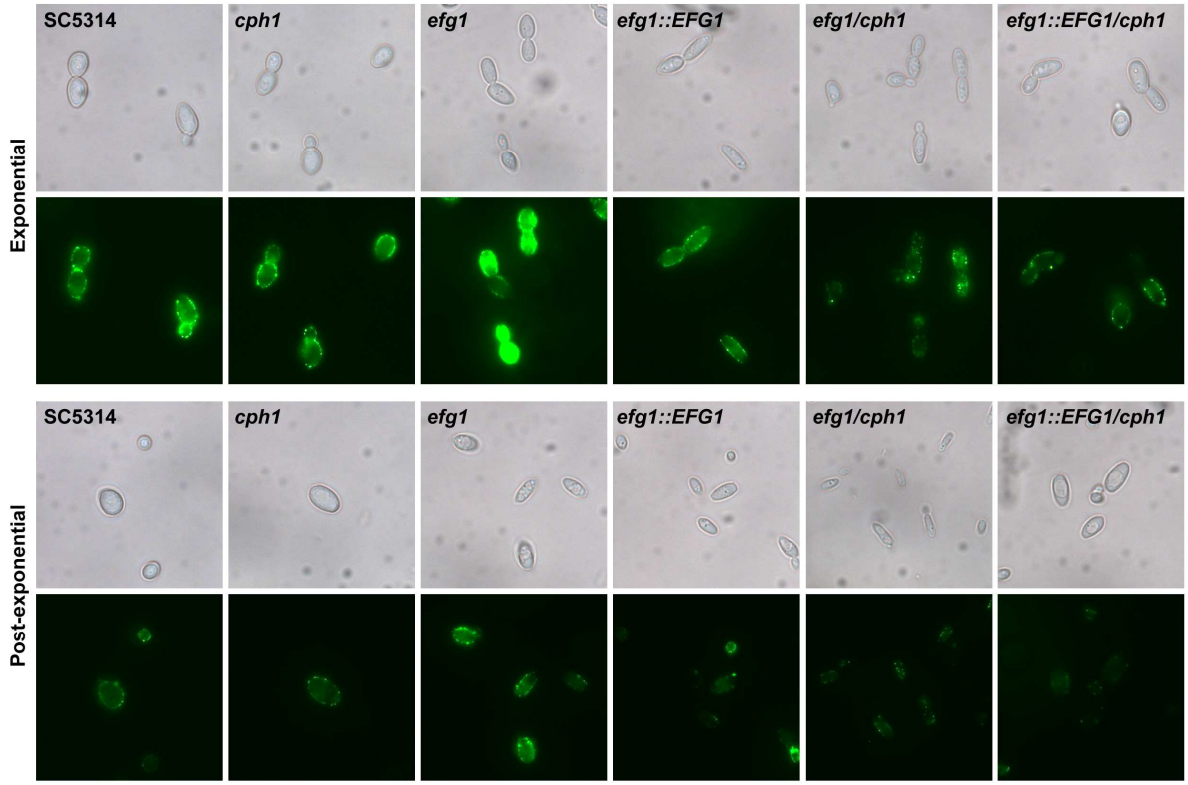


Figure 8. Effect of *efg1* and *cph1* deletion on β-1,3-glucan exposure.

Exponentially and post-exponentially grown cells were stained for β-1,3-glucan, using an anti-β-1,3-glucan antibody. The secondary antibody was labeled with Alexa488. All fluorescent images were taken with constant exposition time in order to compare the signal intensity. In samples labeled only with secondary antibody no visible fluorescence was detected (not shown).

6.1.4 Interaction with Immune System Components

Host defense against fungi ranges from non-specific proteolytic defense to a dedicated adaptive immune response. The first line of host defense against fungal pathogens, including *C. albicans*, relies on fungal recognition by innate immune cells such as dendritic cells, macrophages and neutrophils. On the unopsonized *C. albicans* cell fungal polysaccharides are the ligands for pathogen recognition receptors present on immune cells. The cell wall composition of *C. albicans* varies depending on environmental cues such as temperature, pH, and the presence of serum. The complex recognition of *C. albicans* by innate immune system was reviewed recently (Netea*, et al.*, 2008a). The β-1,3-glucan accessible at the yeast surface but not at the hyphal surface is recognized by Dectin-1 (Heinsbroek*, et al.*, 2005) and is considered to be one of the most important receptors for *C. albicans* yeast cell recognition. Also differential expression of glycosyltransferases, the

enzymes that modify glycan components of the cell surface, can result in differential recognition by the immune system (Netea*, et al.*, 2006). Heavy mannosylation of cell wall proteins with long chains composed of α-linked mannose units is typical for fungi, while in mammals the mannose is only a part of the N-linked polysaccharide core which is ended with sialic acid or fucose. The structures of N-linked polysaccharides can be distinguished by the innate immune system. The mannose receptors recognize oligosaccharides terminated with mannose, glucose, or *N*-acetylglucosamine (Stahl*, et al.*, 1978) and preferentially recognize α-linked branching oligosaccharides (Kery*, et al.*, 1992). In addition to the mannose receptor, dendritic cells can interact with *C*. *albicans* through DC-SIGN (Cambi*, et al.*, 2003). SIGNR1 is another mannan binding receptor present on resident peritoneal macrophages (Taylor*, et al.*, 2004). Finally, Dectin-2 recognizes high-mannose structures (McGreal*, et al.*, 2006) and preferentially binds to hyphae compared to yeast cells (Sato*, et al.*, 2006). Additionally, *C. albicans* can be recognized by interactions with some members of the Toll-like receptor group TLR2 and TLR4. Pro-inflammatory TLR4 recognizes yeast cells (Netea*, et al.*, 2002), while TLR2 recognizes both yeast and hyphal cells and mediates the release of anti-inflammatory cytokines, thus favoring Candida survival (Netea*, et al.*, 2004). Only slight effect of TLR6 and TLR9 on *C. albicans* recognition was reported (Bellocchio*, et al.*, 2004; Netea*, et al.*, 2008b). In the past many host proteins bound by *C. albicans* were described, including complement fragments (Heidenreich and Dierich, 1985), plasminogen (Crowe*, et al.*, 2003), and extracellular matrix proteins such as collagen, fibronectin, and laminin (Gaur and Klotz, 1997). Such ability can lead to partial masking of the immunogenic cell wall and additional modulation of the immune system of the host.

Because of the complexity of the host immune response, it is useful to investigate the behavior of pathogens that interact with isolated elements of the innate immune system. Immune cells, while far from providing a complete picture of the immune response, allow a focus on the initial recognition by the immune system. Therefore, the reaction of macrophages and dendritic cells to strains deficient in *efg1* and *cph1* was tested.

6.1.4.1 Interaction with Macrophages and ROS Release

The significant changes in cell wall composition, especially the lower glucan exposure, of the *efg1*/*cph1* strain could be a reason for its altered immunological properties which have been described previously during contact with immune cells or epithelial and endothelial surfaces (Barker*, et al.*, 2008; Dongari-Bagtzoglou and Kashleva, 2003; Korting*, et al.*, 2003; Lu*, et al.*, 2006; Phan*, et al.*, 2000; Villar*, et al.*, 2004; Yang*, et al.*, 2009). To test the direct effect of mutants defective in *efg1* and *cph1* on immune cells the murine macrophage cell line RAW264.7 was employed in order to analyze release of reactive oxygen species (ROS) in contact with these strains (Figure 9). Production of ROS by innate immune cells is part of the first defense line against pathogens (DeLeo*, et al.*, 1999; Forman and Torres, 2002) and ROS assays thus can be used to evaluate recognition of microbes by the immune system (Gantner*, et al.*, 2005). Although the molecular identities of ligands triggering the response are not fully characterized, the main candidates include β-1,3 and β-1,6-glucans (Gantner*, et al.*, 2003; Rubin-Bejerano*, et al.*, 2007). Zymosan, a crude cell wall preparation from *Saccharomyces cerevisiae*, served as control in the experiment. For the assay exponentially (OD_{600} ~ 1) and post-exponentially (OD_{600} ~ 10) grown cells were used in order to check the immunogenicity of the strains in distinct growth phases.

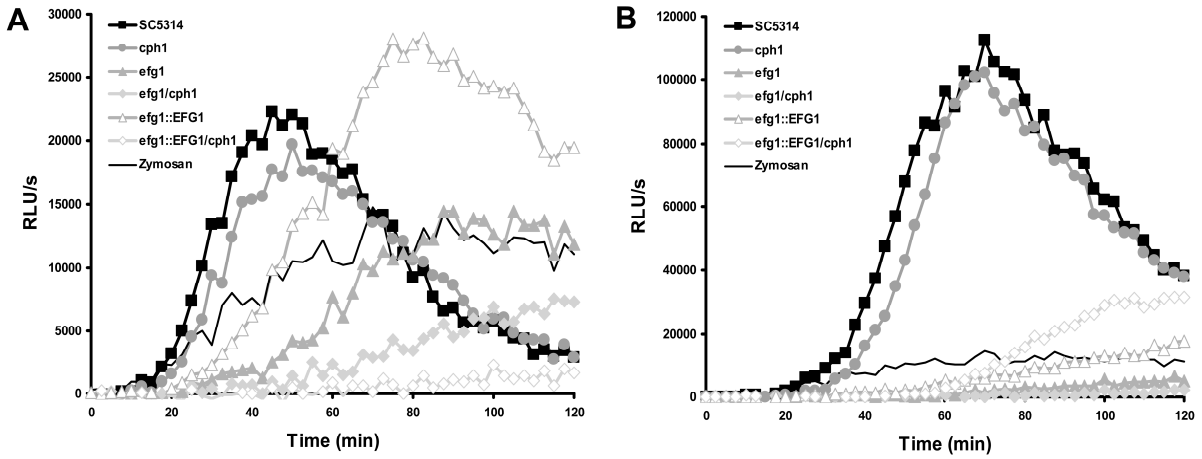


Figure 9. ROS release in contact with strains deleted for *efg1* and *cph1*.

ROS release by macrophages in contact with SC5314, *cph1*, *efg1, efg1*/*cph1, efg1*::*EFG1 and efg1*::*EFG1/cph1* strains grown exponentially (A) or post-exponentially (B). ROS were quantified in real-time with use of peroxidase-luminol coupled reaction. Data were reproduced in three independent experiments as quadruplicates. Average results of representative experiments are presented. Standard deviations of individual time points did not exceed 10% of its absolute values.

For SC5314 and the strain deleted in *cph1* highly similar and rapidly induced strong ROS activity was observed. Post-exponentially grown cells showed about five-fold stronger ROS release, however, the induction was delayed if compared to exponentially grown cells. For the *cph1/efg1* mutant strain only marginal ROS activity was observed under all conditions. Interestingly, for the *efg1* deletion strain a strong difference in ROS activity of the macrophages depending on the growth phase of the cell was observed. In exponentially grown cells the *efg1* mutant strain shows significant ROS activity with a short delay in induction, similar to stationary SC5314 cells. However, in post-exponentially grown *efg1* cells ROS activity is much closer to the *efg1*/*cph1* mutant strain, than to the WT. These data show only partial agreement with β-1,3-glucan exposure observed earlier. In case of *efg1*/*cph1* strain the lower β-1,3-glucan exposure is in agreement with its lower capability to stimulate ROS response, both by exponentially and post-exponentially grown cells. In case of *efg1* strain the situation is different. Compared to the wild-type, *efg1* mutant exposes significantly more β-1,3-glucan on its surface as judged by antibody staining, but the ROS response to the exponentially grown cells is delayed and in case of post-exponentially grown cells the response is strongly reduced. The difference in the immunogenicity of the *efg1* strain, depending on the growth phase, may reflect the relevance of Efg1 gene-regulation during various growth phases as suggested by its influence on metabolism (Doedt*, et al.*, 2004). Additionally, an increase in

immunogenicity of the wild-type during post-exponential growth phase was observed, although it exposes less β-1,3-glucan compared to the exponentially grown cells. This indicates only limited role of β-1,3-glucan exposure, as measured by antibody staining, for *C. albicans* immunogenicity.

For the revertant strains *efg1*::*EFG1* and *efg1*::*EFG1*/*cph1* again only a partial restoration of the phenotype was observed. The ROS response to the *efg1*::*EFG1* strain was reaching WT levels in exponentially grown cells, however with a timely delay, whereas in post exponential cells the ROS levels did not reach the wild-type level. For the *efg1*::*EFG1*/*cph1* strain, in case of the post-exponentially grown cells a significantly increased ROS response was observed, while exponentially grown cells stimulated only very low ROS response, lower than *efg1*/*cph1* strain.

6.1.4.2 Transcriptional Response of Dendritic Cells

To additionally check the differential response of primary murine dendritic cells (DCs) to the different Candida strains on a transcriptional level, qRT-PCR analysis was performed (Figure 10). DCs in general offer faster and stronger transcriptional response to Candida cells than macrophages. Moreover, antigen-presenting dendritic cells, located at mucosal surfaces and in the skin, play an important role in anti-Candidal protective immunity. Dendritic cells function as professional phagocytes to kill *C. albicans* and subsequently present it to the adaptive immune system. The response to *efg1* and *efg1*/*cph1* strains remained significantly reduced for the cytokine transcripts TNFα, IFNβ, IL-4 and IL-23, compared to wild-type and *cph1* strain. For the transcripts of IL-1β, IL-10 and IL-12 no significant changes among the strains were observed. The production of IL-4 is usually linked to ingestion of hyphae (d'Ostiani*, et al.*, 2000), therefore reduction of IL-4 expression in contact with *efg1* and *efg1*/*cph1* strains may reflect the altered filamentation phenotypes of both strains. The reduction in TNFα expression may be linked to changes in mannose structures of the cell wall (Pietrella*, et al.*, 2006). Additionally, IL-23 induction in dendritic cells can be linked to changes β-glucan exposure (Dennehy*, et al.*, 2009). Moreover, these data indicate that the response to the strains is dependent on the cell wall changes after deletion of *efg1* (different response in TNFα, IFNβ, IL-4 and IL-23), but is not affected by the altered cell size after *efg1* deletion, potentially offering lower surface stimulus (similar response among all the strains in IL-1β, IL-10

and IL-12). Addition of the respective *efg1*::*EFG1* and *efg1*::*EFG1*/*cph1* revertant strains in the study could partially restore the immunogenic response.

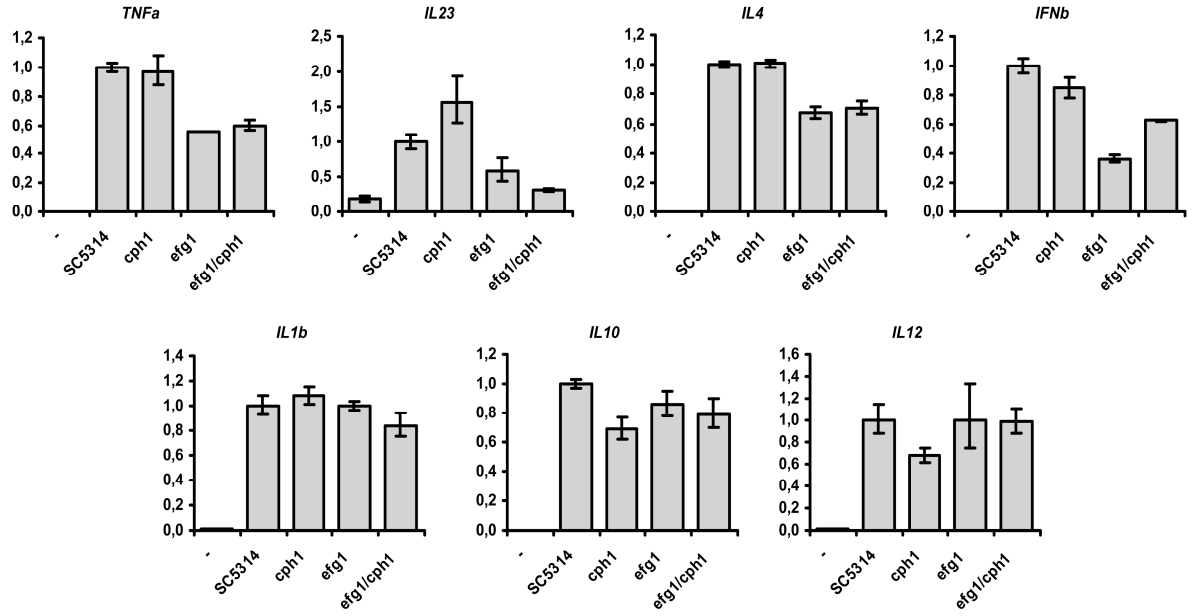


Figure 10. Transcriptional response of murine dendritic cells in contact with *C. albicans* strains deleted for *efg1* and *cph1*.

Transcriptional response of unstimulated DCs (-) and DCs after 4 hours of incubation with SC5314, *cph1*, *efg1* and *efg1*/*cph1* mutant strains. mRNA levels were normalized to GAPDH and related to the absolute levels of DCs stimulated with SC5314, which was set to 1. Experiments were performed in triplicates and average values with standard deviations are presented. Results are representative of two independent experiments.

6.1.5 Effects of *EFG1* Haploinsufficiency

In many cases the reintroduction of one functional *EFG1* allele into the *efg1* and *efg1*/*cph1* mutant strains did not restore the observed phenotypes to the original wild-type levels (ROS response, Zymolyase treatment and amount of cell wall glucans). The revertant strains *efg1*::*EFG1* and *efg1*::*EFG1*/*cph1* were prepared by integration of one functional *EFG1* allele into the *LEU2* locus of strains *efg1* or *efg1/cph1* (Lo*, et al.*, 1997). It seems that the level of expression is sufficient enough to restore their filamentation defects, but insufficient to fully restore all the cell wall defects. Another possibility, to be taken in count, is a position effect of *EFG1* allele in the *LEU2* locus that can also lead to the alterations in phenotype. In order to clarify this, the effects of

haploinsufficiency (presence of single functional copy in diploid organism) of *EFG1* gene in *C. albicans* were tested. For this reason *EFG1* heterozygous strain HLC17 (deleted for only one allele of *EFG1* (Lo, et al., 1997)) was investigated. Additional Ura$^-$ *EFG1* heterozygous strain (HLC46 (Lo, et al., 1997)) reverted for the second functional *EFG1* allele, using plasmid pTD38-HA (Noffz, et al., 2008) and creating new strain HLC17Rev, genotypically identical to the wild type, was used. The genomic DNA of this strain was isolated and Southern blot was performed using restriction enzyme *Bgl*II. Probing against *EFG1* ORF probe (PCR product using oligonucleotides EFG1_F1 and EFG1_R1) proved correct integration of the second functional *EFG1* allele into the originally disrupted *efg1* locus (data not shown).

Wild-type strain SC5314, *efg1* null mutant and *efg1*::*EFG1* revertant were compared to the *EFG1* heterozygous strain HLC17 and HLC17Rev, containing two functional copies of *EFG1*, in terms of Zymolyase sensitivity (Figure 11A), internal and cell wall related glucose amounts (Figure 11B) and finally macrophage ROS response (Figure 11C, D).

While the sensitivity of the revertant strain *efg1*::*EFG1* and *EFG1* heterozygous strain HLC17 to the Zymolyase was moderate and in-between more resistant wild-type and more sensitive *efg1* null mutant, the strain HLC17Rev, containing two functional copies of *EFG1*, showed resistance identical to the wild-type. Thus the deletion of just one *efg1* allele already leads to the weakening of the *C. albicans* cell wall, what is further enhanced by deletion of the second allele. The effect of *efg1* deletion thus can be fully reverted by reintroduction of functional *EFG1* allele into the *efg1* null mutant or HLC17 *EFG1* heterozygous strain.

When the amount of intracellular and cell wall bound glucose units was tested in exponentially grown cells, *EFG1* heterozygous strain HLC17 showed already decreased amounts of both intracellular and cell wall related glucose, like in case of *efg1* null mutant and *efg1*::*EFG1* revertant strain. On the other side, strain HLC17Rev showed both glucose levels comparable to the wild-type strain SC5314. Thus even in this case the deletion of one functional allele of *EFG1* leads to decrease in amounts of cell wall glucans and also intracellular glucose units. Deletion of the second functional *EFG1* allele seems to have an additional effect on the decrease of cell wall glucans. However, there were some slight differences in the cell wall glucan amount between the *efg1*::*EFG1* revertant and HLC17 heterozygote. Its origin is not clear,

but it could be caused by slight differences in growth phases of the strains, when the glucose amounts were determined.

The last phenotype tested was the macrophage ROS response in contact with the strains. The ROS response to the exponentially grown cells in case of wild-type is induced the earliest, the response to the *efg1*::*EFG1* revertant is delayed in time and the response to the *efg1* null mutant is even more delayed in time and slightly reduced. When strains HLC17 and HLC17Rev were tested, the heterozygous HLC17 showed kinetics similar to the *efg1*::*EFG1* revertant, while HLC17Rev to the wild-type, even when the response to this strain appears stronger. In case of stationary grown cells all the strains: *efg1* mutant, *efg1*::*EFG1* revertant and HLC17 heterozygote showed low ROS response. On the other side the HLC17Rev induced ROS response comparable to the wild-type. Thus even in this case a deletion of single functional *EFG1* allele in the wild-type strain has a prominent effect on the *C. albicans* cell wall immunogenicity, which is affected further in the *efg1* null mutant.

Additionally to these strains, the *efg1::EFG1* revertant was compared in terms of ROS response and Zymolyase treatment to the additional *EFG1* revertant strain HLCEEFG1 (Noffz, et al., 2008) with the *EFG1* allele in its own locus. Identical behavior of both strains was further excluding any position effects of the *EFG1* in the *LEU2* locus in case of the *efg1::EFG1* strain (data not shown).

All these data support the insufficiency of one functional *EFG1* allele in the revertant strain for full restoration of the studied phenotypes to the wild type level. These results are further demonstrating a gene-dosis effect for the Efg1 transcription factor, related to several cell wall phenotypes.

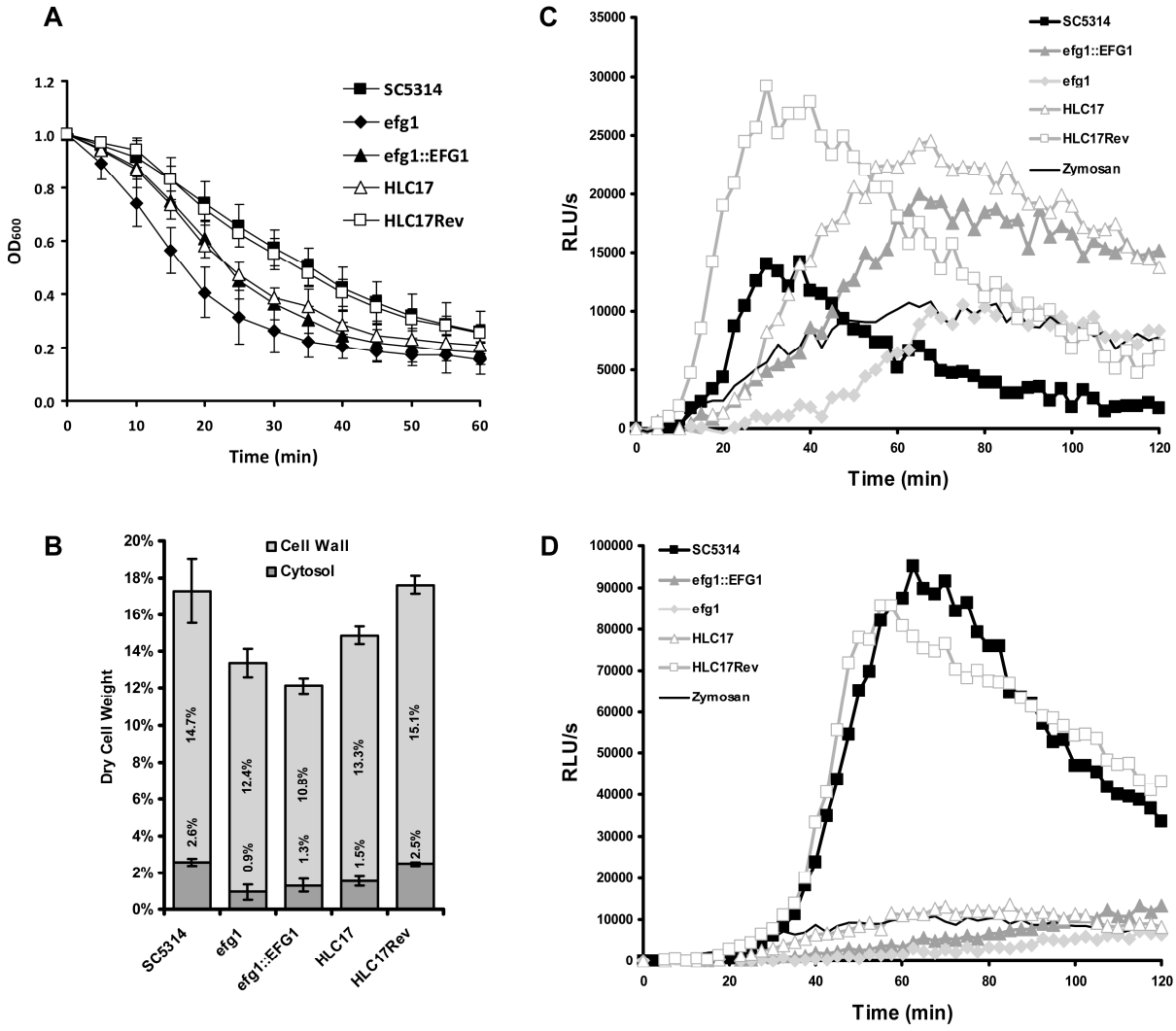


Figure 11. Effects of *EFG1* haploinsufficiency.

Sensitivity of post-exponentially grown cells to the Zymolyase 100T (A). Cells were cultivated in presence of the cell wall degrading enzyme Zymolyase and changes in optical density of the culture starting from OD_{600} = 1 was monitored for 1 hour. Data represent average from three independent experiments. Absolute amounts of glucose released by hydrolysis of total cell dry biomass and cytosolic content of exponentially grown (B) strains. Data represent average of three independent experiments. ROS response to exponentially (C) and post-exponentially (D) grown strains. Data were reproduced in three independent experiments as quadruplicates. Average results of representative experiments are presented.

Deletion of *efg1* has a great impact on the *C. albicans* cell wall, affecting the amount of cell wall β-glucans, polysaccharide cell wall structure and *C. albicans* immunogenicity, whereas deletion of *cph1* alone has only a minor role. Nevertheless, as observed in several other studies, deletion of *cph1* further enhances the *efg1*-bound cell wall changes. These changes in cell wall can partially explain the altered pathogenicity observed for *efg1* and *efg1*/*cph1* strains.

6.2 Investigation of Function of Genes Induced During Adhesion

6.2.1 Identification and Characteristics of *PGA7*, *PGA23* and *PRA1*

C. albicans naturally colonizes various parts of the human body providing environments with various nutrient availabilities, pH and other characteristics. In order to find out whether this organism is able to differentially respond on a transcriptional level to different surface stimuli, experiments of co-incubation of this organism in contact with different surfaces under hypha inducing conditions were performed (Sohn*, et al.*, 2006). Exponentially grown *C. albicans* cells were transferred under hyphae inducing conditions to three different surfaces – abiotic polystyrene surface and two tissue models, Caco-2 and A-431 cell lines. Colorectal adenocarcinoma cell line Caco-2 (Fogh*, et al.*, 1977) offers a model for adhesion to an enterocytic epithelial cell line representing an intestinal model. This cell line has a brush border surface with microvilli structures and very often is used to study intestinal mechanisms *in vitro*. Besides its application for interaction studies with *C. albicans* (Dieterich*, et al.*, 2002), it was described in the literature as a model for studies of adhesion of enterotoxigenic *E. coli* strains (Darfeuille-Michaud*, et al.*, 1990) and for the pathogen *Entamoeba histolytica* (Rigothier*, et al.*, 1991). The second *in vitro* model composed of monolayer of epidermoid vulvovaginal cell line A-431 (Giard*, et al.*, 1973) serves as a vaginal adhesion model and provides a significantly different environment for the adhesion of *C. albicans*. Microvilli structures are missing, instead this cell line growth similar to a stratified squamous epithelium. Candida cells were let to adhere to these surfaces in order to investigate the differences in the transcriptional profile. As a control without any surface stimulus served suspension culture under identical conditions in terms of medium and temperature. The transcriptional profile of *C. albicans* was followed for four hours. Genes *PGA7*, *PGA23* and *PRA1*, described in chapter 3.5.2, were identified in this study as upregulated during the adhesion process to all the three surfaces. All these genes encode for putative cell wall proteins with unknown function in *C. albicans*. While Pga7 and Pga23 belong to the group of GPI-anchored proteins covalently linked to the cell wall, Pra1 is non-covalently associated to the cell surface and is also

found in the medium (Hiller*, et al.*, 2007). How these proteins Pga7, Pga23 and Pra1 contribute to adhesion is not yet clear and was studied as follows.

6.2.2 Transcriptional Profile

In the original experiments (Sohn*, et al.*, 2006) genes *PGA7*, *PGA23*, *PRA1* and also *HWP1*, which serves later in this study as a control for adhesive and invasive conditions, were identified as adhesion upregulated on all the three surfaces (polystyrene surface, Caco-2 and A-431 cell lines). In order to verify the transcriptional profiles from the original work, new infection experiments were performed and transcription of these genes was studied by qRT-PCR (Figure 12).

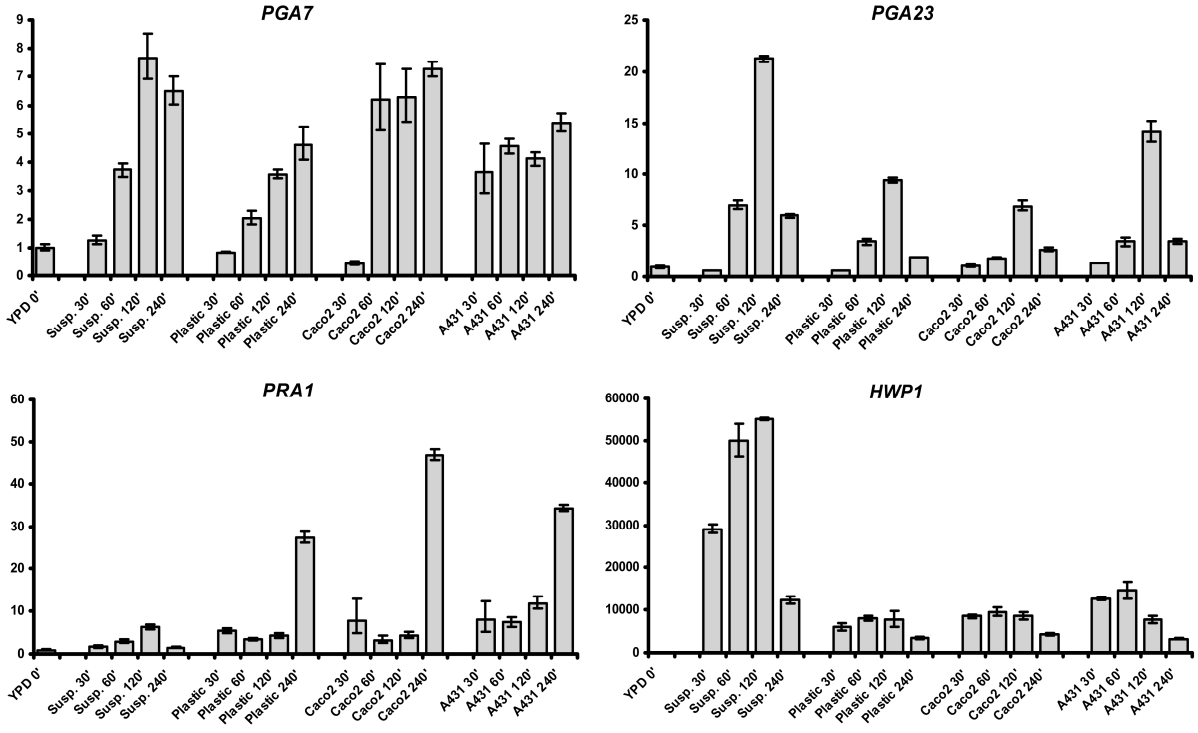


Figure 12. Transcriptional profile *PGA7*, *PGA23*, *PRA1* and *HWP1*.

Expression of *PGA7*, *PGA23*, *PRA1* and *HWP1* in the wild-type strain SC5314 cultivated under hypha inducing condition in suspension culture and adhesion cultures (polystyrene, Caco-2 and A-431 cell lines), focusing on relative expression levels compared to exponentially grown cells in YPD (equal to 1). In total three consistent repetitions of the experiment were performed and data from representative one are presented with error bars from technical triplicates.

In contrast to the original study, genes *PGA7*, *PGA23* and *HWP1* were upregulated also when the wild-type strain SC5314 was cultivated in suspension culture. In case

of *HWP1* the increase in transcription was even significantly higher compared to the adhesion conditions. This difference may have been caused by incubation of the suspension culture in presence of 5% CO_2. Suspension cultures grown in the absence of CO_2 show significantly reduced expression levels of the genes tested (Figure 13). The effect of CO_2 on hyphal formation was reported previously (Sims, 1986). In the original work by Sohn *et al.* the 5% CO_2 was present only in cultures adhering to polystyrene, Caco-2 and A-431 cell lines. Nevertheless, this does not exclude *PGA7*, *PGA23* or *PRA1* to encode for putative cell surface proteins involved in adhesion or invasion process, as known for *HWP1*.

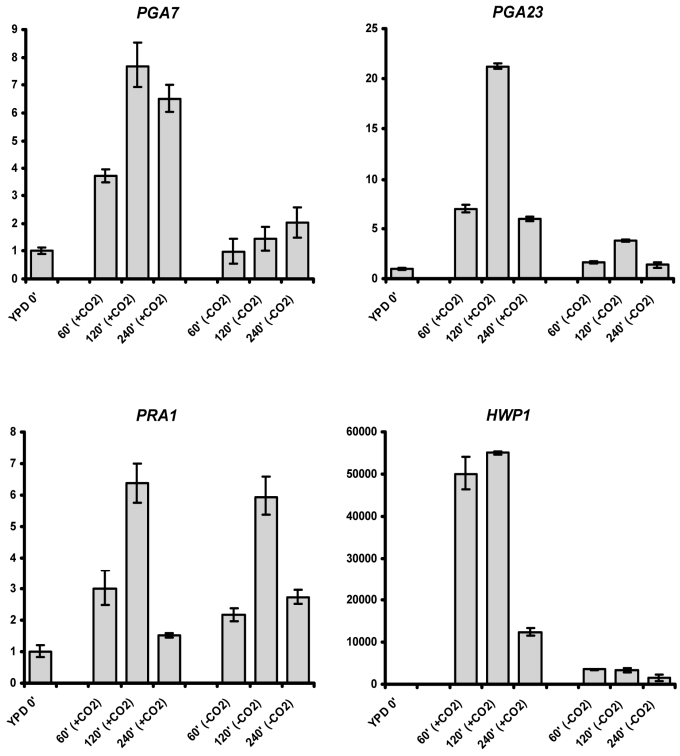


Figure 13. Transcriptional profiles of *PGA7*, *PGA23*, *PRA1* and *HWP1* depend on presence of 5% CO_2

Comparison of transcription profiles of *PGA7*, *PGA23*, *PRA1* and *HWP1* in suspension culture in DMEM at 37℃ with and without presence of 5% CO_2. Data represent qRT-PCR data from representative experiment (out of two) with standard deviations from technical triplicates.

6.2.3 Characterization of Strains Deleted for *pga7*, *pga23* and *pra1*

The first step in characterization of the selected genes was the investigation of the already existing mutant strains lacking *pga7*, *pga23* (Plaine*, et al.*, 2008) derived from BWP17 (Wilson*, et al.*, 1999) and *pra1* (Sentandreu*, et al.*, 1998) derived from CAI4 (Fonzi and Irwin, 1993). In previous studies no changes in the strains deleted for *pga7* or *pga23*, regarding cell wall thickness, Congo Red, Calcofluor White or SDS sensitivity, were reported (Plaine*, et al.*, 2008). This indicates that these predicted

GPI-proteins are not crucial for cell wall stability, or that other proteins with redundant function exist. Also, for *pra1* strain no changes in adhesive or invasive behavior were reported so far. To verify these data the strains *pga7*, *pga23* and *pra1* were checked on their sensitivity to the cell wall disturbing agents Congo Red and Calcofluor White (Figure 14). In case of *pga7* and *pga23* strains no differences were observed in sensitivity to the cell wall disturbing agents, when compared to the BWP17 background strain, confirming the previously reported results. In case of *pra1* strain, there was a slightly increased sensitivity to Calcofluor White, while the sensitivity to Congo Red was unaltered. This increase in sensitivity is reverted in *pra1*::*PRA1* strain, where one functional allele of *PRA1* gene was reintroduced to the genome. A possible explanation for this was reported in literature, where *pra1* strain was described to have an altered chitin distribution in the cell under heat stress conditions (Sentandreu*, et al.*, 1998). To focus on this more in detail, analysis of the relative polysaccharide composition of the cell walls of all the mutant and background strains was performed (presented later in Figure 27, page 104).

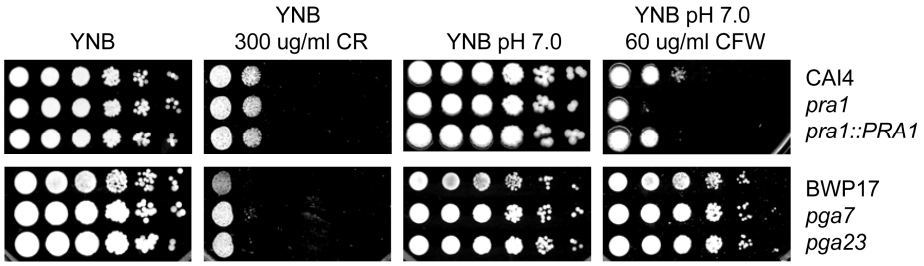


Figure 14. Congo Red and Calcofluor White sensitivity of *pga7*, *pga23* and *pra1* strains.

Plate assay focusing on the sensitivity of the mutant strains employed to cell wall disturbing agents. Cells were spotted on agar plates containing Congo Red or Calcofluor White in dilution series 1:10 starting from OD_{600} = 1.0. Plates were documented 2 days after cultivation at 30°C. While in case of Congo Red no differences of all mutant strains to their respective backgrounds were observed, the *pra1* strains shows higher sensitivity on Calcofluor White which is complemented in the revertant strain *pra1*::*PRA1*.

In order to test the adhesion of these strains on epithelial and biologically inert surfaces, adhesion assays were performed on polystyrene, Caco-2 and A-431 cell lines (Figure 15A). To further support the data from the adhesion assays, wash assay experiments were included (Figure 15B) in order to reveal any changes in cell-to-cell adhesion and eventually leading to flocculation effects. This assay could also reveal

any eventual changes in invasion into the agar medium. Besides the focus on adhesive phenotypes, additional invasion assays were performed in order to characterize eventual changes in invasion behavior of these strains (Figure 15C). The *in vitro* invasion model consists of rat tail tendon collagen type I matrix overlaid by a monolayer of Caco-2 cells and is infected by exponentially grown *C. albicans* cells of the different strains. The model is further incubated under hyphae inducing conditions at 37℃ and 5% CO_2 for 24 hours. After fixation the samples are histologically processed and stained using the Periodic acid-Schiff staining method.

Based on data from adhesion and invasion assays no differences in adhesion or invasion of the deletion mutants compared to their respective background strains were observed. From these data no crucial role of any of these proteins individually for adhesion or invasion can be deduced. Therefore, overexpression of all these genes in two strain backgrounds was performed in order to gather further insight in their function.

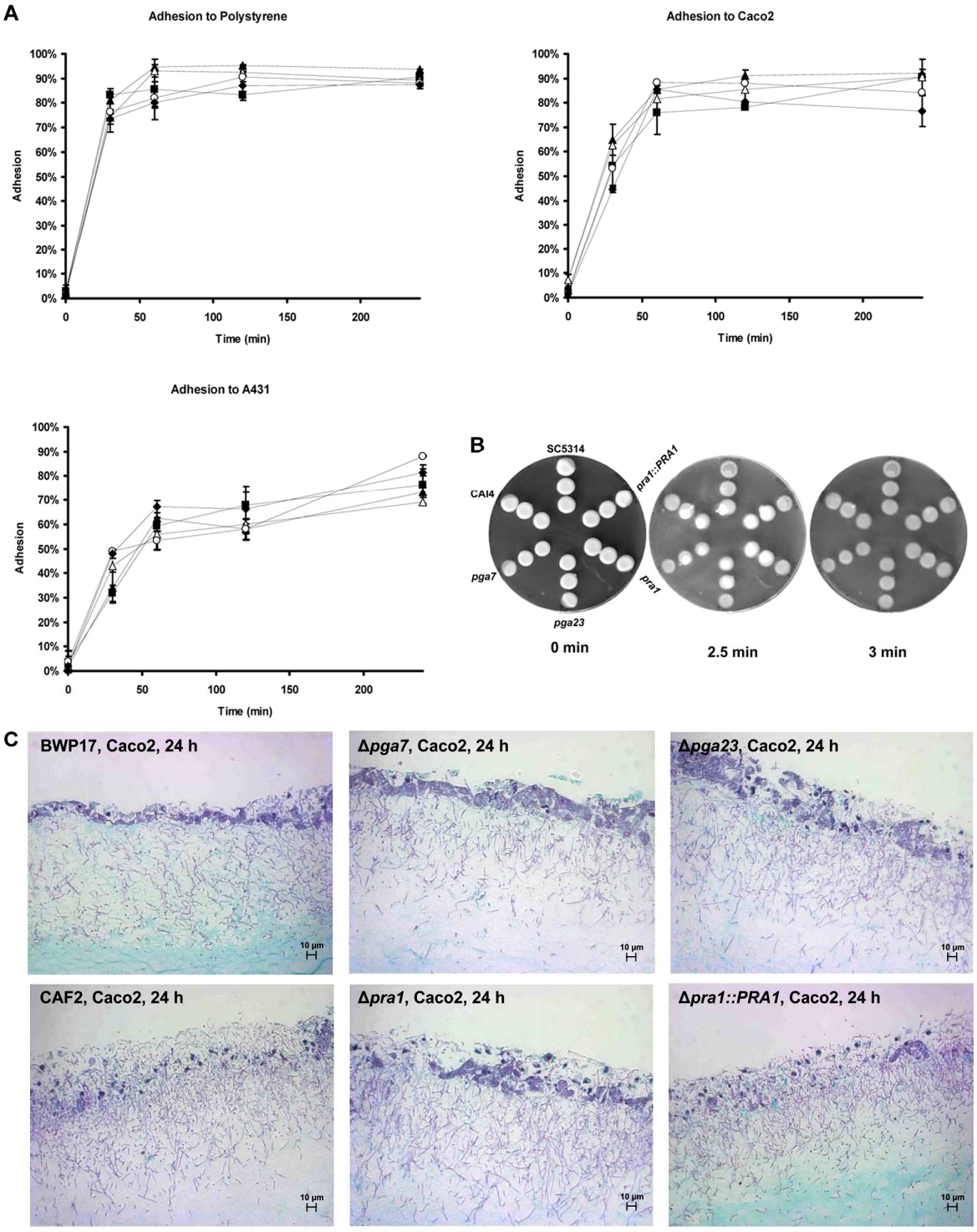


Figure 15. Adhesion and invasion behavior of strains *pga7*, *pga23* and *pra1*.

Adhesion assays (A) performed on three different surfaces: polystyrene, Caco-2 and A-431 cell lines, quantifying adherence on these surfaces over time. The strains are marked as follows: SC5314 (■), *pga7* (○), *pga23* (♦), *pra1* (▲) and *pra1*::*PRA1* (∆). The adhesive patterns of background strains CAI4 and BWP17 are unaltered from the SC5314 wild-type and are not presented. Supplementary wash assays (B) are focused on differences in agar invasion and cell-to-cell adhesion among the strains. After two days of YPD agar-plate cultivation, about 15 ml of water was added and the plate was

incubated on a rotatory shaker with constant rotation 100 rpm. Washing off of the strains was documented over time. CAI4 serves as the auxotrophic control also for strains *pga7* and *pga23*, since the behavior of BWP17 in additional assays was identical. Epithelial invasion assays (C) investigating the invasion behavior of strains deleted for *pga7*, *pga23* and *pra1* with their respective background strains. DMEM media for all the strains were supplemented with additional amino acids and uridine to complement the strain auxotrophies if necessary. The *in vitro* invasion model consisting of a collagen matrix overlaid with Caco-2 cell monolayer was infected with exponentially grown Candida cells, incubated for 24 h, fixed, histologically processed and stained according to periodic acid-Schiff method (Caco-2 dark blue, Candida red and collagen light blue).

6.2.4 An Adhesion Deficient Strain Model to Identify Adhesins by Ectopic Expression

To further characterize gene products of ORFs *PGA7*, *PGA23* and *PRA1*, overexpression of these genes in two strain backgrounds was performed. The first strain chosen for the overexpression was HLC69 (Lo*, et al.*, 1997). This *ura3* auxotrophic strain is lacking genes encoding for the transcriptional factors Efg1 and Cph1 (mentioned previously). Deletion of both of these genes strongly reduces adhesive properties of this strain (Dieterich*, et al.*, 2002). Due to this fact, the *efg1*/*cph1* genetic background is extremely suitable for characterization of genes potentially participating in adhesion. An advantage of using *C. albicans* rather than *S. cerevisiae*, which is a common model for expression of adhesins, is existence of a non-canonical codon in *C. albicans* as well as its thermotolerance, which for example is crucial in studying host pathogen interaction on tissue models. Thus the adhesion can be studied directly on tissue at physiological temperatures (37℃), which results in a severe heat stress response in most *S. cerevisiae* strains (Miller*, et al.*, 1979). The second strain CAI4 (Fonzi and Irwin, 1993) is an *ura3* deficient stain derived from SC5314, a wild-type clinical isolate (Gillum*, et al.*, 1984). This genetic background offers wild-type phenotype for the overexpression. The participation of the overexpressed genes in adhesion and invasion in wild-type can be thus further studied.

Gene *HWP1* (see chapter 3.5.1 on page 11)*,* encoding for a well established adhesin, was used as an internal control. All genes (*PGA7*, *PGA23*, *PRA1* and *HWP1*) were expressed from the *pACT1* promoter after integration of the respective vector at the *RPS1*/*RP10* gene of the strains CAI4 and HLC69 (see Material and Methods). For construction of negative controls served the original vector

(overexpression of *lacZ* gene) and the same vector without *lacZ* gene, in order to complement the background strains for the *ura3* deficiency. The positive transformants were selected on agar plates without uridine. Several clones from each transformed vector were verified using Southern blot. Genomic DNA was restricted by *Bam*HI enzyme and probed on Southern blot against *RP10* ORF, amplified by oligonucleotides RP10_F and RP10_R (Figure 16).

Prior to integration, the inserts of all vectors were sequenced and the *PGA7*, *PGA23*, *PRA1* and *HWP1* gene sequences were checked for potential errors. The results of sequencing were: *PGA7* (100 % aa identity to orf19.5635 and orf19.13080), *PGA23* (identical to orf19.3740 with aa changes V7F and S144L), *PRA1* (identical to orf19.10623 with aa changes D90E and E101D) and *HWP1* (100% identity to orf19.8901 and orf19.1321). *C. albicans* is diploid organism and genes often have two alleles, which can differ in the encoded amino acid sequence. In CGD (Candida Genome Database) these two alleles are distinguished by two ORF numbers. When the sequence of both the alleles is not identical, the cloned gene will sequentially match only to one of the two alleles. There is also a possibility of sequence differences to the CGD caused by errors during Candida genome sequencing and assembly or by genetic differences between the strains accumulated by time, even when they originate from one source (isolated 1984 by Gillum *et al*. (Gillum*, et al.*, 1984)). The main nucleotide changes of interest were the frameshift and nonsense mutations, which could have been created during PCR amplification and creating, shift in reading frame or STOP codon. However, none of them occurred in the sequences.

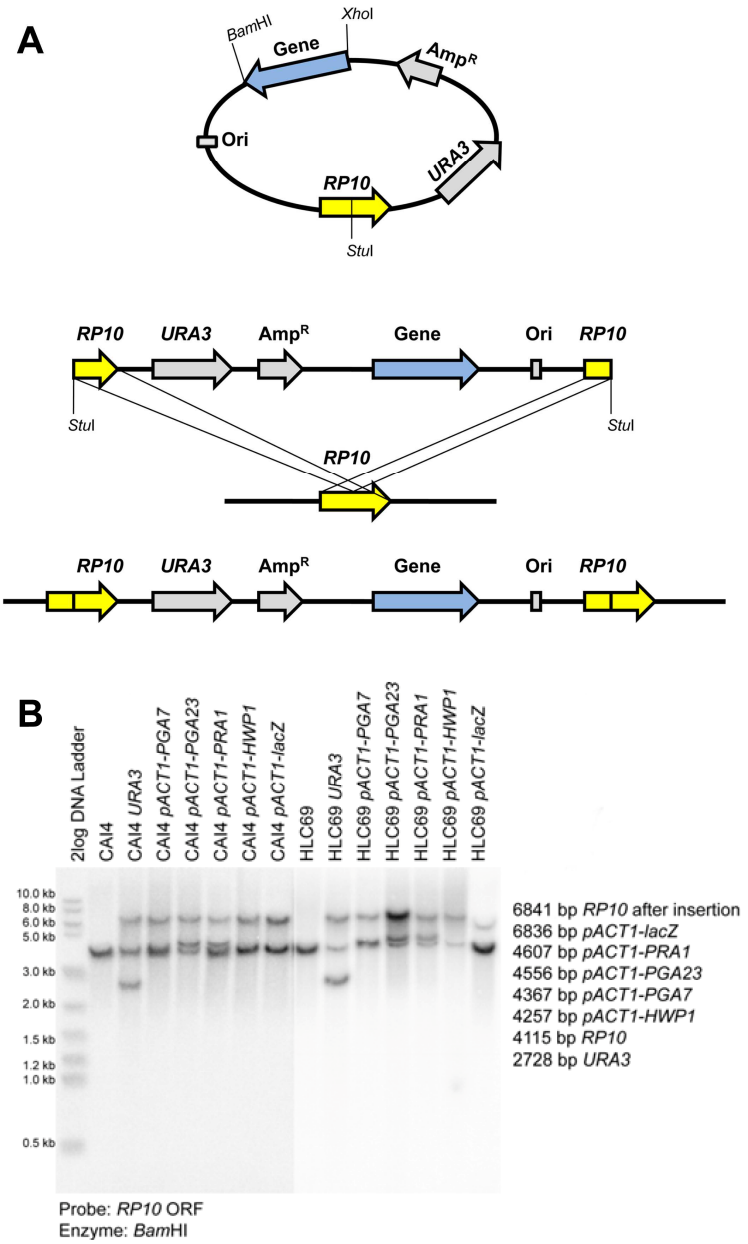


Figure 16. Scheme of construction of overexpression strains.

Schematic representations of the plasmids and their integration into the genome (A). Genes were cloned between *Bam*HI and *Xho*I sites behind the actin promoter. The plasmids were linearized with *Stu*I and transformed into Ura$^-$ auxotrophic Candida strains. In the genome the linearized plasmid integrated into the *RP10* gene and complemented the *ura3* deficiency together with overexpression of desired gene. Southern Blot following the construction of CAI4 and HLC69 strains overexpressing the respective genes (B). About 20 µg of isolated genomic DNA was restricted with enzyme *Bam*HI, run on agarose gel, blotted on nylon membrane and probed against PCR generated *RP10* ORF. After correct integration a new band of 6841 bp appeared, representing an additional *RP10* gene-copy including the vector sequences after insertion.

The efficiency of a correct genome-integration after transformation of these vectors was only about 30%. Positive clones with any inconsistencies in phenotype during cultivation on different media, compared to parallel transformants, were excluded from further studies. When possible, three clones with correctly integrated vector were selected for further characterization of gene expression level by Northern blot. RNA was isolated from strains exponentially grown in YPD and the RNA level was compared to the wild-type strain SC5314. Mutants with the highest expression were used for further work (Figure 17). In the newly prepared strains the expression level of all the genes increased by one to two orders of magnitude, although their expression levels differ between the two strain backgrounds. Additionally, the levels of expression of *PGA7*, *PGA23*, *PRA1* and *HWP1* in a strain lacking *efg1* and *cph1* (HLC54) was checked in microarray data from experiment performed previously by N. Hauser (unpublished results). No upregulation of any of these genes, compared to the wild-type strain, was observed. Also in the literature none of these genes is mentioned to be regulated by Efg1 and Cph1, except for *HWP1* (Sharkey*, et al.*, 1999).

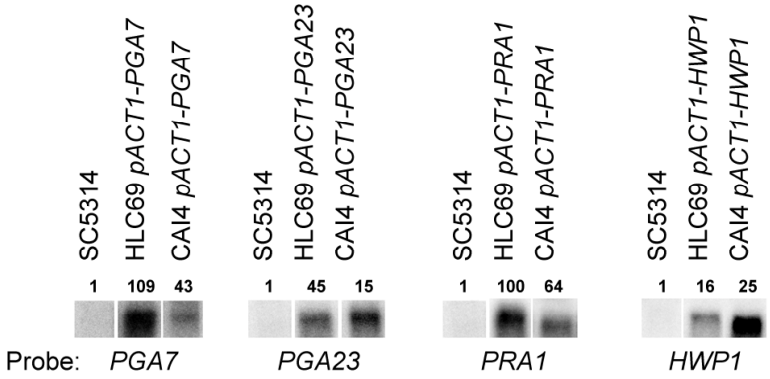


Figure 17. Expression levels of overexpressed genes.

Northern blot analysis following the expression level of overexpressed genes *PGA7*, *PGA23*, *PRA1* and *HWP1* in CAI4 and HLC69 strains compared to the wild-type SC5314. Total RNA was isolated from exponentially growing cells, run on a denaturing agarose gel, blotted on a nylon membrane and probed against radioactively labeled probes generated from ORFs of the respective genes by PCR. Numbers on top reflect the gene expression normalized to the quantity of 26S rRNA and related to wild-type strain set equal to 1.

6.2.5 Phenotypical Characterization

6.2.5.1 Growth

As presented in Figure 18, the overexpression of all the proteins in CAI4 background does not have any significant effect on growth rate in a wild type background. All the strains are slightly delayed in growth, compared to CAI4. However, this difference is still in the error rate of the assay. Actually, there is significant difference between SC5314 wild-type and *URA3* complemented CAI4. These strains are genotypically almost identical and besides the locus and copy number of functional *URA3* gene, there is one additional difference. During construction of the CAI4 strain (Fonzi and Irwin, 1993) also a neighboring gene *iro1* encoding for iron-responsive transcriptional factor was deleted. This together with *URA3* position effect can cause some phenotypical differences from the parental strain SC5314. For strain HLC69 the overexpression of all the genes increases the doubling time significantly. The strongest delay was observed in case of overexpression of *PGA23*, which seems to be detrimental for the cells when overexpressed. The doubling times of HLC54 and *URA3* complemented HLC69 strains are within the error rate. They are identical with regard to their genotype only with a difference in the locus for *URA3* complementation.

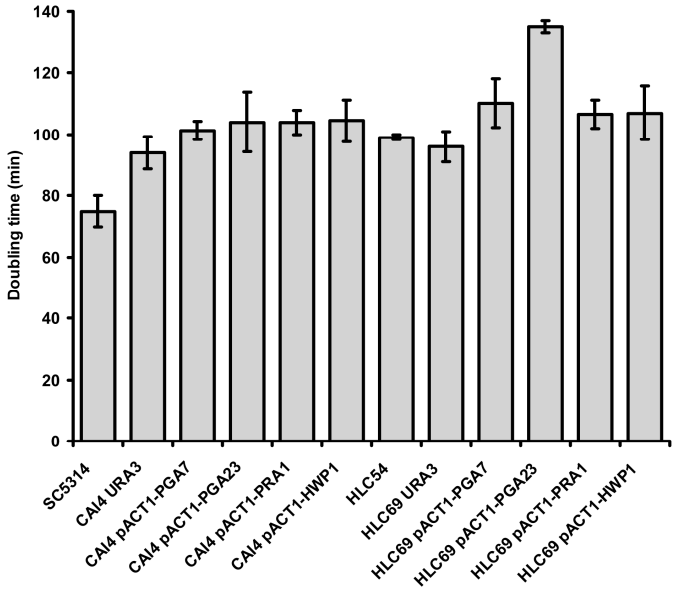


Figure 18. Doubling times of strains overexpressing *PGA7*, *PGA23*, *PRA1* and *HWP1.*

Effect of gene overexpression in both backgrounds CAI4 and HLC69 on doubling time in YPD during exponential phase of growth. Cells were grown in YPD suspension culture from low inoculum (OD_{600} ± 0.01), OD_{600} was measured continuously and doubling times were determined from exponential phase of growth between OD_{600} 0.1 - 2. Doubling times were calculated from linear range of growth curves in logarithmic scale. Graph represents average from 3 independent experiments.

6.2.5.2 Cell Size and Filamentation

Cell shape and filamentation of strains overexpressing genes *PGA7*, *PGA23*, *PRA1* and *HWP1* were investigated as well (Figure 19). For CAI4 derived strains no significant change in cell size, shape or separation was observed. Also no difference among the strains in terms of germ tubes formation or hyphal length was observed (DMEM, N-acetyl-D-glucosamine, serum at 37℃ in pre sence of 5 % CO_2), indicating that the overexpression in a wild-type background does not affect any of these aspects in the cell. Overexpression in the afilamentous HLC69 did not induce filamentation in any case. However, for *HWP1* overexpression the cell size was changed significantly to about double sized cells, when compared to the background strain. Previous reports indicate that Hwp1 may have additional structural function in addition to its adhesive properties due to altered sensitivity of a *HWP1* mutant strain to Calcofluor White (Plaine*, et al.*, 2008).

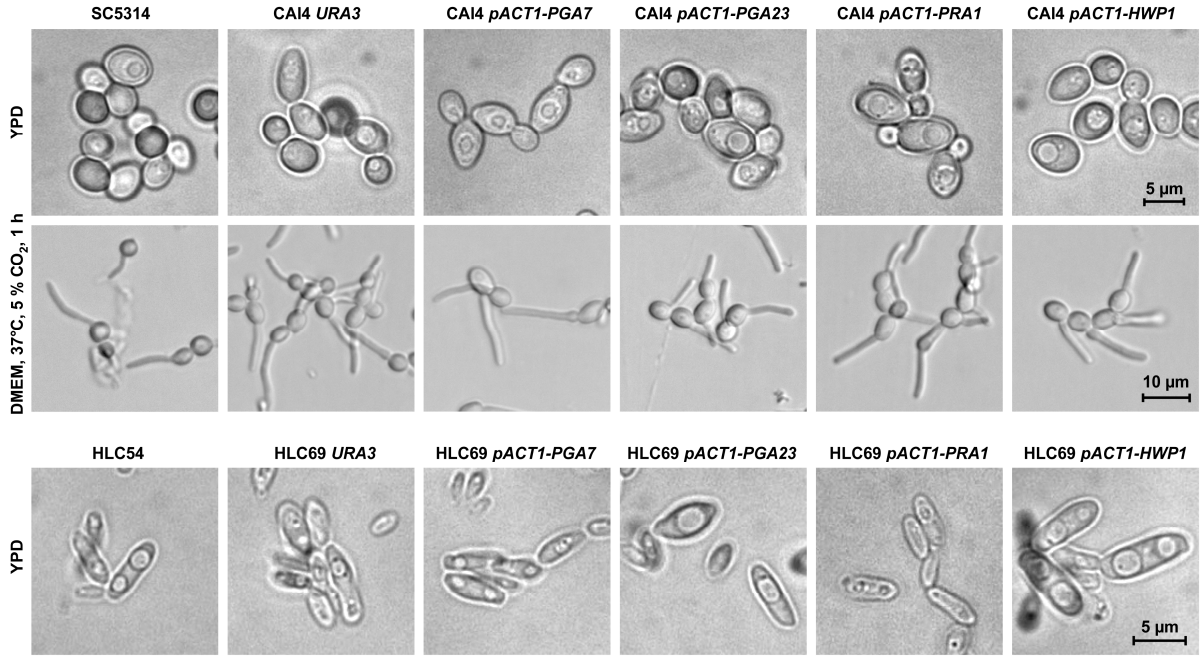


Figure 19. Cell phenotype of strains overexpressing cell wall genes.

Microscopical analysis of CAI4 and HLC69 derived strains including filamentation phenotypes of CAI4 derived strains. Cells were grown exponentially in YPD (blastospores) or incubated 1 h at 37°C and 5% CO_2 in hypha inducing media in order to analyze hypha formation.

In additional experiments testing the CAI4 derived strains in terms of hypha induction and colony morphology on solid media, an effect of *HWP1* overexpression on increased filamentation was observed (Figure 20). The *HWP1* overexpressing strain tends to filament earlier compared to all other strains including SC5314. The effect of earlier agar invasion was observed also at 37°C. Th is filamentation phenotype was verified in additional *HWP1* overexpressing CAI4 clones constructed in parallel. Also, overexpression of *PRA1* on SPIDER agar medium slightly increased the filamentation. Overexpression of the other genes did not have any significant effect on filamentation on the agar media used.

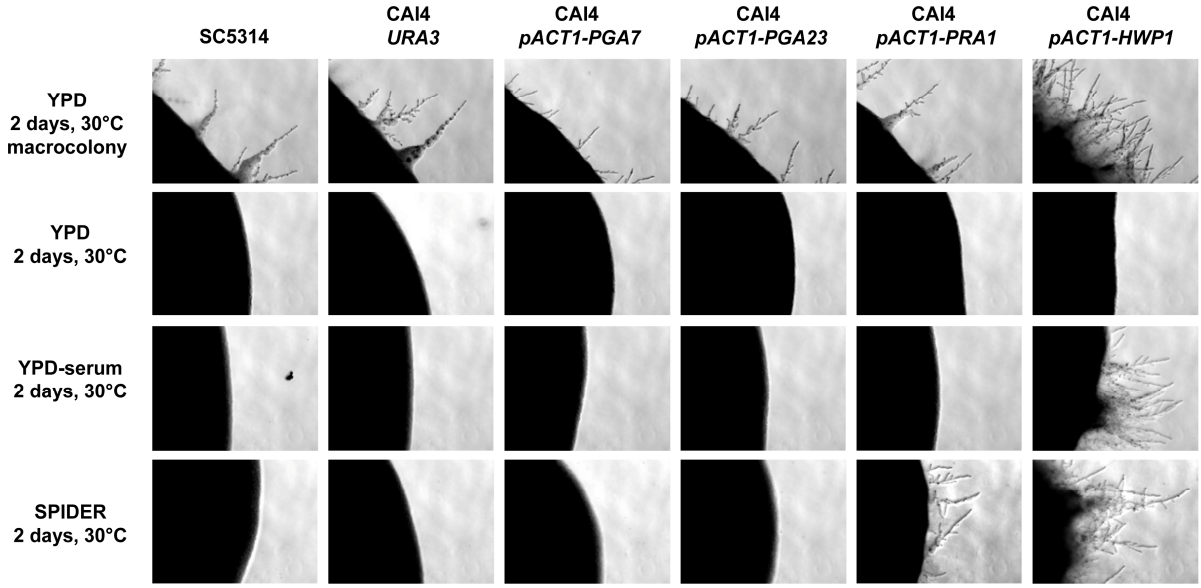


Figure 20. Colony filamentation phenotypes.

Shape of colonies of CAI4 derived strains overexpressing *PGA7*, *PGA23*, *PRA1* and *HWP1* on various media. Cells were spotted on YPD agar plate as 5 µl suspension of OD_{600} = 1 (macrocolony) or plated as individual cells (approximately 100 cells/plate). Colony morphology and its filamentation were documented after 2 days under the microscope (10 x objective magnification).

6.2.5.3 Adhesive and Invasive Properties

The adhesion of both CAI4 and HLC69 strains overexpressing the genes *PGA7*, *PGA23*, *PRA1*, *HWP1* and *lacZ* was tested on all three surfaces: polystyrene, Caco-2 and A-431 cell lines (Figure 21A). In case of CAI4 derived mutants the adhesion was followed up to 4 hours, while in case of HLC69 background only for 2 hours. The reason is that while the CAI4 derived strains start to filament in the DMEM medium and the amount of CFUs increases only slightly, in case of non-filamentous HLC69 derived strains the cells multiply in the medium and after the initial increase in adhesion during first two hours, there is a significant drop in the fraction of the adherent cells in later time points. While in case of CAI4 background strain there was no effect of any of the overexpressed genes on the adhesion, positive or negative, in HLC69 strain significant differences were observed. The *URA3* complemented strain showed moderate adhesion reaching about 30% of adherent cells to polystyrene, 20% to Caco-2 and 15% to A-431 cell lines within two hours of the experiment. The overexpression of *HWP1* in this strain background increased the adhesion of the strain to all three surfaces for additional 10-15%. The increase in adhesion is only partial because the Hwp1 is not the only adhesin of the cell wall responsible for the

adhesion to surfaces. Surprisingly, adhesion to the polystyrene surface increased as well, although Hwp1 was described to be tissue specific and dependent on the host enzyme transglutaminase, which crosslinks this protein to epithelial cells (Staab*, et al.*, 1999). However, indications about its adhesion to plastic were already reported, since *HWP1* overexpressed in *bcr1* mutant increases its adhesion to catheters (Naglik*, et al.*, 2003; Nobile*, et al.*, 2006a; Nobile*, et al.*, 2006b). On the other hand overexpression of *PGA7*, *PGA23*, *PRA1* and also of the negative control *lacZ*, which is not surface exposed, leads to decrease in adhesion, when compared to *URA3* complemented HLC69. The decrease in case of *lacZ* is the smallest compared to other genes, but nevertheless apparent. This fact may be explained by the use of strong *pACT1* promoter that could affect the homeostasis in the cell in terms of the ratios of expressed proteins and thus affecting the remaining adhesive properties of this strain. The overexpression of *PGA7*, *PGA23* and *PRA1* leads to decrease in adhesion, indicating that none of these genes is participating directly or indirectly in cell adhesion process like *HWP1*. The overexpression of *PGA23* additionally has a strong effect reducing the adhesion of the HLC69 strain and having kind of anti-adhesive effect. This effect was not observed in the adhesion assays in case of the *PGA23* overexpressing CAI4 strain.

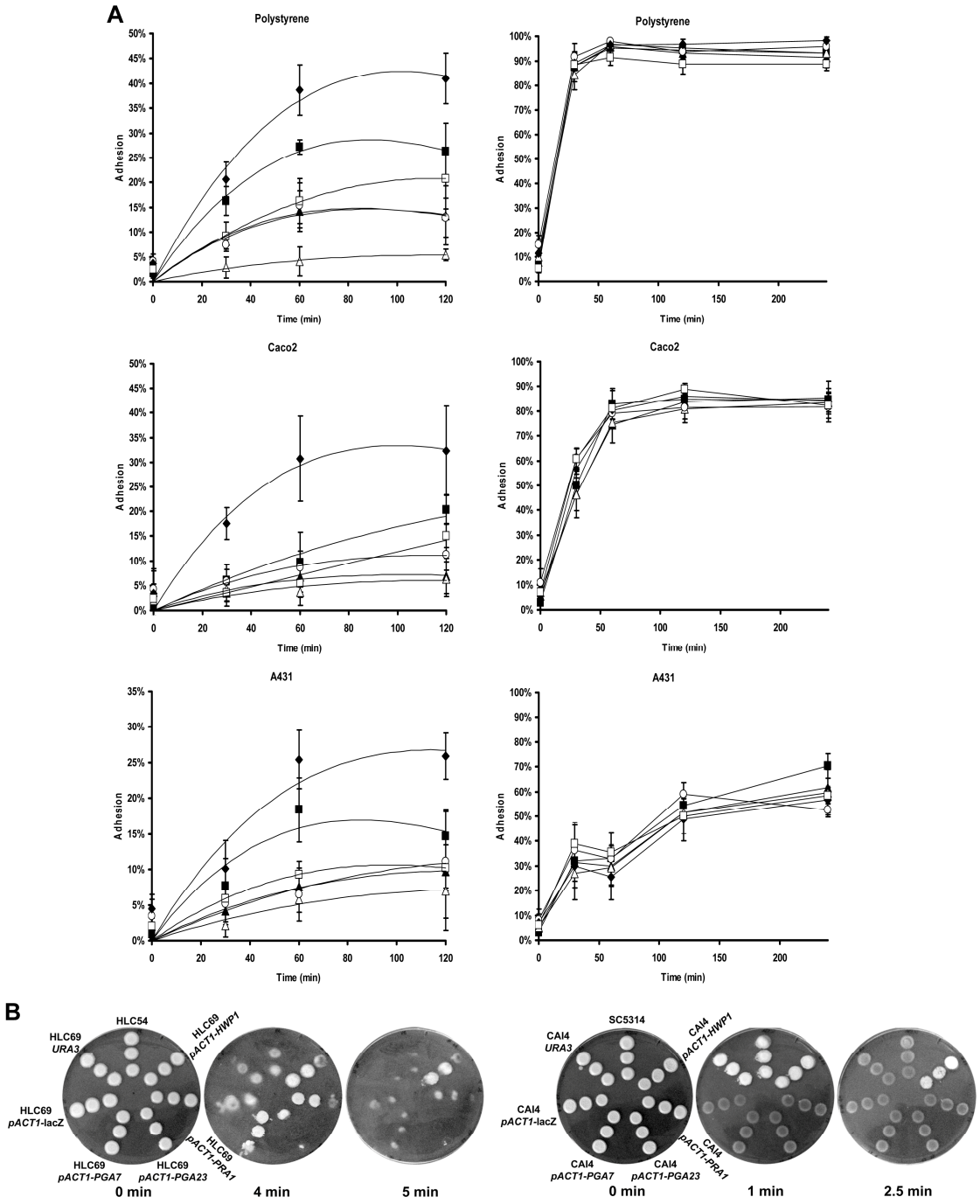


Figure 21. Effect of overexpressed genes on strain adhesion.

Adhesion assays (A) of HLC69 (left column) and CAI4 (right column) derived strains overexpressing *PGA7*, *PGA23*, *PRA1*, *HWP1* and *lacZ*. The progress of adhesion on three different surfaces was monitored. Strains are marked as follows: *URA3* complemented background strain (■), overexpression of *PGA7* (▲), *PGA23* (∆), *PRA1* (○), *HWP1* (♦) and *lacZ* (□). Wash assay (B) of strains derived from HLC69 (left) and CAI4 (right) backgrounds overexpressing *lacZ*, *PGA7*, *PGA23*, *PRA1* and *HWP1*. Strains were spotted on YPD agar plates and cultivated for two days at 30℃. 15 ml of water was added and plates were cultivated on rotatory shaker at constant rotation 100 rpm. Washing off of the strains was monitored. Increase in adhesion in case of overexpression of *HWP1* can be observed in both strain backgrounds, while the *PGA23* leads to faster release of such strain from the agar medium.

In the wash assay experiments (Figure 21B) a positive effect of *HWP1* overexpression was observed. In both background strains CAI4 and HLC69 the overexpression of *HWP1* increases the strain resistance to washing from the agar. In case of HLC69 derived strains, they were all washed out with the same efficiency like the HLC54 and the *URA3* complemented HLC69 with exception of *HWP1* and *PGA23*. While HLC69 strain overexpressing the *HWP1* is released from the agar as last, the overexpression of *PGA23* has an opposite effect, confirming the results observed for adhesion to epithelia and polystyrene. In case of CAI4 derived strains, the *HWP1* overexpressing strain was not washed out due to stronger invasion into the medium, compared to the other strains. A strongly wrinkled surface of the colonies furthermore indicates a higher filamentation and adhesion within the colony. On the other side, no effect on adhesive behavior was observed for the overexpression of *PGA7*, *PRA1* or *lacZ*. Even in this case, *PGA23* overexpression led to a faster release of the strain from the agar medium, what is in agreement with the anti-adhesive effect observed in adhesion assays performed in HLC69 background strain. Interestingly, the CAI4 strains overexpressing *lacZ*, *PGA7*, *PGA23* and *PRA1* were released from the agar prior to the *URA3* complemented CAI4 strain, partially paralleling the effect of overexpression of these genes in HLC69 strain background.

In epithelial *in vitro* invasion assays none of the genes *PGA7*, *PGA23*, *PRA1* and *HWP1*, when overexpressed in HLC69, was capable to induce invasion in this non-invasive strain and cross the Caco-2 barrier (Figure 22A). Compared to HLC69, in CAI4 strain background the overexpression of all four putative cell wall proteins led to alteration of the invasion phenotype. While the *URA3* complemented CAI4 strain invaded the model efficiently, the overexpression of *PGA7*, *PGA23*, *PRA1* and *HWP1* led to reduced invasion with altered pattern of the invaded sites. While the wild-type and *URA3* complemented CAI4 strain developed long hyphae invading the collagen matrix, all the overexpressing strains formed more clusters of mixed hyphal, pseudohyphal and blastospore cells. Interestingly, when the strains invaded a collagen layer without epithelia, these differences were not observed. The histological decrease in Caco-2 cell damage was confirmed by additional quantitative LDH assays (Figure 22B), focusing on tissue damage connected to release of LDH from the lysed human cells. Overexpression of all genes, *PGA7*, *PGA23*, *PRA1* and

HWP1 leads to a decrease of Caco-2 cell damage, while the *URA3* complemented CAI4 strain is equal to SC5314 wild-type. This further supports the qualitative invasion data and indicates an effect related to the protein overexpression rather then to their amino acid sequences.

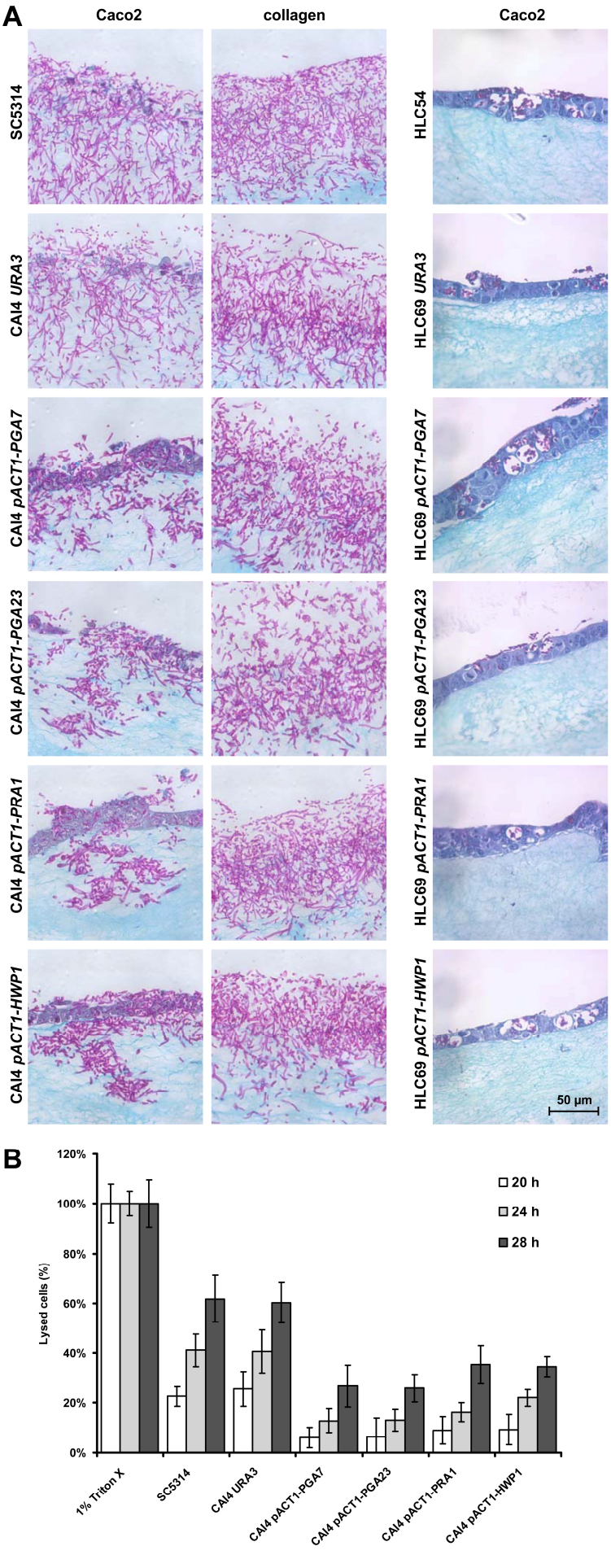


Figure 22. Invasion of CAI4 and HLC69 derived strains into *in vitro* invasion models and quantification of human cell damage.

Invasion assay 24 h post-infection (A) comparing the CAI4 derived strains invading an *in vitro* model containing a Caco-2 monolayer on a collagen matrix (left column) or a pure collagen matrix (middle column) under identical conditions. In the right column invasion of strains derived from non-invasive strain HLC69 incapable of crossing of the Caco-2 cell barrier is represented. *In vitro* models were infected by exponentially grown cells, incubated for 24 h, fixed, histologically processed and stained according to the periodic acid-Schiff method (Caco-2 dark blue, Candida red and collagen light blue). Quantification of Caco-2 cell-line damage (B) by CAI4 derived strains overexpressing genes *PGA7*, *PGA23*, *PRA1* and *HWP1* and compared to the *URA3* complemented CAI4 strain and SC5314 wild-type. The medium was checked for released LDH by damaged Ccao2 cells 20, 24 and 28 hours post-infection and quantified by coupled chemical reaction.

6.2.5.4 *PRA1* and Adhesion to Fibrinogen

Originally, Pra1 was described as a fibrinogen binding protein (Casanova*, et al.*, 1992). In order to verify the function of Pra1 as a fibrinogen binding factor, *PRA1* overexpressing strains derived from CAI4 and HLC69 backgrounds were used for adhesion assays performed on 24-well plates coated with the fibrinogen using standard method for ELISA assays. Additional strains *pra1*, *pra1*::*PRA1* and SC5314 wild-type were used for the assay as well (Figure 23). In case of strains SC5314, *pra1*, *pra1*::*PRA1*, CAI4 *URA3* and CAI4 *pACT1-PRA1* there was no difference in adhesion to fibrinogen coated polystyrene 24-well plate. The result of strains HLC69 *URA3* and HLC69 *pACT1-PRA1* was more surprising. While *URA3* complemented strain HLC69 reached about 50 % of adherent cells within two hours, the strain HLC69 *pACT1-PRA1* reached hardly 10 %. Because the *URA3* complemented strain HLC69 adhered with higher efficiency to fibrinogen coated polystyrene (50 % in 2 hours) than to untreated polystyrene (30 % in 2 hours) it seems that on the cell surface of HCL69 there is an adhesive molecule responsible for this phenomenon, however, Pra1 is not the cause. *PRA1* overexpressing HLC69 adhered to fibrinogen coated polystyrene surface with about the same efficiency as it adheres to untreated polystyrene 24-well plates.

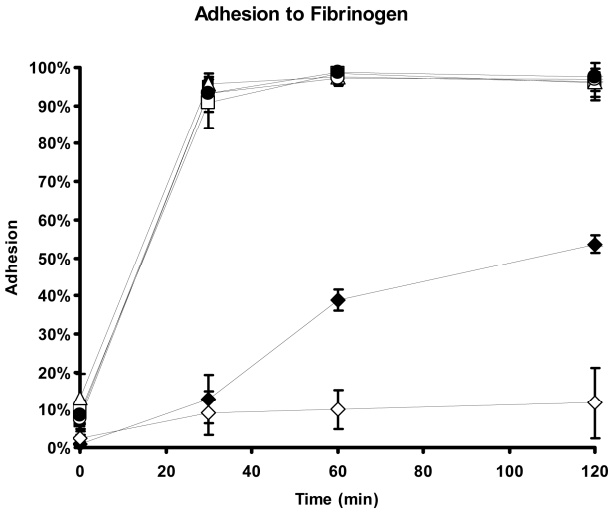


Figure 23. Effect of fibrinogen on adhesion of strains deleted for or overexpressing *PRA1*.

Adhesion of various strains over time to polystyrene surfaces coated with fibrinogen. Strains are represented as follows: HLC69 *URA3* (♦), HLC69 *pACT1-PRA1* (◊), CAI4 *URA3* (□), CAI4 *pACT1-PRA1* (Δ), *pra1* (○), *pra1*::*PRA1* (●) and SC5314 (■). The graph represents the average of three independent experiments.

6.2.5.5 *PGA7* and Iron Metabolism

Additionally, there were some indications that Pga7 could be involved in iron metabolism. Besides its slight sequence homology to *PGA10* and *RBT5*, it shares

similar regulation in response to ketoconazole, oxidative stress and pH (Hromatka*, et al.*, 2005; Lee*, et al.*, 2005; Liu*, et al.*, 2005). In addition, *PGA7* is localized in the genome between the predicted ferric reductase *FRP1* and *RBT5* involved in haemoglobin utilization. For this reason the sensitivity of *pga7* strain to sublethal concentrations of the iron chelator ferrozine was tested, however, no difference to the background strain was observed. The same results were obtained with CAI4 and HLC54 strains overexpressing the *PGA7* (data not shown).

6.2.5.6 Cell Wall Biosynthesis

Since all of the studied proteins are putatively cell wall associated, the cell wall stability of the strains was analyzed. To test the general changes in the cell wall, the sensitivity to Congo Red and Calcofluor White was determined using strains both derived from the CAI4 and the HLC69 backgrounds (Figure 24). In case of CAI4 derived strains, their sensitivity to Congo Red increased more significantly in case of overexpression of *PGA7*, *PGA23* and *PRA1*, while it was less affected in case of *HWP1*. The sensitivity to Calcofluor White was slightly affected by the overexpression of any of the genes with an exception for *HWP1* which actually increased resistance. For HLC69 derived strains the effect of overexpression of the selected genes to Congo Red sensitivity can be observed as well. Surprisingly, the Calcofluor White sensitivity in this strain background overexpressing *PGA7* and *PGA23* was affected more significantly than in case of CAI4. However, the positive effect of *HWP1* expression can be observed even in this background.

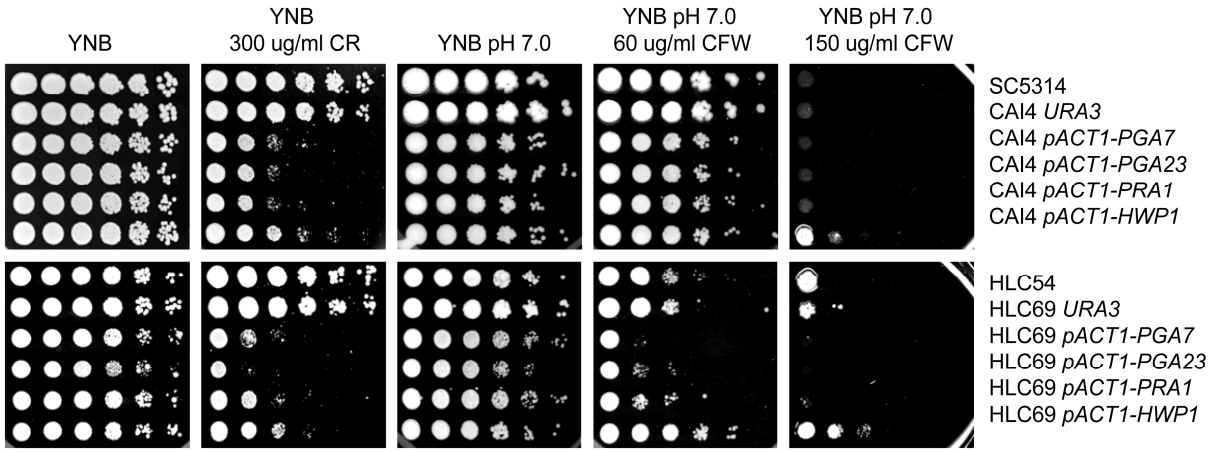


Figure 24. Sensitivity to Congo Red and Calcofluor White of CAI4 and HLC69 derived strains.

Drop tests focusing on CAI4 and HLC69 derived stains overexpressing genes *PGA7*, *PGA23*, *PRA1* and *HWP1* and their sensitivity to cell wall disturbing agents. Cells were spotted on agar plates containing cell wall disturbing agents in dilution series 1:10 starting from OD_{600} = 1.0 and documented after 2 days. The overexpression of *PGA7*, *PGA23*, *PRA1* and *HWP1* has a general effect on the strains' sensitivity to Congo Red and in case of HLC69 derived strains the overexpression of *PGA7* and *PGA23* has also strong effect on the Calcofluor White sensitivity. Overexpression of *HWP1* in both strain backgrounds on the other side increases the resistance to Calcofluor White.

6.2.5.7 Cell Wall Stability

In order to confirm the effects on cell wall stability, exponential cultures of all the strains were diluted to OD_{600} = 1.0 and their sensitivity to the Zymolyase was examined (Figure 25). All the strains overexpressing the *PGA7*, *PGA23*, *PRA1* and *HWP1* derived from CAI4 background showed increased sensitivity to Zymolyase treatment, which is in agreement with the increased sensitivity to Congo Red. The variations among the strains, however, are within the experimental error rate. In the case of strains derived from HLC69, the increased Congo Red sensitivity was not reflected in an enhanced sensitivity to Zymolyase 100T. This might be due to the general change in cell wall architecture of the *efg1*/*cph1* deletion mutant resulting in decrease in the cell wall thickness and decreased absolute amount of glucans in the cell wall without any significant changes in mannans and chitin in the exponentially grown cells (see chapter 6.1.2).

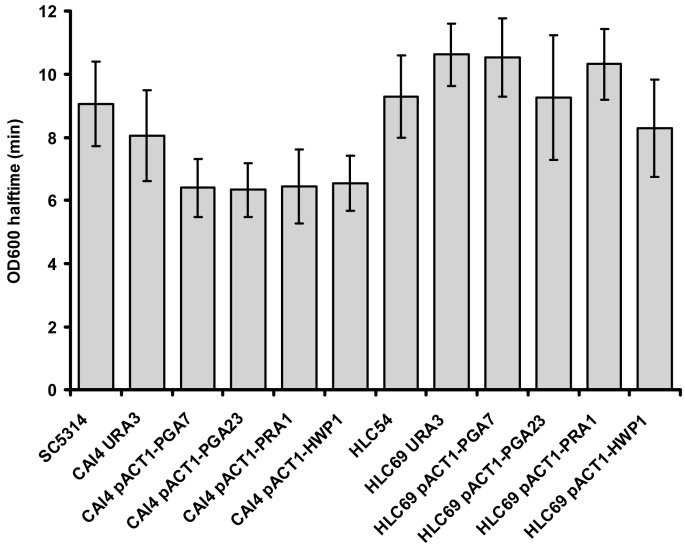


Figure 25. Zymolyase sensitivity of CAI4 and HLC69 derived strains overexpressing cell wall genes.

Half life of cells (measured as OD_{600}) during treatment with Zymolyase. Zymolyase was added to the exponentially grown cells and the OD_{600} starting from 1.0 was monitored over time. The half life of the cells was determined by OD_{600} measurements. The graph represents an average value from five independent experiments.

6.2.5.8 Cell Wall Thickness

To monitor the causes of the enhanced sensitivity to cell wall damaging agents, two independent experiments focusing on total cell wall mass were performed. The first experiment was using transmission electron microscopy in order to determine any changes in the cell wall thickness related to the parent strain. Exponentially grown cells of all studied strains were fixed and processed for transmission electron microscopy imaging. The relative changes in the cell wall thickness are presented in Figure 26A. The absolute cell wall thickness may be altered due to all the cell processing steps required, but its changes can be represented in relation to the background strain. An increase of cell wall thickness in case of overexpression of *PGA7*, *PGA23* and *PRA1* in CAI4 background was observed, while *HWP1* overexpression had only minimal effect. The highest increase in the cell wall thickness appeared in CAI4 strain overexpressing the *PGA23* (Figure 26B), which in case of HLC69 derived strain had a significant effect on cell growth indicating some toxic effects. Overexpression of the genes *PGA7* and *PRA1* in HLC69 background did not have a significant effect on the cell wall thickness but in case of *PGA23* and *HWP1* overexpression slight increase was observed.

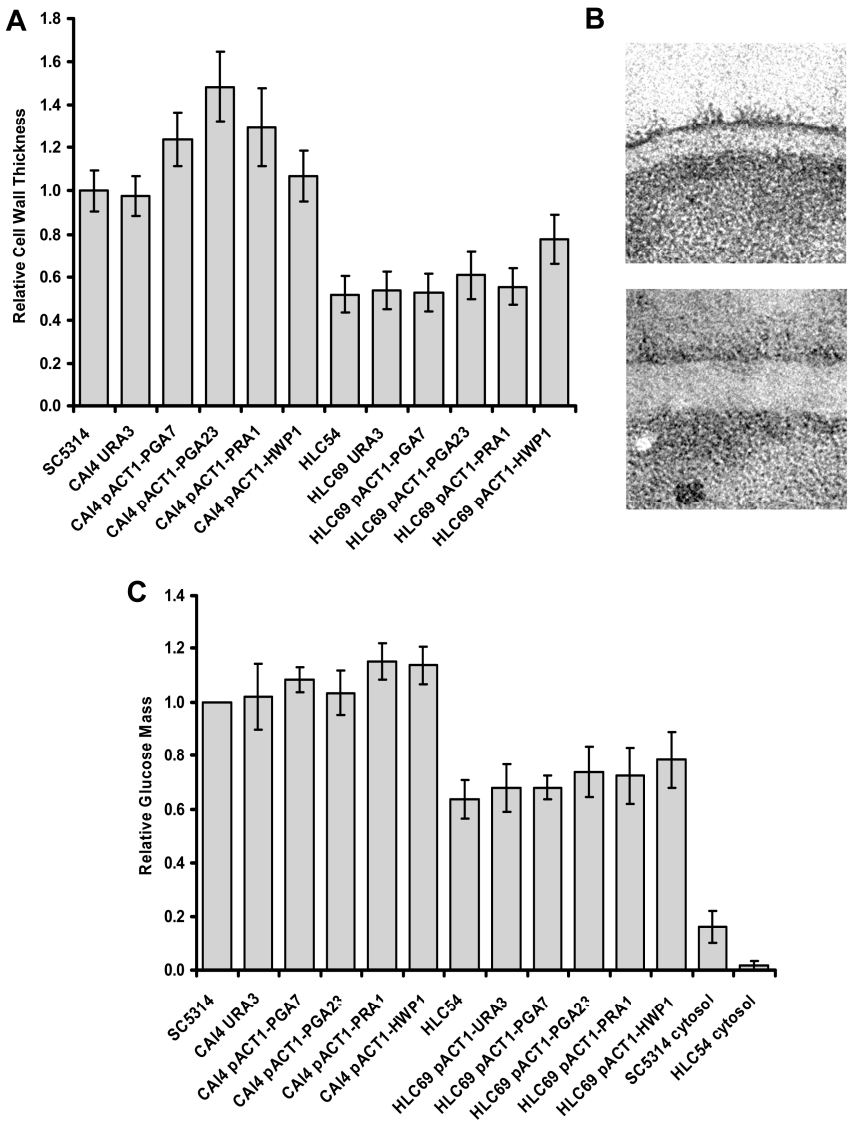


Figure 26. Cell wall changes in CAI4 and HLC69 derived strains.

Comparison of cell wall thickness (A) determined by transmission electron microscopy and related to wild-type SC5314 (equal to 1). Exponentially grown cell were fixed, processed for transmission electron microscopy imaging and the thickness of the cell walls for different strains was determined. The average cell wall thickness for the wild-type strain SC5314 was 63 nm. Representative pictures of cell walls (B) of wild-type SC5314 (upper) and CAI4 strain overexpressing *PGA23* (lower). Pictures were taken at 16,000 x magnification and represent the same area size. Relative amount of glucose units released by hydrolysis of different strains (C) compared to wild-type SC5314 (equal to 1). Exponentially grown cells were lyophilized, hydrolyzed with sulphuric acid and the released glucose units were related to the original dry biomass weight. The amount of glucose in this case reached 19 % of the dry cell weight for the wild-type. Intracellular glucose units were determined as well from glass bead-disrupted cells and are presented for strains SC5314 wild-type and HLC54 *efg1*/*cph1* mutant. Overexpression of *PGA7*, *PGA23*, *PRA1* or *HWP1* did not affect the amount of intracellular glucose units in the background strains and is not further presented.

The absolute amount of the glucans related to the total cell biomass was determined in order to describe changes in the amount of this most abundant polysaccharide component of the Candida cell wall (Figure 26C). All strains were grown to exponential phase, harvested and the dry cell biomass was hydrolyzed using sulphuric acid. The amount of released glucose was related to the biomass. Also the total amount of glucose released from the cytosolic fraction was determined. The difference between these two values represents the glucose units present in the cell wall. None of the overexpressed genes affected the amount of the intracellular glucose units (data not shown). Interestingly, for both strain backgrounds the glucan amount did not follow the increase in the cell wall thickness determined by the transmission electron microscopy. This may actually indicate changes in cell wall structure resulting in looser density in case of overexpression of *PGA7*, *PGA23* and *PRA1* in CAI4 strain which might explain their strongly increased sensitivity to Congo Red.

6.2.5.9 Cell Wall Composition

The relative cell wall composition in terms of polysaccharide components was further determined. For this purpose the cell walls of all strains overexpressing the respective cell wall proteins, as well as from the respective deletion mutants were isolated and after their hydrolysis the relative amounts of all three polysaccharides were determined: glucans, mannans and chitin (Figure 27). In case of the *pga7*, *pga23* and *pra1* mutant strains there was no difference in relative representation of the polysaccharides compared to the wild-type. In case of CAI4 derived strains overexpressing *PGA7*, *PGA23*, *PRA1* and *HWP1* there is slight general decrease in mannan in the cell wall accompanied by a general increase of glucans. However, these differences are within the error rate of the assay. For strains derived from HLC69 there is no difference to the background strain, only the deletion of *efg1* and *cph1* increases the mannan to glucan ratio as reported previously in chapter 6.1.2. The most interesting result was a significant decrease in relative representation of chitin (about 20-30 % decrease in absolute amount) in case of *HWP1* overexpression in both backgrounds CAI4 and HLC69. In both cases this could offer an explanation for the increased resistance to Calcofluor White in the drop tests. A decrease in the chitin amount in the cell wall is usually followed by enhanced resistance to Calcofluor White. For example, *S. cerevisiae* mutants with a defective *chs3* gene, which

encodes the major chitin synthase, and with defective *chs4–chs7* genes, which are required for proper Chs3 activity, are more resistant to Calcofluor White and Congo Red (Imai*, et al.*, 2005; Roncero and Duran, 1985). However, increased Congo Red resistance in case of *HWP1* overexpression was not observed.

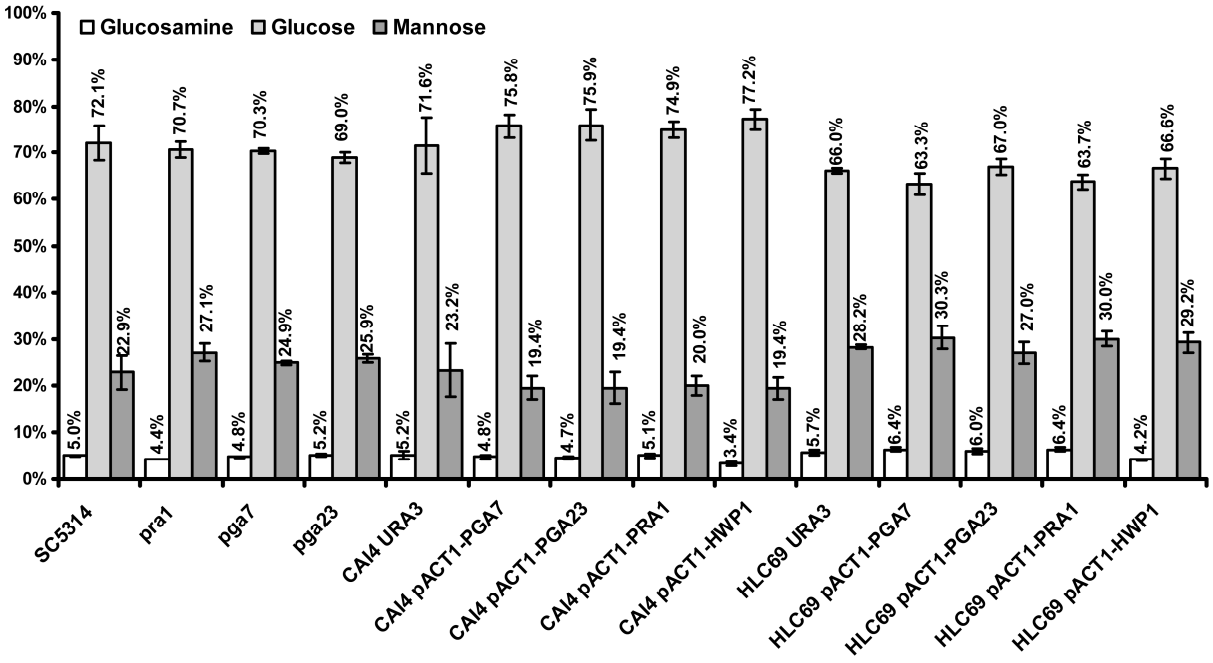


Figure 27. Relative polysaccharide cell wall composition.

Relative composition of the cell wall of *pga7*, *pga23* and *pra1* mutants and strains overexpressing *PGA7*, *PGA23*, *PRA1* and *HWP1* derived from CAI4 and HLC69 backgrounds. Cell walls from exponentially grown Candida cells were purified and hydrolyzed by sulphuric acid. Released monosaccharide units were determined by HPIC measurement. Glucosamine stands for chitin, glucose for the glucans and mannose for mannosylation of covalently linked cell wall proteins.

In order to transform the relative representations to absolute numbers, the relative polysaccharide concentrations were combined with the data from the absolute glucose concentrations related to the dry cell biomass. This is resulting in absolute numbers of the individual polysaccharides related to the dry cell biomass (Figure 28). It is evident that only the glucan amounts are changing among the strains.

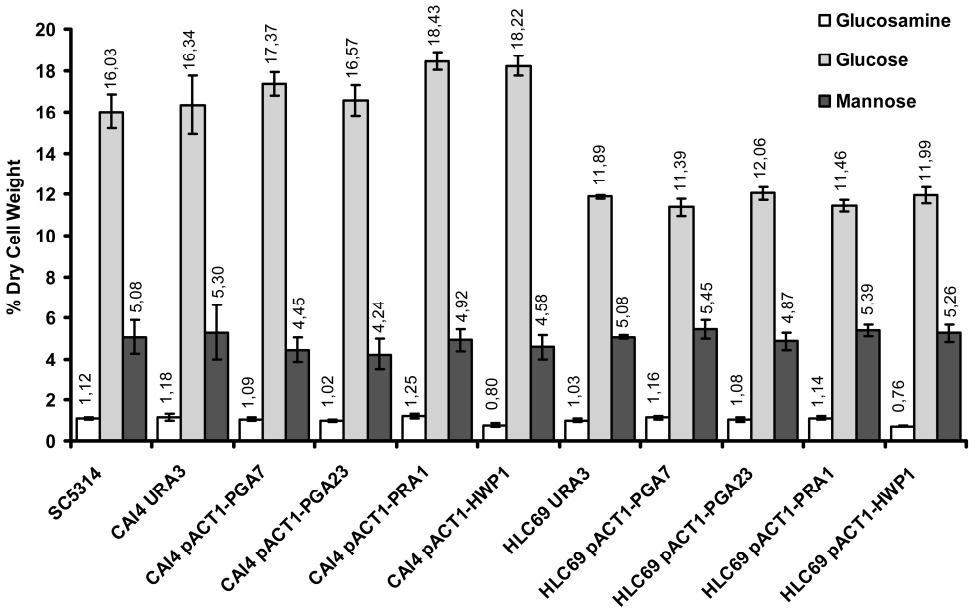


Figure 28. Absolute polysaccharide cell wall composition related to dry cell biomass.

Absolute amounts of polysaccharide cell wall components related to dry cell biomass of CAI4 and HLC69 derived strains overexpressing *PGA7*, *PGA23*, *PRA1* and *HWP1*. Data were combined from results of absolute amount of cell wall related glucose to the dry biomass and relative cell wall composition. Glucosamine reflects chitin, glucose the glucans and mannose the mannosylation of covalently linked cell wall proteins.

6.2.5.10 Interaction with Macrophages and Dendritic Cells

Strains overexpressing genes *PGA7*, *PGA23*, *PRA1* and *HWP1* derived from CAI4 and HLC69 backgrounds were used in order to investigate any changes in immunological response to these strains by incubation with murine bone marrow derived macrophages (Figure 29). This assay was focusing on Candida recognition and release of reactive oxygen species (ROS). Any differences in recognition of *C. albicans* strains can thus be connected to change in ROS response. Strains that were derived from the HLC69 (*efg1*/*cph1*) background did not induce any significant ROS response (reported previously in chapter 6.1.4.1 for RAW264.7 macrophage cell line) and are not presented. The overexpression of *PGA7*, *PGA23*, *PRA1* and *HWP1* in CAI4 background did lead to significant ROS response, however, the overexpression of all the putative cell surface proteins led to a decrease of the ROS response, when compared to the *URA3* complemented CAI4. This could have been caused by the general cell wall protein overexpression that may mask the immunogenic polysaccharide cell wall determinants. When the same experiment was repeated wit RAW264.7 macrophage cell line, no difference between *URA3*

complemented CAI4 and CAI4 overexpressing *PGA7*, *PGA23*, *PRA1* and *HWP1* have been observed (data not presented). It seems that the observed effect is rather specific for the bone marrow derived macrophages. However, such difference is not surprising, since differences in response among various macrophage cell lines and isolated macrophages are often reported (Stojkovic*, et al.*, 2008).

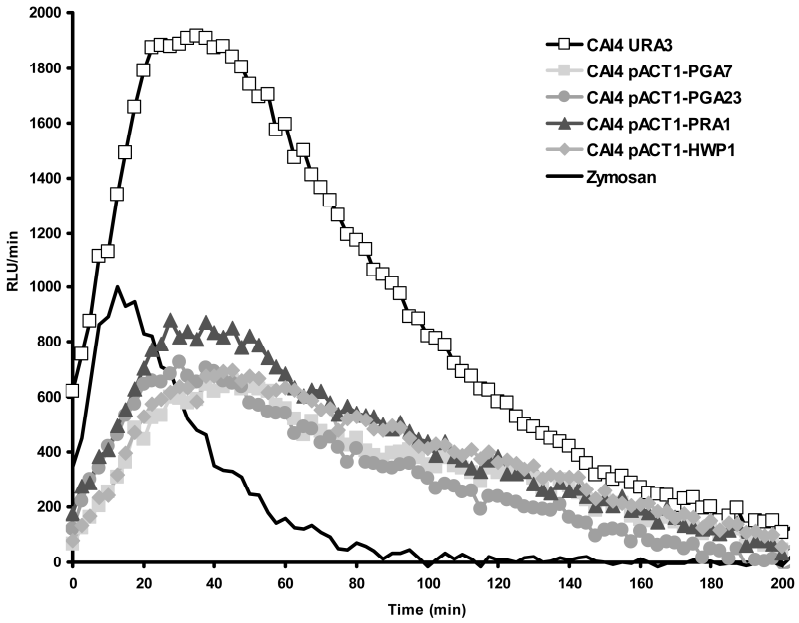


Figure 29. ROS release by macrophages in contact with CAI4 derived strains.

ROS release by bone marrow derived macrophages in contact with various *C. albicans* strains derived from CAI4 background, followed for 200 minutes and quantified by peroxidase-luminol coupled reaction. Graph represents average values of one experiment performed in triplicates. In total three consistent repetitions were made. The error rates of individual time points did not exceed 10% of its absolute value.

Similar results were obtained in contact with myeloid dendritic cells, which as professional phagocytes modulate the immunological response in contact with various pathogens. Transcription of various signaling molecules was studied (pro-inflammatory IL-1β, IL-12 and TNFα; anti-inflammatory IL-4 and IL-10; and additional IL-23 and IFNβ), however, overexpression of *PGA7*, *PGA23*, *PRA1* and *HWP1* in both strain backgrounds led only to general slight decrease in transcription of all the signaling molecules, which could have been caused by the non-specific effect of cell-wall protein overexpression (Figure 30).

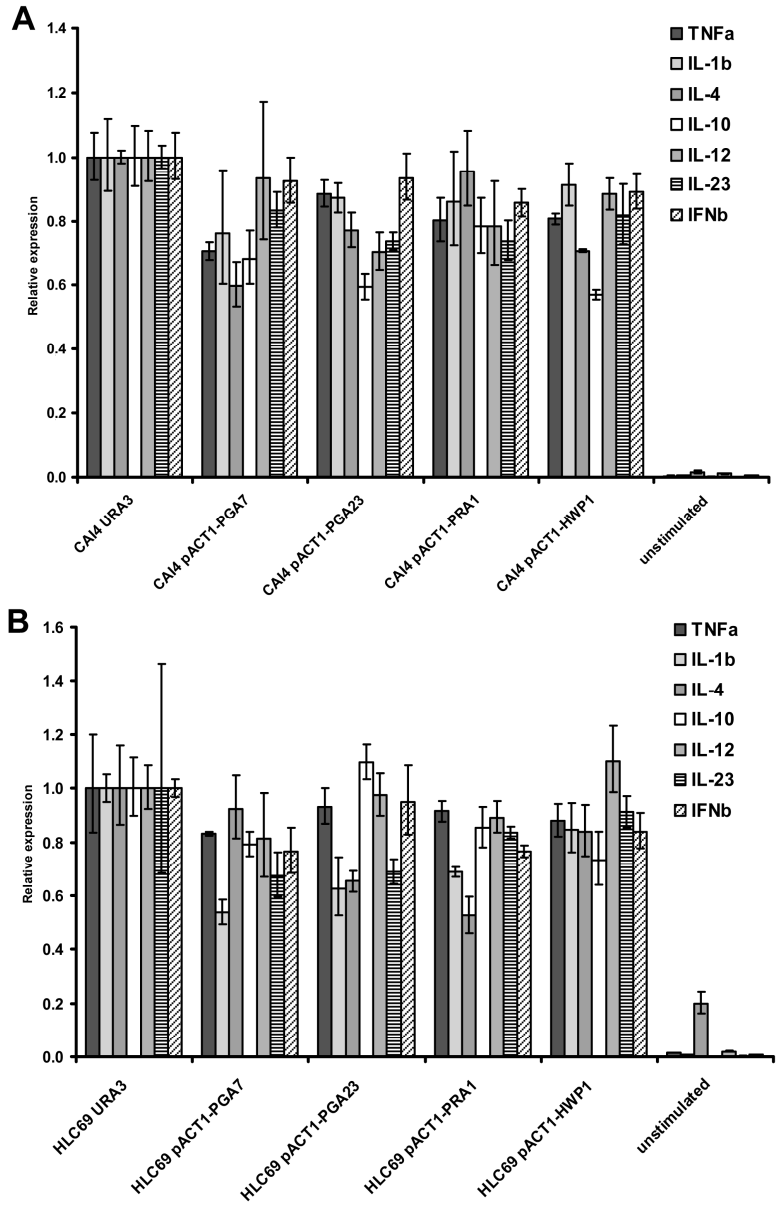


Figure 30. Transcriptional response of dendritic cells.

Transcriptional response of DCs in contact with CAI4 (A) and HLC69 (B) derived strains overexpressing *PGA7*, *PGA23*, *PRA1* and *HWP1*. Murine dendritic cells were cultivated for 4 h in presence of various *C. albicans* strains. Total RNA was isolated and strains specific cytokine response was quantified on transcriptional level by qRT-PCR. No tendency in transcriptional response dependent on specific gene expression can be observed.

Strains defective in *efg1* and *cph1* also show significant reduction in survival in contact with macrophages (Lorenz*, et al.*, 2004). Therefore, survival of HLC69 derived strains in contact with bone marrow derived macrophages was studied in order to see effects of all the overexpressed proteins on survival of *C. albicans*. However, none of the overexpressed genes improved the survival of the HLC69 strain (Figure 31). Actually, the overexpression of the *PGA7*, *PGA23*, *PRA1* and

HWP1 increase the susceptibility of the strains to macrophage killing. This effect could be the result of the weakening of the fungal cell wall caused by the protein overexpression and following the pattern of the Congo Red sensitivity (page 100), which leads in result to more effective clearance by the immune cells.

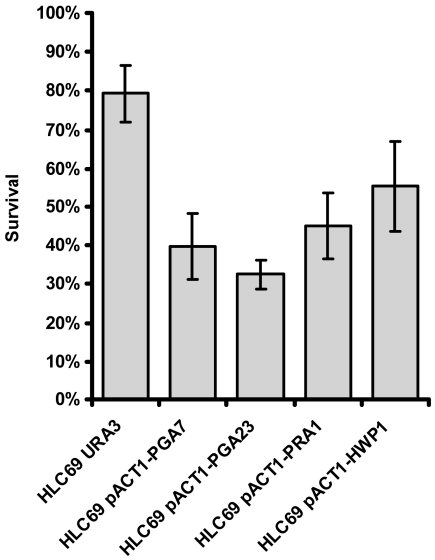


Figure 31. Survival assay.

Susceptibility of HLC69 derived strains overexpressing *PGA7*, *PGA23*, *PRA1* and *HWP1* to macrophage killing. Strains were cultivated with/without presence of murine bone marrow derived macrophages in 96 well plates as static culture. Killing efficiency was measured from the difference in optical density of parallel wells with/without macrophages.

Functional studies of genes *PGA7*, *PGA23* and *PRA1* characterize them more as structural elements of the cell wall rather than adhesins. Their overexpression in two strain backgrounds did not reveal any adhesive properties or any importance for invasion behavior as well as interaction with immune system.

6.3 *AUF* Genes

6.3.1 Identification and Characterization

ORF19.3908 (named *AUF8* – Adhesion Upregulated Factor 8) was identified in the microarray studies, besides *PGA7*, *PGA23* and *PRA1*, to be upregulated during adhesion process (Sohn*, et al.*, 2006). The strongest expression of *AUF8* appeared after two hours of adhesion with the strongest evidence on Caco-2 cell line as verified by Northern blot (Figure 32). This regulation was further confirmed by qRT-PCR experiments (data not shown). *AUF8* encodes a protein consisting of 204 aa and is predicted to carry 4 transmembrane domains using computational analysis (Krogh*, et al.*, 2001). By additional *in silico* analysis no N-terminal secretion domain was identified in the sequence, but using available prediction methods (SoftBerry ProtComp V8 prediction application) the localization was predicted with the highest probability to the plasma membrane. So far no characterization of this ORF was reported in the literature.

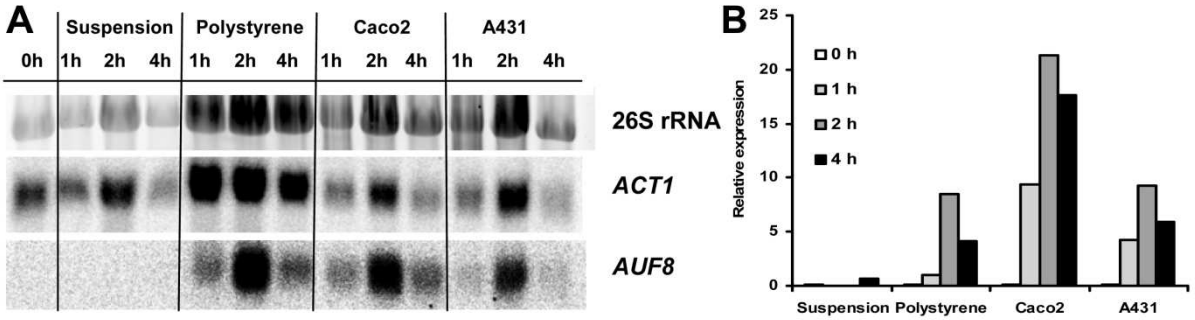


Figure 32. Analysis of *AUF8* expression.

Northern blot (A) following *AUF8* expression. Exponentially YPD grown cells of wild-type strain SC5314 were cultivated in DMEM or let to adhere to polystyrene surface, Caco-2 or A-431 cell line. The isolated mRNA was probed against *AUF8* ORF. The highest expression appears after two hours of adhesion to the Caco-2 cell line, while in suspension culture no significant increase in expression can be observed. The *AUF8* signal was normalized to the amount of *C. albicans* 26 S rRNA and plotted into the graph (B). The expression level of *AUF8* after 1 hour of adhesion to polystyrene surface was set equal to 1.

Interestingly, *AUF8* has several homologues in the genome. Based on protein blast, 6 additional ORFs encoding for proteins with significant similarity to Auf8 were identified. These are: ORF19.4691 (*AUF1*, 201 aa), ORF19.3902 (*AUF2*, 248 aa), ORF19.3903 (*AUF3*, 195 aa), ORF19.3904 (*AUF4*, 256 aa), ORF19.3905 (*AUF5*,

213 aa) and ORF19.3906 (*AUF6*, 196 aa). *AUF2-8* together are localized on chromosome 5 in one 10 kb gene-cluster, while *AUF1* is localized separately on chromosome 4 (Scheme on Figure 33A). None of the seven ORFs was characterized except for *AUF6*, which was deleted, but without any effect on phenotype that was studied (Nobile*, et al.*, 2003). The expression levels of *AUF* genes during two hours of the original microarray experiment are presented in Figure 33B. Obviously only the *AUF8* responds strongly to these conditions by increase in its transcription. For *AUF2* and *AUF5* some transcriptional induction during adhesion was observed as well. These two ORFs together with *AUF4* were also found in literature to be transcriptionally active under various conditions, indicative of active genes, not pseudogenes.

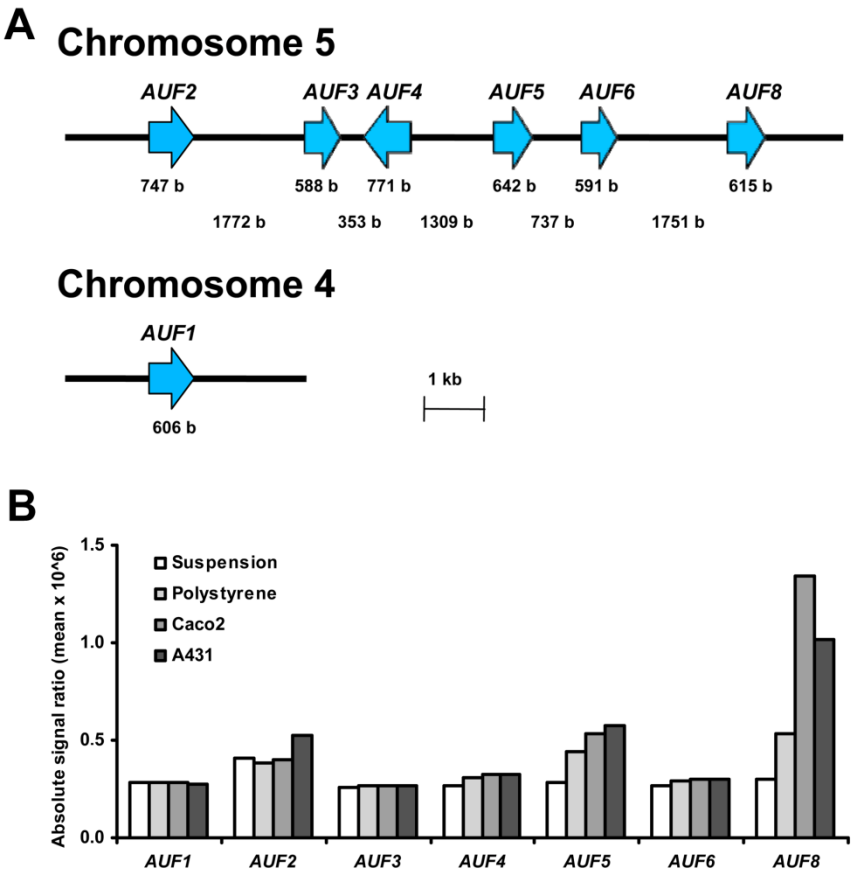


Figure 33. Localization of *AUF* genes in the *C. albicans* genome and their expression activity measured by microarray data.

Scheme of 10 kb cluster of consisting of genes *AUF2-8* is present on chromosome 5 and the separate ORF *AUF1* on chromosome 4 (A). Microarray data generated originally by Ilknur Senyürek representing mean spot intensity of all the *AUF* genes after two hours of cultivation in suspension culture or adhesion cultures on polystyrene, Caco-2 and A-431 cell line under identical hyphae inducing conditions (B).

The amino acid identity among Auf proteins is highly variable and ranges between 17 and 54 % (Table 15). All of the proteins were predicted to carry 4 transmembrane domains. No conserved amino acid regions or motives were identified among the translated proteins (Figure 34A). Additional 5 ORFs encoding for homologous proteins, with even reduced similarity, were identified in the *C. albicans* genome (ORF19.1334, ORF19.3057, ORF19.3600, ORF19.7043 and ORF19.7556), but none of them with known function. All of them carry 2-4 predicted transmembrane domains and their identity, among themselves and to Auf proteins, ranges between 5 and 20 % (Table 15). Phylogenetic tree (Figure 34B) additionally demonstrates the high sequence variability of the Auf proteins and their separation from the other five predicted and sequentially related proteins. Except for the closely related *C.*

dubliniensis, where 4 homologues of the *AUF* genes were found in one gene cluster (Cd36_53890 with the highest homology to Auf2, Cd36_53900 to Auf4, Cd36_53910 to Auf5 and Cd36_53920 to Auf8), no significant homologies to these proteins in other organisms were identified in the databases. Only for Auf2 a limited homology to *S. cerevisiae YNR061c* (21% identity, 38% similarity) was identified. This non-essential protein of unknown function has 4 predicted transmembrane domains and is localized to the vacuole (Huh*, et al.*, 2003). However, its cellular function is unknown.

Table 15. Similarity and identity among Auf proteins and five uncharacterized sequentially related proteins.

***AUF* / ORF19.xxxx (% similarity)**	***AUF* / ORF19.xxxx (% identity)**												
		AUF1	***AUF2***	***AUF3***	***AUF4***	***AUF5***	***AUF6***	***AUF8***	**3057**	**7043**	**7556**	**1334**	**3600**
	AUF1	100	21.0	38.2	21.3	27.0	32.9	54.4	18.5	13.0	12.2	4.9	18.2
	AUF2	31.7	100	22.4	31.4	23.2	17.0	18.9	16.6	8.1	20.1	5.4	17.6
	AUF3	55.9	36.9	100	20.8	25.7	27.0	35.6	20.2	16.1	16.5	6.4	14.4
	AUF4	33.6	47.4	33.6	100	17.7	18.1	18.4	14.2	13.1	13.4	8.6	17.7
	AUF5	40.4	37.5	42.6	33.2	100	16.6	27.6	19.4	13.2	21.4	5.6	16.7
	AUF6	48.1	29.2	45.6	33.6	32.0	100	47.4	15.0	10.2	13.1	8.3	13.1
	AUF8	69.6	29.1	52.7	30.9	43.4	59.6	100	16.9	14.7	15.7	5.6	13.7
	3057	32.0	26.8	37.5	25.2	33.0	26.0	26.2	100	14.9	13.2	6.9	11.4
	7043	22.8	16.5	32.1	22.7	24.4	15.5	26.5	27.0	100	20.2	4.2	6.5
	7556	27.8	32.4	29.2	26.2	36.9	25.8	27.0	27.3	31.6	100	9.6	16.7
	1334	11.2	9.8	11.3	16.5	11.5	13.3	10.1	12.3	9.1	15.1	100	6.9
	3600	28.5	31.8	25.6	32.7	26.7	22.2	22.0	20.1	12.5	23.5	12.3	100

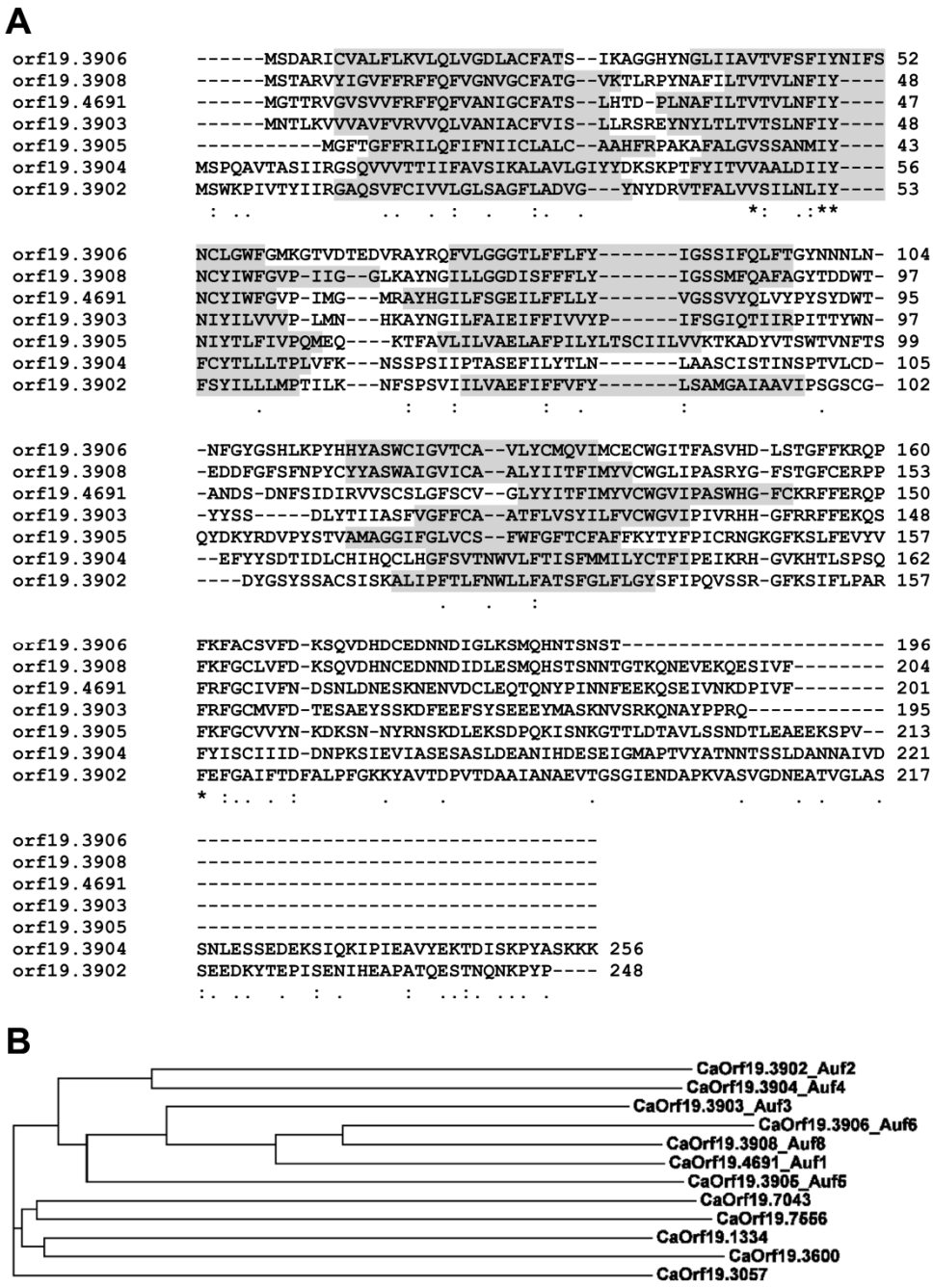


Figure 34. Sequential analysis of predicted Auf proteins.

ClustalW multiple sequence alignment of the predicted Auf proteins (A). No significantly conserved motifs can be seen. The regions with the highest similarity correspond mostly to predicted membrane-spanning regions (shaded). Phylogram presenting evolutional relation of Auf and Auf-related proteins (B) based on comparison of protein sequences of Auf proteins with predicted protein products encoded by sequentially related ORFs.

6.3.2 Construction of Deletion and Revertant Strains

To further characterize the function of the *AUF* genes, the genes were deleted in order to analyze differences in mutant phenotypes compared to the respective wild

type strain. The phenotypical differences should indicate the function of the gene and its encoded protein. Several deletion mutants deleted for *auf1*, *auf8*, *auf2-8* and *auf1-8* were constructed and the revertant strains *auf1*::*AUF1*, *auf8*::*AUF8*, with complementation of functional alleles, were constructed as well. In the case of deletion of the genes *AUF2*-8 located on a 10 kb fragment, cloning of this DNA fragment into a plasmid was not possible due to recombination of the DNA in *E. coli* (not shown). In such case only *AUF8* was reintroduced in the deletion strain (*auf2-8*::*AUF8*). In the strain deleted for all the *AUF* genes, just *AUF1* or *AUF8* (*auf1-8*::*AUF1* and *auf1-8*::*AUF8*) were reintroduced separately.

Plasmids were constructed and the *AUF* genes were deleted as mentioned in Material and Methods (chapter 5.26.1). In order to delete both alleles of the diploid *C. albicans*, after integration of the cassette the mutants were examined using Southern blot and the positive clones were further incubated in YCB-BSA medium in order to excise the deletion cassette. Positive clones were again verified on Southern blot. The correct integration of the cassette occurred in more than 90% of all the transformants. Verified heterozygous strains were transformed again with the same deletion cassette. In this case the correct integration efficiency into the second allele was lower than 20%. The rest of the colonies selected on the nourseothricine mostly integrated the cassette into the allele that has been already deleted. In order to distinguish these two types of mutants, multiplex colony PCR was performed using oligonucleotides URA3_F and URA3_R amplifying about 500 bp region of *URA3* gene and serving as positive control for the PCR reaction. Additional two oligonucleotides specific for upstream and downstream regions of the gene subject to deletion (*AUF2-8* and *AUF8* using oligonucleotides 3908dU_F and 3908dD_R or *AUF1* using oligonucleotides 4691dU_F and 4691dD_R) were used. These oligonucleotides were supposed to amplify bands only of following size in case of successful deletion: *AUF8* 1000 bp (band of wild-type allele 2000 bp was missing) and *AUF1* 1000 bp (band of wild-type allele 2000 bp was missing). The 1000 bp bands were supposed to be amplified from the already deleted alleles that were not replaced again with the deletion cassette. The 2000 bp bands were produced only in case that the intact ORFs were present in the genome. The DNA of the alleles with the integrated cassettes was not amplified due to the amplicon size over 5 kb. For deletion of *AUF2-8* no other DNA besides the 500 bp amplified from *URA3* was expected (band of wild-type allele 2000 bp had to be missing). Clones with an

amplified *URA3* region and a band of correct size from the deleted gene were further verified by Southern blot. The deletion cassette was excised and the final homozygous mutants were verified by Southern blot again. In total three parallel strains were constructed for each mutant in order to exclude any change in phenotype caused by other factors than deletion of the desired gene. The phenotypes of these strains were compared on various media and any strains showing inconsistence with the other mutants generated in parallel were excluded from further studies. Revertant strains had to be constructed as well in order to reintroduce a functional allele into the mutant strains.

An additional strain overexpressing *AUF8* was constructed. For this purpose the predicted *AUF8* promoter was replaced with the promoter of the *TDH3* gene, encoding for the highly expressed glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase. Southern blots following the *auf* gene deletion, reintroduction and overexpression are presented in Figure 35. The amount of *AUF8* transcript in the overexpression strains was determined by northern blot. The successful deletions show that the *AUF* genes are not essential for *C. albicans*.

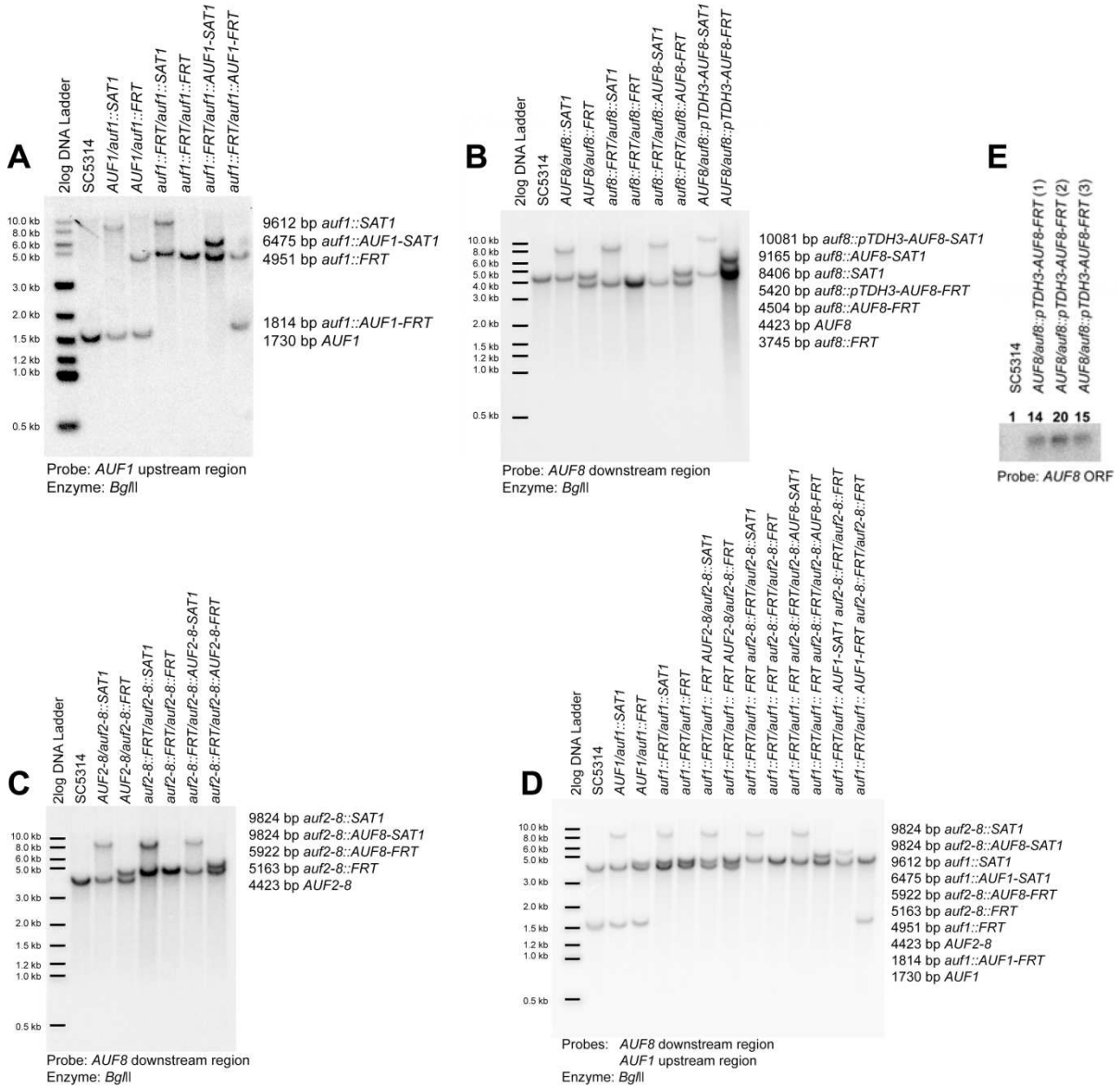


Figure 35. Southern blots confirming construction of the *AUF* mutant and revertant strains.

AUF1 (A) and *AUF8* (B) mutant and revertant strains, *auf2-8* mutant strains with *AUF8* reversion (C) and *auf1-8* mutant strain with reversion of *AUF1* or *AUF8* (D). About 20 µg of isolated genomic DNA was restricted with enzyme *Bgl*II, run on agarose gel and blotted on nylon membranes. The membrane was probed against PCR generated DNA sequences radioactively labeled with ^{32}P-dCTP. Northern blot (E) presenting expression of *AUF8* in three independently constructed strains *pTDH3-AUF8* overexpressing *AUF8* and grown exponentially in YPD. Isolated mRNA was run on a denaturing agarose gel, blotted on nylon membrane and probed against radioactively labeled *AUF8* ORF sequence. Numbers evaluate the gene expression normalized to rRNA quantity and related to wild-type strain (equal to 1).

6.3.3 Adhesive and Invasive Properties of Mutant Strains

After construction of all the mutant and *AUF8* overexpressing strains, adhesion and invasion assays were performed in order to investigate the function of the *AUF* genes related to the adhesion and invasion process, since the *AUF8* transcriptional activity was identified under these conditions. However, deletion of *auf1*, *auf8*, *auf2-8*, *auf1-8* or overexpression of *AUF8* did not have any significant effect on the ability of these strains to adhere to the Caco-2 cell line or abiotic polystyrene surface (Figure 36A). Revertant strains were not included in the assay, since there was no evident difference in adhesion among all the mutant strains and the wild-type strain SC5314. No changes in adhesion phenotype were observed in wash assays either (Figure 36B).

Additionally, biofilm formation by various strains deleted for *auf* genes was tested. Since *AUF8* is induced during the first hours of the adhesion process, it could contribute to the process of biofilm formation, even if it is not directly involved in adhesion itself. However, the deletion of *auf1*, *auf8*, *auf2-8* or *auf1-8* did not reveal any changes in the biofilm formation (data not shown).

Finally, in order to detect changes in invasive behavior of the strains, invasion assays were performed (Figure 37A), but no effects of *AUF* gene deletion or overexpression to the invasion of these strains have been observed. The results of the invasion assays were additionally confirmed in a parallel experiment, a quantitative LDH assay (Figure 37B), where any effect of the deletion of any *auf* gene or overexpression of the *AUF8*, on the invasive behavior of *C. albicans*, was detected neither.

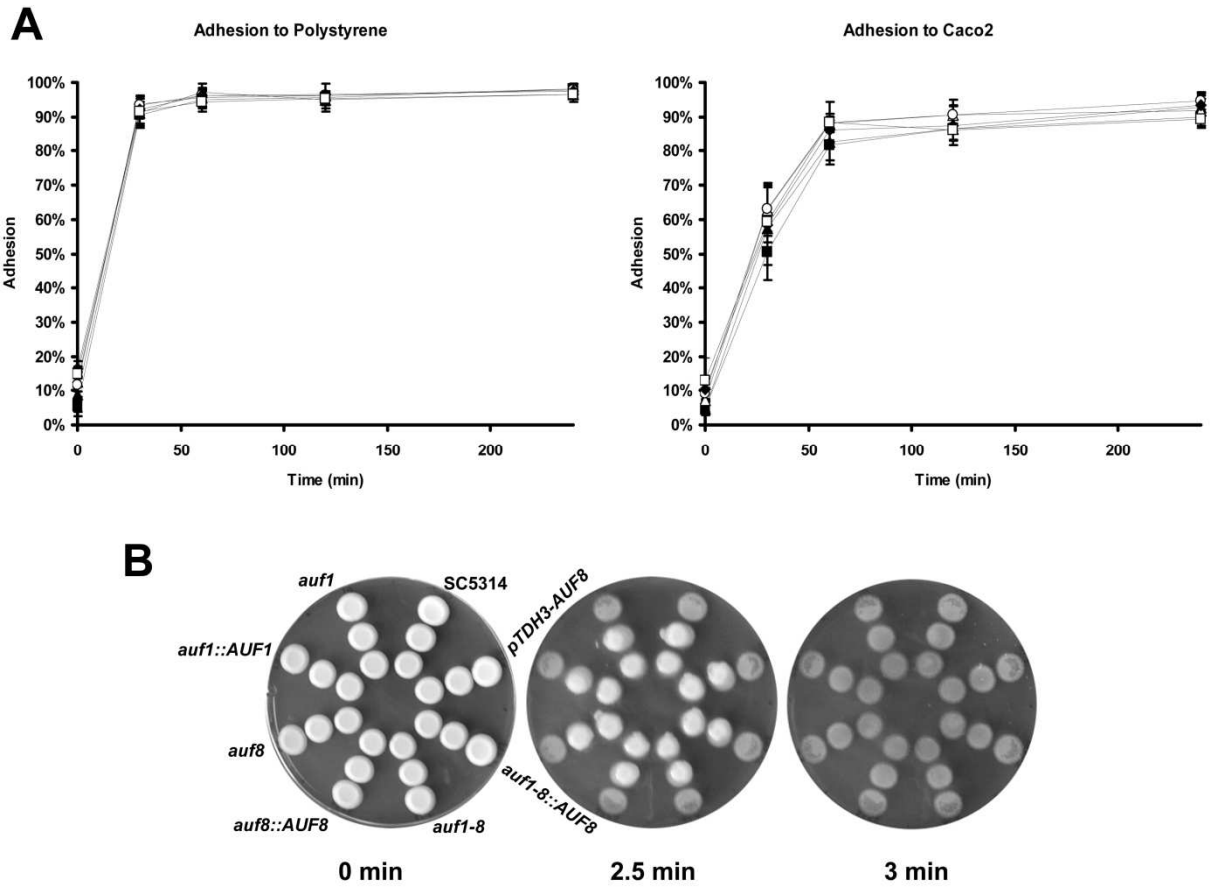


Figure 36. Characterization of adhesive properties of auf mutant strains.

Adhesion assays employing *auf* mutant strains and strain overexpressing *AUF8* on a polystyrene surface and the Caco-2 cell line (A). The graph presents progression of adhesion of different strains on defined surfaces. Strains are marked as follows: SC5314 (■), *auf1* (▲), *auf8* (∆), *auf2-8* (○), *auf1-8* (♦) and *pTDH3-AUF8* (□). Wash assay employing *auf* mutant strains, revertants and strain overexpressing *AUF8* (B). 2 days after inoculation of the YPD agar plate and cultivation at 30℃ the plate was incubated for period of time with 10 ml PBS buffer and constant rotation 100 rpm in order to observe differences in washing off the presented strains.

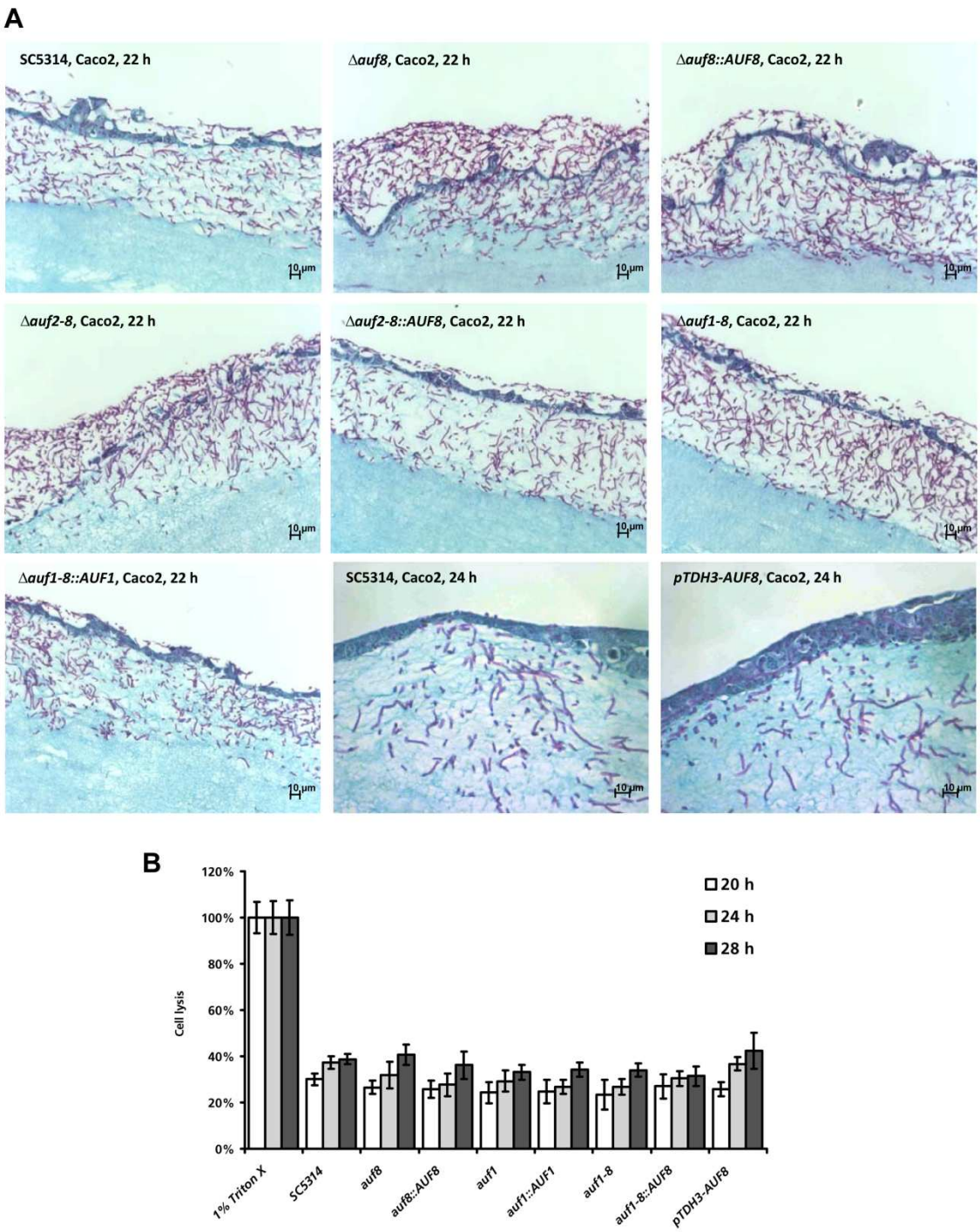


Figure 37. Invasion assays and cell damage quantification.

Qualitative invasion assays employing various *auf* mutant strains and their respective revertants (A). *In vitro* epithelial models consisting of a collagen matrix overlaid by Caco-2 cell monolayer were infected with wild-type strain SC5314 and selected representative *auf* mutant strains with their respective revertants. Additional experiments comparing strain *pTDH3-AUF8* with the wild-type control strain are presented in the bottom row. Samples were histologically processed and stained according to periodic acid-Schiff method (Caco-2 dark blue, Candida red and collagen light blue). Basically no difference among all the strains and the wild-type in terms of invasion can be observed. Additionally damage of Caco-2 cells was quantified by measurement of release of the intracellular enzyme lactate dehydrogenase (B) using a chemical reaction coupled to this enzyme. 100% of possible cell damage

was determined by detergent-induced cell lysis. Time points 20, 24 and 28 hours post-infection of the Caco-2 monolayer are presented. The graph represents an average of three independent experiments.

6.3.4 *AUF8 GFP* Tagging

For additional characterization of *AUF8*, its localization was analyzed. For this reason *GFP* tagging of *AUF8* at its chromosomal locus was performed. In order to be able to do so in a wild-type strain, a *GFP* tagging cassette containing a dominant selective marker, *SAT1*, was constructed for this purpose.

6.3.4.1 Construction of pXFP-SAT1 Plasmids

Based on a set of *XFP* tagging vectors pXFP-HIS1 and pXFP-URA3 (X stands for G, Y or C - green, yellow or cyan fluorescent protein) (Gerami-Nejad*, et al.*, 2001) containing *HIS1* or *URA3* marker for selection of positive transformants a set of vectors containing the dominant selection marker *SAT1* was constructed (see chapter 5.26.3). The original set of vectors can be used for strains defective in *ura3* or *his1*, but it is not suitable for wild-type strains without such auxotrophies. First, the plasmid pGFP-SAT1 was created. Scheme of the cassette after construction is presented in Figure 38. From this plasmid additional colored variants pCFP-SAT1 and pYFP-SAT1 were constructed. The tagging cassettes contain two *FRT* recombination regions at both sides of the *SAT1* gene with the *pACT1* promoter in order to additionally excise the resistance marker if necessary. In such case the recombinase will have to be introduced into the strain separately from the tagging cassette. The recombinase gene was not introduced into the cassette in order to keep it as small as possible. Cassettes significantly larger than 3 kb could cause problems during its PCR amplification. If the cassette is introduced into a strain expressing the recombinase gene, several colored variants for different genes can be created.

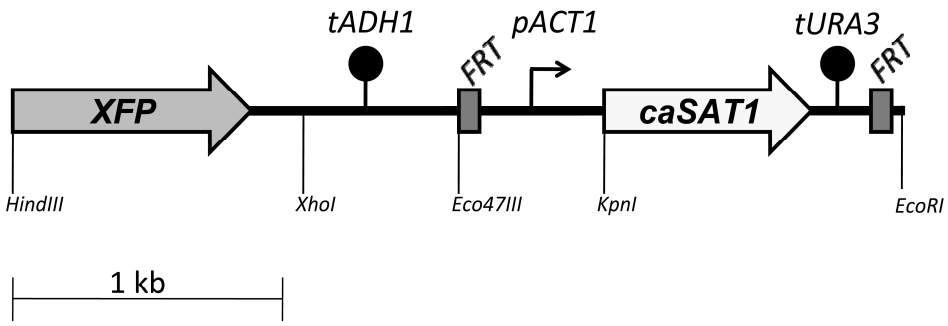


Figure 38. Scheme of constructed GFP-SAT1 cassette.

The presented cassette in the vector pUC119 is roughly 3 kb in size. Amplified by PCR with the use of specific oligonucleotides it can be used for further transformation of *C. albicans* and integrated into sequence specific site in the genome.

6.3.4.2 Tagging of *AUF8* in *C. albicans* Wild-Type

GFP tagging of *AUF8* on chromosome in the wild-type strain was performed in order to confirm localization of *AUF8 in vivo*. The tagged gene was expressed from its own promoter in order to avoid any localization artifacts caused for example by gene overexpression. The *GFP* tagging cassette from vector pGFP-SAT1 was amplified by PCR using oligonucleotides 3908GFP_F and 3908GFP_R2. The oligonucleotide 3908GFP_F contains 80 nt homologous to end of *AUF8* gene excluding the STOP codon (region for the homologous recombination), three GGT repetitions encoding for a linker code for 3 glycines between the Auf8 and GFP sequences, and 20 nt homologous to the beginning of *GFP* ORF for the purpose of amplification of the cassette. The oligonucleotide 3908GFP_R2 contains sequences homologous to the *AUF8* downstream region starting with the STOP codon of the *AUF8* ORF, and 20 nt homologous to the end of the *GFP* tagging cassette for its PCR amplification. The PCR product of the cassette with additional 80 bp upstream and downstream flanking regions (placed on oligonucleotides) was used for homologous recombination into the *C. albicans* genome. After the transformation, positive transformants were selected on YPD plates containing nourseothricine and the recombination was verified by Southern Blot (Figure 39). This resulted in construction of the *AUF8GFP* strain. Several positive transformants were tested, but none of them showed any GFP expression in level visible under microscope. Changing the strategy, *GFP* tagging of the *AUF8* in a strain overexpressing this ORF from the *pTDH3* promoter was performed. In this case the *pTDH3-AUF8* strain was transformed with the *GFP* tagging cassette (plasmid pGFP-SAT1) as described for the wild-type strain. There

were two possibilities of integration of the cassette. First, the cassette could have been integrated behind the *AUF8* allele expressed from its own promoter. In this case the result would have been similar to tagging in wild-type. The second possibility of integration was behind the allele under *pTDH3* promoter. This later possibility was the goal of the tagging. In order to distinguish these two tagging possibilities, colony PCR was performed using oligonucleotides pTDH3_F_SacII and GFP_Ca_R. Since the oligonucleotides were specific for *pTDH3* promoter and *GFP* ORF, the PCR product could be produced only in the case of integration of the *GFP* tagging cassette behind the *AUF8* allele under the *pTDH3* promoter. Positive clones were isolated and verified by Southern Blot (Figure 39). For microscopical observation several independent transformants were used again. However, even in this case no fluorescence associated to overexpressed *AUF8GFP* has been observed. The higher *AUF8* expression was proven previously in Northern blot, but the *GFP* tagging of the overexpressed allele did not result in visualization.

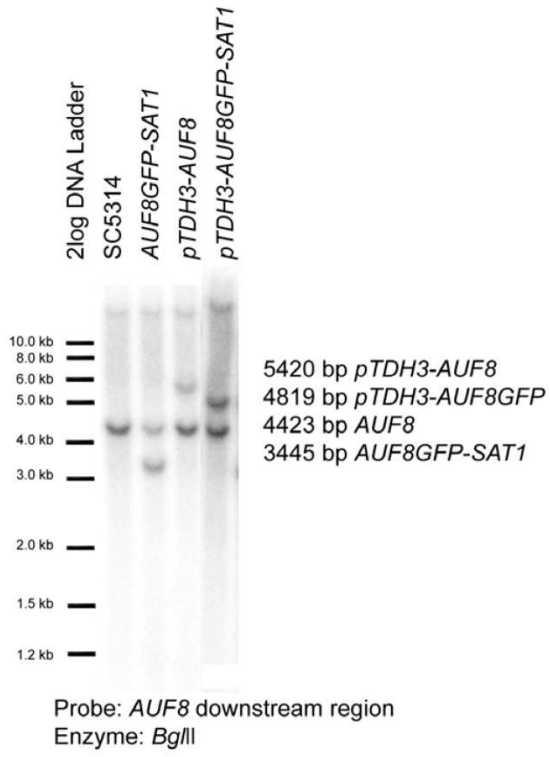


Figure 39. Southern blot of strains with *GFP* tagged *AUF8*.

Southern blot obtained from strains containing the *GFP* tagged *AUF8* gene and strain pTDH3-AUF8GFP overexpressing *AUF8GFP* construct from *pTDH3* promoter. About 20 µg of isolated genomic DNA was restricted with enzyme *Bgl*II, separated on agarose gel, blotted on nylon membrane and probed against a PCR generated *AUF8* downstream non-coding region.

6.3.4.3 Expression of *AUF8GFP* Construct in *S. cerevisiae*

To visualize the *GFP* tagged *AUF8* gene, the *AUF8GFP* construct was heterologously expressed in *S. cerevisiae* from a strong *pTEF2* promoter of

translational elongation factor 2 on a high copy plasmid. This organism is related to *C. albicans* and is commonly used for expression of several genes of *C. albicans* and other fungi in order to characterize their function (Li and Palecek, 2003; Sheppard*, et al.*, 2004). For this purpose plasmid pRS425A8G expressing *GFP* tagged version of *AUF8* was created (chapter 5.26.2) by ligation of *AUF8GFP* construct amplified by PCR from AUF8GFP strain. As a negative control served the plasmid pRS425A8 containing *AUF8* gene without any tag amplified from the SC5314 wild-type strain. Plasmids pRS425A8G and pRS425A8 were transformed into *S. cerevisiae* strain 10560-14A together with the empty original vector pRS425TEF and positive transformants were selected on SC agar plates. All three strains were grown exponentially in liquid SC medium and examined under a microscope in order to determine Auf8GFP localization (Figure 40). This study shows that Auf8GFP is localized into the plasma membrane, as predicted previously *in silico*.

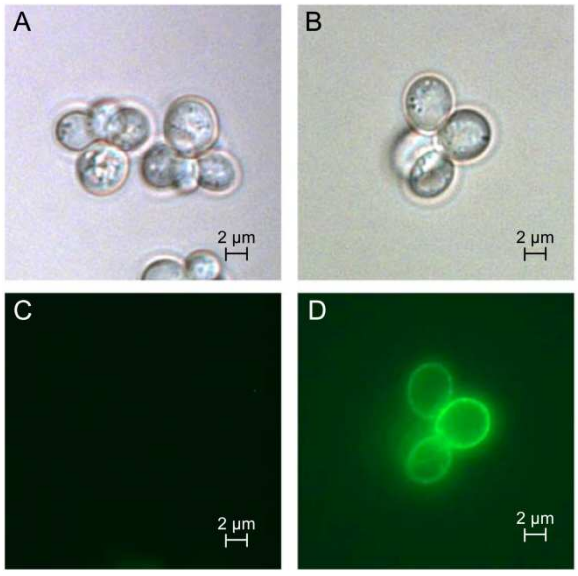


Figure 40. Fluorescent imaging of Auf8GFP heterologously expressed in *S. cerevisiae*.

S. cerevisiae expressing *GFP* tagged *AUF8* gene (B, D) from vector pRS425A8G compared to a control strain (A, C) transformed with empty vector pRS425TEF. Cells were exponentially grown in YNB medium and examined under visible spectrum (A, B) and UV light with appropriate GFP filter (C, D).

6.3.5 Additional Phenotypical Characterization

Since the *AUF* genes have no impact on adhesion or invasion, additional phenotypic characterization was performed in order to reveal a function for the *AUF* genes. The growth rate in terms of the duplication time among all of the deletion strains was determined and compared to the wild-type. However, it was not affected (data not shown). This indicates that these genes are not crucial for general metabolism or cell growth under the conditions tested. Additionally drop tests on various media were performed in order to characterize any defects in the *auf* deletion strains. A list of

conditions used for phenotypic characterization is given in Table 16. The concentrations of compounds inducing stress conditions were chosen to be sub-lethal for the wild-type strain SC5314, in order to see both decrease and increase in resistance. Two different temperatures were used for cultivation of the assays, 30°C for optimal growth and 37°C inducing hyphae formation. However, this assay employing different media and stress conditions did not lead to any significant phenotype of the *auf* deletion strains. Colony morphology and filamentation of the mutant strains were affected neither.

Filamentation in liquid DMEM at 37°C in presence of 50 µM, 100 µM and 250 µM farnesol was tested as well. Farnesol is a quorum sensing molecule produced by stationary *C. albicans* cultures. From certain concentrations in medium on this molecule prevents cells from germ tube formation, while it does not affect already existing hyphae (Mosel*, et al.*, 2005). Again, the mutant strains were inducing the germ tubes with efficiency comparable to wild-type (not shown).

Table 16. Conditions used for testing phenotypes of various *auf* mutant strains.

Condition	Medium	Compound and concentration
Growth, filamentation	SPIDER	-
Growth, filamentation	αMEM	-
Growth, filamentation	Lee's medium	-
Growth, filamentation	SLAD	-
Growth, filamentation	YCB-BSA	-
Growth, filamentation	YPD	-
Growth, filamentation	YPD	10% serum
pH stress conditions	YNB	Unbuffered
		pH 4.0, 100 mM MES
		pH 9.0, pH 10.0, 100 mM Tris
Alternative carbon sources	YNB w/o Glc	2% succinate
		2% ethanol
		2% maltose
		2% lactose
		2% glycerol
		2% sorbitol
		2% galactose
		2% N-acetylglucosamine
Cell wall stress	YNB	200, 300, 500 μg/ml Congo Red
		50, 100 μg/ml Calcofluor White (pH 7.0)
Cell wall/membrane stress	YNB	0.05% SDS
Antifungal	YNB	2, 4 μg/ml fluconazole
		0.3 μg/ml Amphotericin B
Oxidative stress	YPD	2 mM H_2O_2
		2 mM, 5 mM diamide
		5 mM, 10 mM, 15 mM paraquat
Reductive stress	YPD	20 mM DTT
Cupper stress	YPD	1 mM, 10 mM $CuSO_4$
Zinc stress	YPD	5 mM ZnCl
Heavy metal stress	YPD	100 μM $CdCl_2$
Low metal stress	YPD	5 mM EDTA
Cell signaling stress	YPD	5 mM, 10 mM caffeine
Osmotic stress	YNB	2 M NaCl
		2 M KCl

To identify any relevance of growth for the *AUF* genes, competition assays were performed. This kind of assay is focused on the comparison of the fitness of two different strains. For this purpose wild-type and mutant strains are cultivated together in co-culture and their relative representation is measured over time. No change in strain representation means no difference in fitness between the strains under the condition tested, while a change in representation displays higher fitness of strain that is increasing at the expense of the competing strain. In this case separate co-cultivations of wild-type strain SC5314 with mutants deleted for *auf1*, *auf8*, *auf2-8*,

and auf1-8 as well as the strain overexpressing *AUF8* were performed. Both strains were inoculated in equal amounts into YNB medium and incubated for 24 hours at 30℃ with continuous shaking at 160 rpm. Each 24 hours the culture was re-inoculated into fresh YNB medium. The experiment took 10 days in total. In case of SC5314 wild-type competing with *auf1* or *auf8* deletion strains there was no difference in the strains representation (data not shown). No difference was also observed when *auf1* or *auf8* strains were competing with their respective revertants. Surprisingly, in case of *auf1-8* competing with the wild-type, there was an increase of *auf1-8* mutant strain at the expense of the wild-type (Figure 41A). An increase of fitness of the mutant was not expected. However, this result was verified by using a second *auf1-8* strain constructed in parallel (same genotype, independent deletion steps). Since re-integration of all the *AUF* genes into this mutant strain was not possible, reversion of this phenotype could not have been studied. Experiments exploiting strains *auf1-8*::*AUF1* and *auf1-8*::*AUF8* competing with the *auf1-8* mutant strain were performed. However, the phenotype of *auf1-8* strain was not reverted by the re-introduction of a single allele of *AUF1* or *AUF8* genes (Figure 41C, D). These observations indicate that reversion of *AUF1* or *AUF8* single allele alone is not sufficient to reverse the observed phenotype and cooperation of several of the *AUF* genes on this phenotype is most probable.

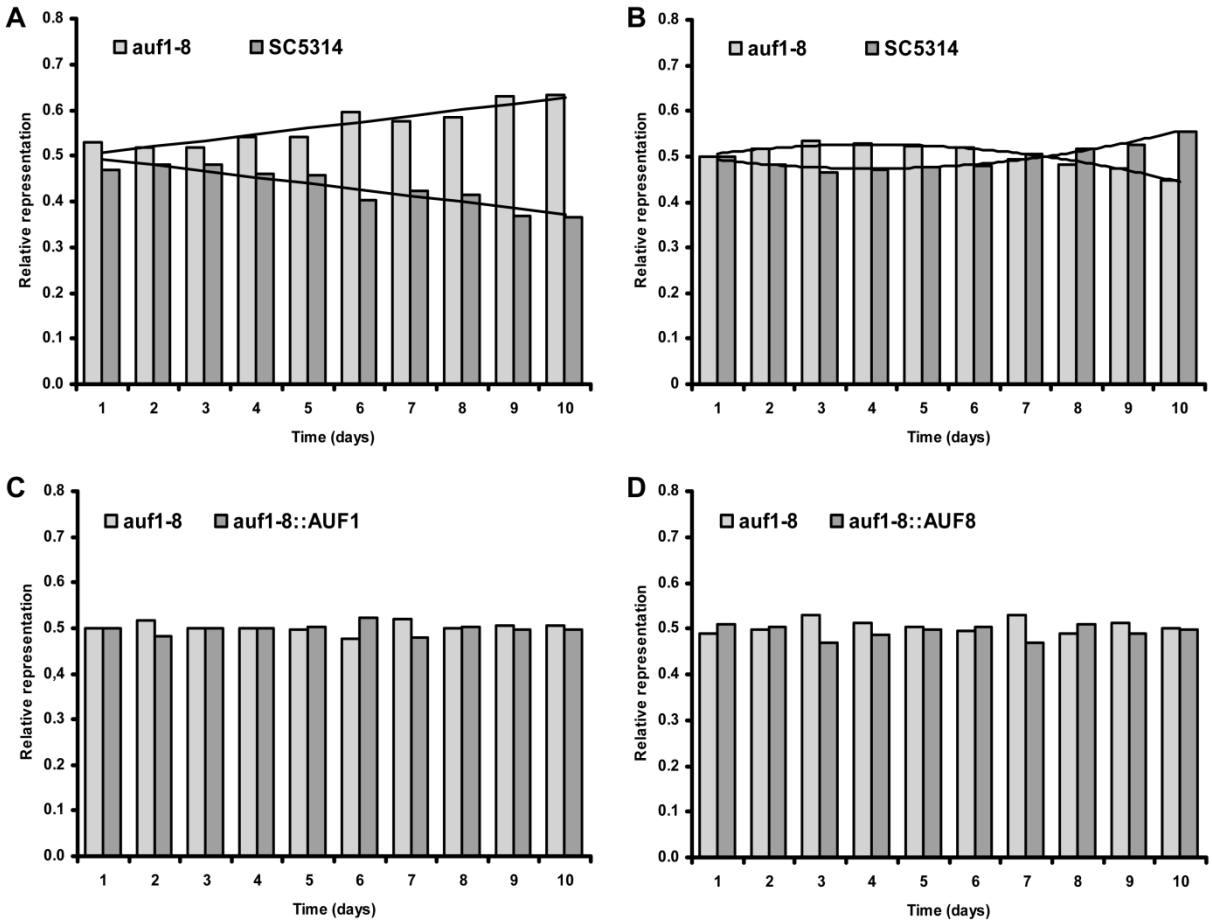


Figure 41. Competition assays.

Competition assays in YNB during 24 hour re-inoculation (A) and stationary culture cultivation (B) comparing wild-type strain SC5314 with *auf1-8* deletion mutant. Competition assays between *auf1-8* deletion mutant and its *auf1-8*::*AUF1* (C) and *auf1-8*::*AUF8* (D) revertants with 24 hour re-inoculation. Strains were co-cultivated in YNB suspension culture and each 24 hours a sample was recovered. From the samples genomic DNA was isolated, restricted with enzyme *Bgl*II, separated on gel, blotted to nylon membrane and hybridized against radioactively labeled probe providing different band size for each of the strains. Strain specific bands were quantified and compared. Changes in quantitative representation of the strains in the co-culture are presented in the graphs. While in the 24 hour re-inoculated culture the *auf1-8* mutant is increasing in expense of the wild-type strain, in stationary phase kept culture, after initial increase, the *auf1-8* mutant is decreasing from the day 5.

For further characterization a competition assay of SC5314 with strain *auf1-8* in YNB for 10 days of stationary culture without re-inoculation was performed. The suspension culture was cultivated at 30℃ with 160 rpm shaking for 10 days and each 24 hours a sample of the suspension was recovered. From this experiment the changes in strain representation were measured as well (Figure 41B). In this case an increase in *auf1-8* strain until day 5 post-inoculation occurred. After this time point the *auf1-8* strain was constantly decreasing. Again, this phenotype was not observed for

strains *auf1*, auf1::*AUF1*, *auf8* and *auf8*::*AUF8*. And again, the observed phenotype for strain *auf1-8* was not reverted in strains *auf1-8*::*AUF1* and *auf1-8*::*AUF8*. These data indicate that the *auf1-8* strain gains an advantage in early stationary phase (until day 5) while in later stationary phase it tends to be eliminated in the co-culture with the wild-type.

Due to the possible alterations in the exponential, post-exponential and stationary growth of the *auf* mutants, differences in OD_{600} of cultures of the *auf* mutants and revertant strains were monitored for 11 days (Figure 42). Evidently, there are no significant differences among the strains in the maximal OD_{600} reached, which could indicate changes in growth during stationary phase. In addition, all the strains tend to decrease in OD_{600} at the same time, excluding any severe changes in stability in stationary phase among the strains.

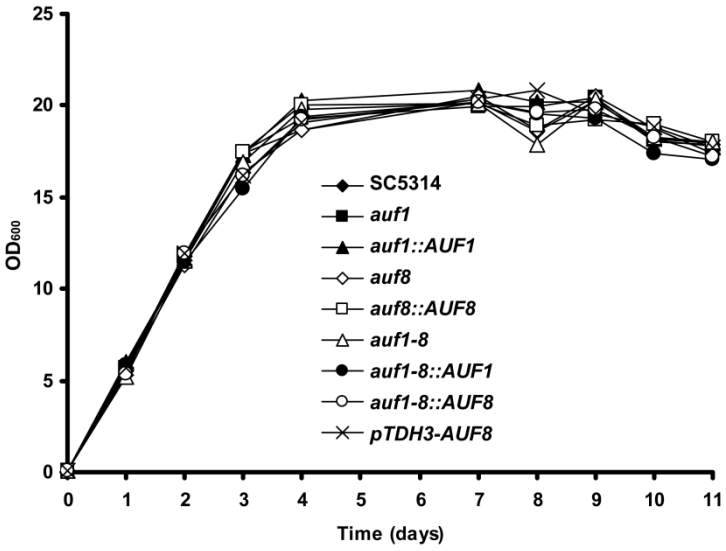


Figure 42. Growth of *auf* mutants and respective revertant strains. Optical density of the YPD cultures of various *auf* mutant strains and their respective revertants focusing on maximal optical density reached by every strain and time point of decrease.

6.3.6 Metabolism of Stationary Cells and *AUF8* Regulation

Due to the results indicating a role of the *AUF* genes in stationary phase of growth, transcriptional profiling of the *AUF8* gene in wild-type strain in YNB and YPD medium was performed together with monitoring differences in metabolism (Figure 43). SC5314 was cultivated for 5 days in complex YPD and minimal YNB medium and besides the expression level of *AUF8*, determined by qRT-PCR, the levels of glucose, ethanol and acetate were measured every 12 hours. Additionally, the optical density and the pH of the medium were measured and plotted in a graph.

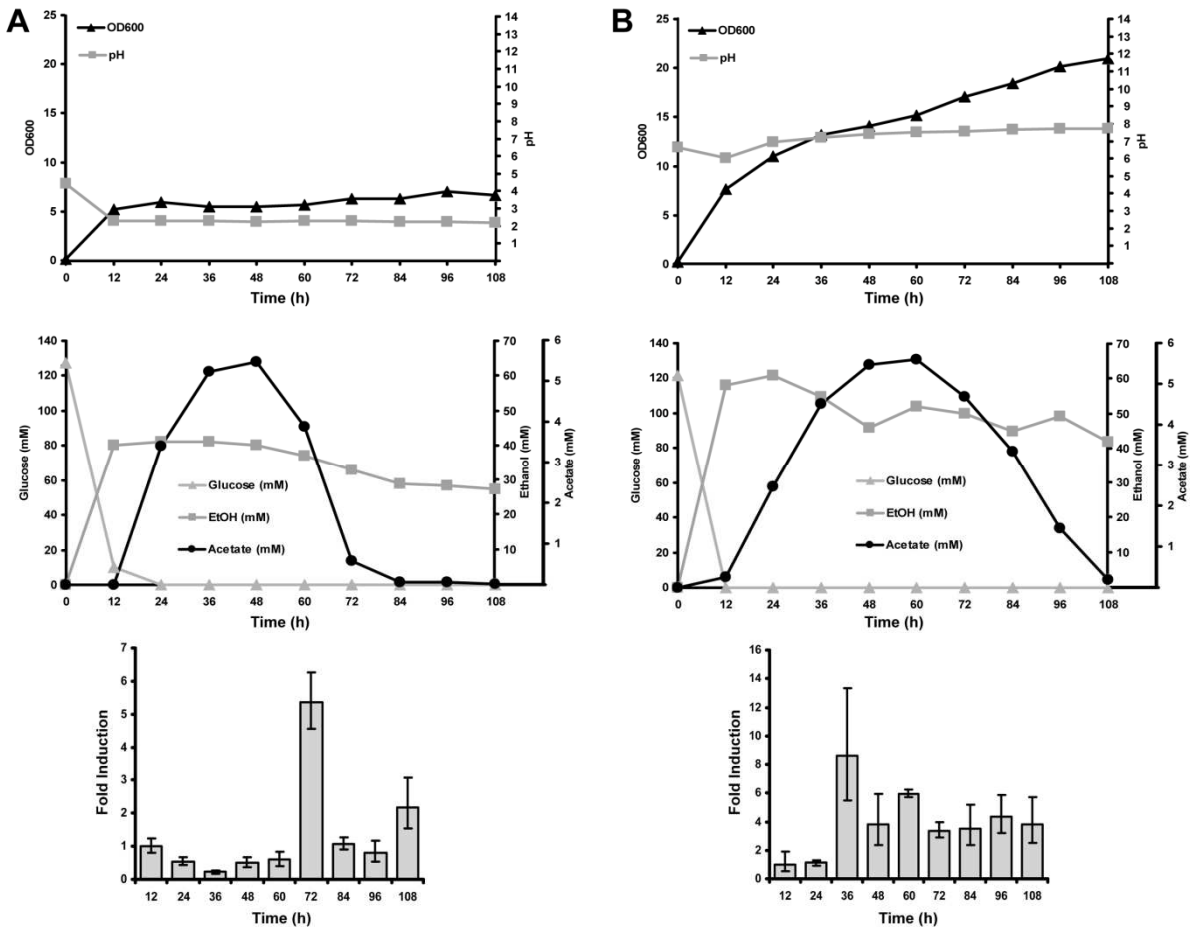


Figure 43. Metabolic activity and *AUF8* expression.

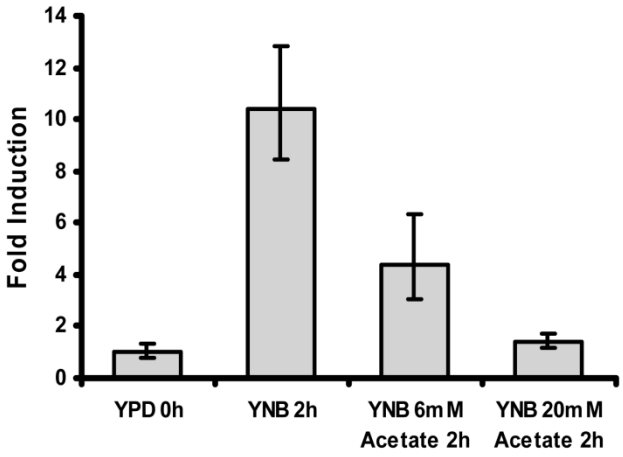
Growth and metabolic activity of wild-type strain SC5314 grown in YNB (A) and YPD (B) medium and transcriptional analysis of the *AUF8* gene under these conditions followed for 5 days with measurements each 12 hours. Cultures were inoculated to initial OD_{600} = 0.1 and cultivated for 5 days. Each 12 hours sample of the cultures was recovered, pH and optical density was measured. Glucose, acetate and ethanol concentrations in the medium were measured. From the cells RNA was isolated, transcribed into the cDNA and *AUF8* gene expression was compared to the rRNA internal control.

Interestingly, SC5314 grew at completely different pH in the minimal (YNB) and complex (YPD) media. While in YNB it grew at pH 2 after initial drop from pH 4.5 during first 12 hours, in YPD the strain grew at pH 7 and increasing to the pH 8 during the experiment. The metabolic profiles were similar in both media. Initial decrease in glucose was followed by increase of ethanol and delayed increase of acetate. While the acetate was probably metabolized, the level of ethanol was decreasing only slowly. In the YNB culture the *AUF8* expression increased at 72 hours and dropped again, but in the YPD culture the expression increased at 36 hours and remained significantly higher from then on. Since these are the time points where the level of the acetate is changing, additional effects of acetate on *AUF8*

expression was investigated. Exponentially grown cells from YPD culture were transferred into fresh YNB medium containing different concentrations of acetate and *AUF8* expression level was determined after 2 hours (Figure 44). From the experiment it seems that the presence of acetate in YNB medium leads to a decrease of *AUF8* expression. The same effect was observed also in case of the YPD medium (not shown).

Figure 44. Affect of acetate on *AUF8* transcription.

Relative expression of *AUF8* in exponential YPD culture (absolute mRNA level equal to 1) and after 2 hours of cultivation in YNB medium in presence of different acetate concentrations. Graph represents qRT-PCR data of one of two biological replicas and the error bars represent variation in technical replicates.



6.3.7 Interaction with Macrophages

In order to test any effect of the *AUF* genes during contact with immune system, survival assays were employed in order to check a susceptibility of the *auf1-8* strain and its respective *AUF8* revertant to killing by murine bone marrow derived macrophages (Figure 45A). However, no effect of deletion of all the *auf* genes was observed. Survival of the wild-type strain in contact with macrophages was higher than 90% and the deletion of the *auf* genes did not affect this rate, excluding any crucial importance of the *AUF* genes for survival in contact with the macrophages.

Additionally, a ROS assay employing murine bone marrow derived macrophages was performed (Figure 45B). The changes in ROS release could indicate alterations in the cell wall structure and strain immunogenicity or reveal additional changes in the interaction with macrophages. However, also in this case no differences among the *auf* mutant strains and the wild-type were observed. Thus it seems that deletion of *auf* genes does not have any effect on contact of *C. albicans* with macrophages.

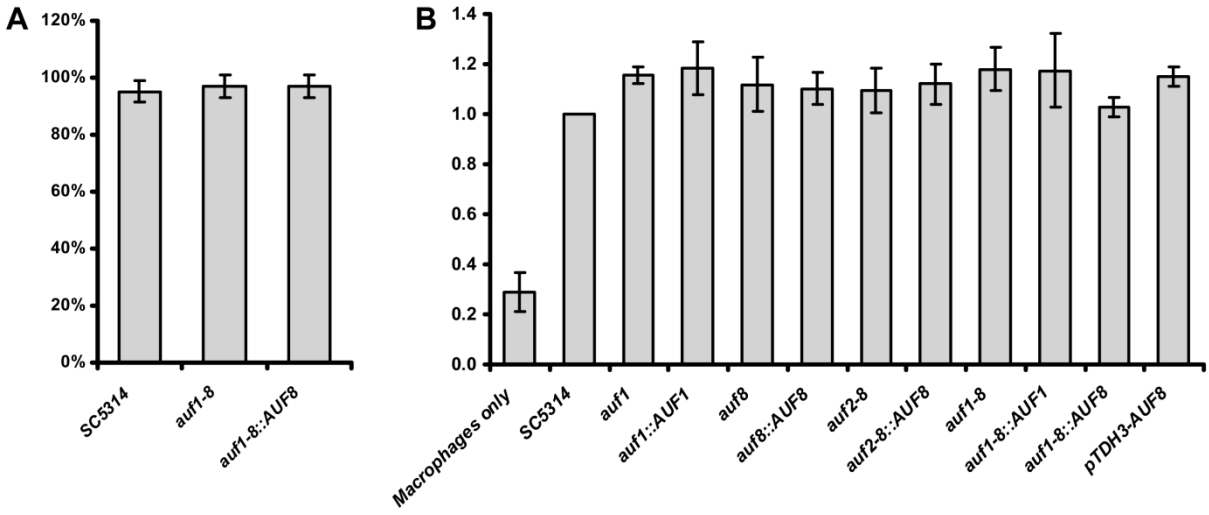


Figure 45. Interaction of *auf* mutants with macrophages.

Survival assays monitoring the susceptibility of *auf1-8* strain and its *AUF8* revertant to the elimination by murine bone marrow derived macrophages (A). Strains were cultivated with/without presence of murine bone marrow derived macrophages and killing susceptibility was determined from the difference in counts of colony forming units. Basically all the strains show high resistance to macrophage killing that is not changed even in case of *auf1-8* mutant. Comparison of relative ROS release by murine bone marrow derived macrophages (B) in contact with different *auf* mutant strains and their respective revertants, related to the wild-type (set to 1). Strains were cultivated in the presence of BMDMs and ROS release between 20 and 100 minutes was quantified. The graph represents average from three independent experiments of Candida-macrophage interaction performed in duplicates.

The *AUF* gene family is a novel and previously uncharacterized set of 7 genes expressed under various conditions. *AUF8*, induced during the adhesion process, encodes for protein that localizes to plasma membrane when heterologously expressed in *S. cerevisiae*. However, the importance of Auf8 for adhesion and invasion behavior for *C. albicans* could not be confirmed as well as for the other members of the family. Any additional phenotypical effect was not revealed either. However, the gathered data are indicating a role of the *AUF* genes during stationary phase of growth.

7 DISCUSSION

Candida albicans is a commensal organism and opportunistic pathogen living on skin and mucosal surfaces of humans and warm blooded animals, where it colonizes various host niches. The cell surface of the fungus is the site of direct interaction of Candida with the host, mediating environmental sensing, adhesion and also interaction with the host immune system. Since the cell wall is not present in humans its components are a prime target for drug development.

This work was characterizing cell wall changes after deletion of two transcription factors affecting not only its protein composition, but also its polysaccharide structure and further leading to an alteration of its immunological properties. Additionally it was focused on characterization of several genes encoding for proteins putatively localized to the cell surface and induced during interaction with *in vitro* infection models.

7.1 Cell Wall Effects of *efg1* and *cph1* Deletion

As reported in the literature, besides other phenotypical effects, deletion of genes *efg1* and *cph1* has a prominent effect on cell wall protein composition and adhesive characteristics of *C. albicans* (Dieterich*, et al.*, 2002; Doedt*, et al.*, 2004; Harcus*, et al.*, 2004; Sohn*, et al.*, 2003). However, changes in the cell wall polysaccharide composition were not studied so far. In order to characterize them as a possible origin of different immunogenic behavior of *efg1* and *efg1*/*cph1* mutant strains (Barker*, et al.*, 2008; Dongari-Bagtzoglou and Kashleva, 2003; Korting*, et al.*, 2003; Lu*, et al.*, 2006; Phan*, et al.*, 2000; Villar*, et al.*, 2004; Yang*, et al.*, 2009), a study focusing on their cell walls was performed.

Transcription factor Efg1 by itself and in combination with Cph1 has a strong effect on cell wall protein composition, adhesion, invasion and metabolism and therefore is one of the major virulence factors of *C. albicans* (Biswas*, et al.*, 2007). The influence of these genes on cell wall architecture and immunogenicity was further investigated. In experiments focusing at cell wall stability, significant weakening was observed in strains deleted for *efg1*, resulting in increased sensitivity to Congo Red as well as to the treatment by cell wall degrading enzyme Zymolyase.

By employing a plate analysis of strains defective in *cph1*, *efg1* or *efg1*/*cph1* stronger sensitivity to glucan related Congo Red of the *efg1* deleted strains was

observed, whereas the strain defective only in *cph1* showed no change in Congo Red sensitivity, compared to its respective background. In strains *efg1* and *efg1*/*cph1* reverted for functional allele of *EFG1* also the observed phenotype was reverted. Strain *efg1*/*cph1* showed little stronger resistance to the Congo Red than the *efg1* strain does. How deletion of *cph1* in an *efg1* mutant background ameliorates the sensitivity to this cell wall perturbing agent is currently not understood. Also increased sensitivity to the cell wall disturbing agent Calcofluor White in strain deleted for *efg1*, as observed previously (Harcus*, et al.*, 2004), was confirmed. However, this phenotype was not observed in *efg1*/*cph1* mutant nor reverted in *efg1*::*EFG1* strain. Thus it is not clear whether this phenotype can not be reverted be reintroduction of just one *EFG1* functional allele in *efg1* strain or is caused by any additional change in genome.

These cell wall effects observed can be explained by the significantly altered regulation of genes required for cell wall biogenesis including a group of cell wall synthesis and remodeling enzymes (Sohn*, et al.*, 2003). It has been shown that not only the cell wall protein expression levels are disturbed, but also the polysaccharide structure of the cell wall itself is significantly altered in strains deleted for *efg1* and *cph1*. TEM images showed that the cell wall of the *efg1/cph1* strain is reduced to about 50% of the original wild-type thickness. This also correlates with the reduced amounts of β-glucans observed for *efg1* and *efg1/cph1* strains. The glucose amounts released by hydrolysis of the cell walls, however, did not correspond directly to the cell wall thickness. The explanation for this may be a higher density of the glucan fibrils in the cell wall of *efg1* mutant strains to support stability of this reduced scaffold. An additional explanation for the differences between the transmission electron microscopy data and glucan levels may be the fact that cells of the strains *efg1*, *efg1*/*cph1*, *efg1*::*EFG1* and *efg1*::*EFG1*/*cph1* are generally smaller, resulting in an altered cell-surface to volume ratio. The effect of *efg1* deletion on cell morphology was already described (Lo*, et al.*, 1997; Stoldt*, et al.*, 1997). With regard to the cell size, smaller cells would need relatively more cell wall components, related to cell biomass, in order to keep the same cell wall strength. Actually, the cell size of these strains is even decreasing with the transition of the culture from the exponential to the post-exponential phase (observed qualitatively under microscope and verified by CFUs related to OD_{600} of the cultures). It additionally offers an explanation for the lower differences in glucan levels among all the strains in post-exponential phase

compared to the cells grown exponentially. The decreasing cell size of strains lacking a functional *efg1* during transition to the post-exponential phase offers an additional evidence for importance of Efg1 regulation in different growth phases (Doedt*, et al.*, 2004). Interestingly, compared to the glucans, the levels of mannans and chitin are basically unaffected by the deletion of *efg1* and *cph1* in the exponentially grown cells. In case of *efg1*/*cph1* strain grown post-exponentially, strong increase in mannans in the cell wall was observed and reaching almost the level of glucans. In strain *efg1*::*EFG1*/*cph1* this increase was partially reverted. However, deletion of *efg1* alone did not affect the mannan content at all, although that in the *efg1*::*EFG1* revertant moderately elevated levels were observed, possibly related to the *EFG1* haploinsufficiency.

The amount of glucose released by hydrolysis of the cytosolic content was also significantly reduced in strains carrying a deletion in *efg1*, and showing a smaller impact of *cph1* deletion. This massive difference in glucose/glucan content in the different strains may be explained by the altered metabolism reported for strains deleted in *efg1* (Doedt*, et al.*, 2004; Harcus*, et al.*, 2004). Deletion of *efg1* favors oxidative metabolism and reduces the expression level of the gluconeogenesis pathway. This effect seems to be even more prominent in case of *efg1*/*cph1* double mutant indicating a contribution of *cph1* deletion as well. Only a partial reversion of internal glucose levels occurred in revertant *efg1*::*EFG1* and *efg1*::*EFG1*/*cph1* strains. In this case an effect of *EFG1* haploinsufficiency was proven, when in strain deleted for one functional *EFG1* copy already reduced levels of both internal and cell wall bound glucose units were observed, corresponding to the *efg1*::*EFG1* revertant.

Deletion of *cph1* by itself in the wild-type strain did not reveal any significant effect on the cell wall, while deletion in an *efg1* background significantly altered some of the *efg1* phenotypes. Using microscopical analysis the *efg1*/*cph1* strain has a lower amount of β-1,3-glucans accessible for antibody staining, compared to all other strains tested. This result is in agreement with the lower amount of glucans in its cell wall in general. However, the glucan could be also masked better by the relatively higher amounts of the mannose present in the cell wall presumably originating from mannosylated proteins. In case of *efg1* strain the staining was revealing higher β-1,3-glucan exposure. In both cases this phenotype was at least partially reverted by reintegration of the functional *EFG1* allele.

Changes in the cell wall structure often result in altered immunogenicity. Indeed, significant changes both in ROS response of murine macrophage cell line as well as cytokine response in murine DCs was observed. Interestingly, a general difference in ROS response depending on the growth phase of *C. albicans* was observed. For the wild-type and *cph1* strain a stronger, but slightly delayed ROS response was observed in cells originating from post-exponential phase of growth. Strains deleted in *efg1* also showed ROS response; however, it was delayed and in post-exponentially grown cells the ROS response dropped significantly, indicating further different functions of Efg1 for cell wall structure during different growth phases. The *efg1*/*cph1* strain provided low ROS response in general, independent of the phase of growth.

Interestingly, only partial relation between the strain immunogenicity and β-1,3-glucan exposure was shown. Besides a higher β-1,3-glucan exposure in the *efg1* strain, which was not reflected by stronger ROS response, also an increase in immunogenicity of the wild-type during post-exponential growth phase was not accompanied by higher β-1,3-glucan exposure. Thus it seems that although the β-1,3-glucan is important for the *C. albicans* immunogenicity, it is only a part of more complex stimuli inducing the ROS response.

In contact with dendritic cells, both the *efg1* and *efg1/cph1* mutant strains induced significantly lower expression levels of IFNß, TNFα, IL-4 and IL-23 in agreement with the alterations in the cell walls of these strains, expressed mostly by reduced amounts of β-glucans. The results indicate that the changes in cell wall structure and composition of the *efg1* and *efg1*/*cph1* strains contribute significantly to the reduced immunogenicity during contact with the dendritic cells. In this case the production of IL-4 is usually linked to ingestion of hyphae, while production of IL-12 favoring Th-1 response is observed with yeast stage cells (d'Ostiani*, et al.*, 2000). Therefore, reduction of IL-4 expression in contact with *efg1* and *efg1*/*cph1* strains may reflect the altered filamentation phenotypes of both strains. The reduction in TNFα expression may be linked to changes in mannose structures of the cell wall, since TNFα expression has been shown to be connected to stimulation of DCs by mannoproteins (Pietrella*, et al.*, 2006). Additionally, IL-23 induction in dendritic cells was linked to β-glucan exposure (Dennehy*, et al.*, 2009). Additionally, no significant changes were observed among the strains in case of IL-1β, IL-10 and IL-12 transcription, indicating that there is no generally lower response to *efg1* and

efg1/*cph1* strains due to their altered cell size. Compared to the ROS assay the dendritic cells have more time in the assay (4 h) for ingestion and analyzing of the complex cell wall structure of the strains. This could be one of the explanations for the similarities in transcriptional response to the *efg1* and *efg1*/*cph1* strains which is not evident in the ROS assay.

In many cases the phenotypes of strains *efg1*::*EFG1* and *efg1*::*EFG1*/*cph1* were not fully recovered. It seems that the level of *EFG1* expression in these strains is sufficient enough to restore their filamentation defects, but insufficient to fully restore all the cell wall defects. In order to verify the indicated *EFG1* haploinsufficiency, *EFG1* heterozygous strain HLC17 (deleted for only one allele of *EFG1*) and additional HLC17Rev (heterozygote reverted for the second functional *EFG1* allele) were investigated. The *EFG1* heterozygous strain HLC17 was corresponding to the *efg1::EFG1* in terms of ROS response, Zymolyase treatment, internal and cell wall related glucose amounts, while the HLC17Rev in these terms was similar to the wild-type. These data support the insufficiency of one functional *EFG1* allele to fully restore the studied phenotypes to the wild type level and additionally reveals a gene-dosis effect for the Efg1 transcription factor.

Although the *efg1* and *efg1/cph1* strains are recognized by immune cells *in vitro* (Korting*, et al.*, 2003; Lo*, et al.*, 1997), the immune response is significantly reduced, especially in the *cph1/efg1* mutant strain. The lower overall immunogenicity of the *efg1/cph1* strain could be one of the explanations for limited recognition and clearance of this strain during mouse systemic infection *in vivo*, and causing organ infiltration, as described previously (Bendel*, et al.*, 2003; Yang*, et al.*, 2009). The lack of filamentation together with reduced expression of many known virulence factors under the control of Efg1 and Cph1, as well as the identification of the severe changes in cell wall structure, help to explain the exceptionally low virulence of this strain.

7.2 Characterization of the Genes *PGA7*, *PGA23* and *PRA1*

C. albicans naturally colonizes various parts of the human body providing environments with various nutrient availabilities, pH and other characteristics. In order to find out whether this organism is able to differentially respond on a

transcriptional level to different surface stimuli, transcriptional profiling was performed previously (Sohn*, et al.*, 2006). Transcriptional profiles of *C. albicans* incubated on three different surfaces – abiotic polystyrene surface and two tissue models, Caco-2 and A-431 cell lines were followed for 4 hours. Genes *PGA7*, *PGA23* and *PRA1*, described, were identified in this study as upregulated during the adhesion process to all the three surfaces. Their transcriptional profile indicated a possible importance for host-pathogen interaction. How these genes contribute to the host-pathogen interaction was the aim of their functional study.

In contrast to the original studies (Sohn*, et al.*, 2006), genes *PGA7*, *PGA23* as well as *HWP1* were shown to be upregulated in response to DMEM and the presence of 5% CO_2, which is strong hypha inducer, rather than in contact with surface stimuli. Thus these genes seem to be induced by CO_2 rather than by contact with a surface. *PGA23* and *HWP1* on the other hand seem to be even slightly repressed by contact with a surface, which can be also caused by limited nutrient accessibility in the static adhesion cultures. On the other side, expression of *PRA1* in conditions offering a surface stimulus, either abiotic polystyrene or Caco-2 and A-431 cell lines, was confirmed.

By examination of the *pga7*, *pga23* and *pra1* null mutants no effect on adhesion or invasion behavior has been observed. However, in many cases the effect of deletion of a single gene can not be observed directly due to overlapping functions of other proteins that are still presented in the cell. It is common that several ORFs in genome, encoding for proteins sharing similar function, guarantee vital functions by their redundancy. For example in the case of genes encoding for adhesins Als1 or Als3 (Zhao*, et al.*, 2004) the mutant strains still carry partial adhesive properties originating from presence of other adhesive proteins that are still present on the cell surface. Additionally, in case of *pra1* mutant, increased sensitivity to Calcofluor White was observed. As reported, *pra1* strain has an altered chitin distribution in the cell under heat stress conditions (Sentandreu*, et al.*, 1998). This indicates that the cell wall structure may be partially altered even under optimal conditions. In order to further characterize these genes, they were overexpressed in two different strain backgrounds.

A model for characterization of adhesive properties of unknown proteins was established by overexpression in a *C. albicans* mutant strain defective in *efg1* and *cph1*, which both encode for transcription factors and their deletion alters adhesive

properties of *C. albicans* (Dieterich*, et al.*, 2002). Overexpression of the *HWP1* gene served as a positive control for the assay and improved the adhesion of this strain to various surfaces by 10-15%. However, the adhesion did not reach levels of the wild-type Candida strains. Characterization of the strains overexpressing *PGA7*, *PGA23* and *PRA1* did not reveal any contribution to adhesion for any of these proteins. Overexpression of all these genes in the wild-type genetic background implied their functions more in contribution to the cell wall structure. While deletion of *pga7* or *pga23* does not affect significantly the cell wall and *pra1* has slight effect on chitin structure, their overexpression leads to thickening of the cell wall with the largest increase for *PGA23* overexpression. The data indicate an involvement of Pga7, Pga23 and Pra1 in cell wall glucan structure. Whether this is their primary role is not clear. In case of Hwp1, previously postulated cell wall functions (Plaine*, et al.*, 2008) were confirmed in this study (relation to chitin in the cell wall), although this protein acts primary as an adhesin.

Interaction of strains CAI4 and HLC69 overexpressing *PGA7*, *PGA23*, *PRA1* and *HWP1* with murine bone marrow derived macrophages and myeloid dendritic cells did not reveal any enhanced immunogenicity for the encoded proteins during host-pathogen interaction. Their overexpression in a wild-type background led to general decrease in ROS release by BMDMs. This was probably caused by general cell wall changes altering the immunogenicity of the strains after cell wall protein overexpression and may not be connected to any specific protein sequence. Also transcriptional response of mDCs did not reveal any specific changes among the CAI4 and HLC69 strains overexpressing *PGA7*, *PGA23*, *PRA1* and *HWP1*. Thus it seems that none of the encoded proteins has a prominent direct involvement in interaction with macrophages or dendritic cells *in vitro*.

Overexpression however caused additional alterations in the strains in terms polysaccharide cell wall composition. In case of CAI4 derived strains overexpressing *PGA7*, *PGA23*, *PRA1* and *HWP1* a general decrease in mannan in the cell wall at the expense of glucans was observed. This result was surprising, since due to overexpression of putative cell wall proteins an increase in mannans would have been expected due to their glycosylation pattern. However, this phenomenon can originate in an overload of the secretory pathway and incomplete glycosylation again due to use of the strong actin promoter. Additionally, alteration in the invasion behavior of all these strains in an *in vitro* invasion model was observed. While wild-

type and *URA3* complemented CAI4 strain invaded into *in vitro* invasion models as hyphae, the strains overexpressing *PGA7*, *PGA23*, *PRA1* and *HWP1* invaded less efficiently as mix of hyphae, pseudohyphae and yeast cells. On the other side overexpression of *HWP1* led to hyper-invasion into the agar medium and the other strains did not differ from the wild-type. In fact the conditions for the agar invasion were different, 30℃ and medium not inducing hyphae was used. Moreover, the hyphae induction *in vitro* in DMEM and other media inducing filamentation did not reveal any differences in the efficiency of the germ tube induction or the hyphal length. In case of the *in vitro* invasion assays the use of a strong *pACT1* promoter, causing altered structure of the cell wall by the unusually high amount of the overexpressed proteins, may have led to the difficulties in crossing of the Caco-2 monolayer barrier and invading the collagen matrix. This seemed more like a general effect of cell-wall protein overexpression than a defined action of the proteins overexpressed. The presence of the Caco-2 monolayer seems to be also critical, since there was no difference in invasion of all the CAI4 derived strains into the Caco-2-cell-free collagen matrix under identical condition. Oral and vaginal epithelia were reported to have certain cell-contact specific antifungal effect (Yano*, et al.*, 2005), which could actually explain these differences. The cell wall defects thus could lead to higher sensitivity of these strains to the antifungal effects and thereby reduce the *in vitro* model invasion.

When genes *PGA7*, *PGA23*, *PRA1* and *HWP1* were overexpressed in HLC69 strain, none of these genes was capable to induce invasion in this non-invasive strain and cross the Caco-2 barrier. Thus the individual genes tested are not sufficient to induce invasion in HCL69. Probably the only way to induce invasion in this non-invasive strain would be induction of filamentation, which as a result is developing mechanical forces aiding penetration of the Caco-2 cell monolayer and invading the collagen matrix.

Additionally, some flocculation effects for *HWP1* overexpression were revealed. In general, strains overexpressing *HWP1* (both CAI4 and HLC69) showed during cultivation in liquid media increased flocculation. However, the effect of Hwp1 on flocculation was already indicated in the literature. Not only that *hwp1* strain makes thinner biofilm with a lot of planktonic cells and the overexpression of *HWP1* in *bcr1* mutant strain improves its biofilm formation (Nobile*, et al.*, 2006b), but it also promotes adherence of *S. cerevisiae* strain heterologously expressing Hwp1 to the

cells of *C. albicans* wild-type (Nobile*, et al.*, 2008a). Thus these findings support the function of Hwp1 in cell-to-cell adhesion. Such an effect was not observed for any other of the overexpressed genes. Moreover, overexpression of *HWP1* reduced the amount of chitin in the cell wall followed by increased resistance of the strains to Calcofluor White. The deletion of *hwp1* increases chitin sensitivity (Plaine*, et al.*, 2008), thus it is not surprising that the overexpression has an opposite effect.

Common transcriptional regulation together with heme-iron utilization involved *RBT5* or *PGA10* in response to oxidative stress, azoles, ciclopirox, Rim101 dependence or during protoplast regeneration (Barelle*, et al.*, 2008; Bensen*, et al.*, 2004; Castillo*, et al.*, 2006; Hromatka*, et al.*, 2005; Lee*, et al.*, 2005; Liu*, et al.*, 2005; Sigle*, et al.*, 2005), as well as genome localization of *PGA7* ORF between predicted ferric reductase *FRP1* and *RBT5*, puts forward a question of its role in iron metabolism. The importance of Pga7 for iron metabolism could not be confirmed, since the deletion or overexpressing strains did not show altered sensitivity to the iron-chelator ferrozine, when compared to their respective backgrounds. However, deletion and overexpression genotype phenotypically masked by presence of other genes involved in iron utilization should be still taken into account.

This study did not reveal a defined function for cell wall biosynthesis of Pga23 either. Therefore the function of this protein, besides a general function in cell wall structure, remains unclear. Its overexpression had the most prominent effect on the cell wall of all the studied genes. In strain *efg1*/*cph1* it led to significantly reduced adhesion, compared to the other genes involved in the study. Moreover, its overexpression in both strain backgrounds had the strongest effect on the cell growth, indicating some toxic effects caused by its overproduction. Its overexpression led to the most significant thickening of the cell wall in case of wild-type genetic background, not followed by increase of glucans related to the cell biomass. This significant change in the cell wall structure directly correlates to the observed growth defects.

Regarding *PRA1*, involvement in adhesive or invasive behavior was shown neither. Contradictory to the originally described function of Pra1 (Casanova*, et al.*, 1992) no effect of Pra1 for binding to fibrinogen could be confirmed in *in vivo* adhesion assays, where fibrinogen coated polystyrene plates were used. This is in agreement with an additional study published during process of this work, (Marcil*, et al.*, 2008). In general, an interaction of Pra1 with the immune system was reported

recently (Luo*, et al.*, 2009; Marcil*, et al.*, 2008). The specificity assigned to this interaction for an opportunistic pathogen, however, does not correspond to the existence of homologues in many other fungal species, including those that are not involved in interaction with mammalian hosts. None of its homologues was functionally characterized so far. The absence of GPI anchor also seems to be *C. albicans* specific, since many of its homologues in other organisms contain it, including its *S. cerevisiae* homologue *ZPS1*. However, involvement in interaction with the immune system could have evolved later with the loss of the GPI anchor. This fact also supports its upregulation in contact with macrophages and presence of serum in the cultivation medium (Marcil*, et al.*, 2008) as well as cultivation in blood samples (Fradin*, et al.*, 2003). On the other side, *PRA1* seems to have a structural function in the cell wall, indicated by its expression which is linked to presence of the cell-wall glycosidase Phr1 (Choi*, et al.*, 2003). This is further supported by alteration of the chitin structure reported for the *pra1* deletion strain (Sentandreu*, et al.*, 1998) and verified in this study.

Specialization of *C. albicans* as a pathogen colonizing various environments was postulated to be connected with the unusual amount of GPI-anchored proteins. The number of putative GPI proteins identified in *C. albicans* (115) is almost twice as high as identified in non-pathogenic *S. cerevisiae* (58). 66% of them in *C. albicans* are still with unknown function (Richard and Plaine, 2007). Besides a possible role in masking of the immunogenic β-glucan in the cell wall, one of the explanations for this high numbers could be involvement of many of the GPI anchored proteins in maintenance of β-glucan structure, which is different in *C. albicans* from *S. cerevisiae* (Mio*, et al.*, 1997). Supporting for this could also be the fact, that the overexpression of *PGA7*, *PGA23* and *PRA1* in *efg1*/*cph1* strain, containing lower amounts of glucans in the cell wall, did not affect significantly its cell wall thickness. However the cell wall of this strain seems to be aberrant and the changes in its structure do not replace the wild-type. When overexpressed in CAI4 background strain, the *PGA7*, *PGA23* and *PRA1* also loosened the cell wall structure, indicated by higher Zymolyase sensitivity, and the increased thickness which was not followed by the increase of glucans related to the cell wall biomass. The results shown here indicate that these genes are induced during initiation of germ tube formation and participate in the changes of the cell wall structure, rather than in the interaction with the host. Due to reduced invasion of the CAI4 strain overexpressing *PGA7*, *PGA23* and *PRA1* as well as

HWP1, none of the gene products seem to participate actively in invasion of the *in vitro* models.

7.3 *AUF* Genes

AUF8 was also identified as upregulated during infection of the *in vitro* infection models and during adhesion to polystyrene surface, as observed for the genes *PGA7*, *PGA23* and *PRA1* (Sohn*, et al.*, 2006). It encodes for a protein predicted to carry four transmembrane segments and being localized to the plasma membrane. In the *C. albicans* genome six additional homologous genes were identified, describing a novel gene family. None of the genes were previously characterized on a functional level.

The *AUF8* gene is induced under hypha inducing conditions in contact with three surfaces, polystyrene as well as Caco-2 and A-431 cell lines. Its expression is increasing during two hours of interaction, with the strongest evidence on the Caco-2 cell line. During later time points it starts to decrease again. During cultivation in suspension culture under identical conditions, *AUF8* expression does not change significantly. Thus this gene is upregulated clearly as a response to contact of *C. albicans* with a surface and is not induced primarily in response to filamentation.

In the *C. albicans* genome six homologous genes are present: *AUF1*, *AUF2*, *AUF3*, *AUF4*, *AUF5* and *AUF6*. While *AUF1* is located on chromosome 4, a 10 kb cluster of genes involving *AUF2-8* is localized on chromosome 5. The identity among the encoded proteins is rather low, between 17 and 54%, and provides high sequence variability. Additional 5 ORFs encoding for slightly homologous proteins were identified in *C. albicans* genome (5 - 20% identity to any of the Auf proteins), but none of them was characterized either. Except for the closely related fungus *Candida dubliniensis*, where 4 homologous genes in one gene cluster are present, no significantly homologous proteins were identified in other fungal species to any of the Auf proteins. Only *AUF2* has a weak homologue YNR061c in *S. cerevisiae*, a gene without known function. By prediction the Auf proteins contain 4 membrane spanning regions that are actually the parts with the highest protein homology among the Auf members. However, no highly conserved regions among the predicted proteins, which could suggest its biological importance, are present. In various studies, transcriptional activity of several of the genes has been observed. *AUF2* was found to be regulated in pH dependent manner and induced in alkalic pH (Bensen*, et al.*,

2004), upregulated in opaque stage cells compared to the white stage (Lan*, et al.*, 2002) and together with *AUF5* they are induced during adhesion process on polystyrene (early phase biofilm) and even in planktonic cells (Murillo*, et al.*, 2005). On the other side, *AUF8* was downregulated in this study under both of these conditions, opposite to *AUF2* and *AUF5*, which is contradictory to our data. However, the differences could have been caused by differential experimental settings like cultivation media, growth phase of cells and the experimental controls. *AUF2* and *AUF4* were also identified to be upregulated in *hgt4* mutant strain lacking a glucose sensor (Brown*, et al.*, 2006). Most interestingly, *AUF2* was found in *in vivo* studies to be upregulated in rabbit kidney lesions during infection by *C. albicans* (Walker*, et al.*, 2009).

Auf8 localization to the plasma membrane, predicted by *in silico* analysis, was analyzed by tagging of *AUF8* using an integrative *GFP* tagging cassette at its chromosome locus. Neither the *AUF8GFP* construct nor the same construct under regulation of strong *pTDH3* promoter did result in *C. albicans* in fluorescence visible under the microscope. By expression of *AUF8GFP* heterologously in *S. cerevisiae*, its predicted localization to the plasma membrane was confirmed. Although the localization of heterologously expressed proteins in *S. cerevisiae* usually correspond to the localization in its original fungal species, the proper localization in *C. albicans* would need to be verified by additional studies.

Using deletion studies, several deletion mutants were constructed. Besides the fact that none of the studied genes is essential for *C. albicans*, no obvious phenotype involving growth, filamentation or cell wall defects have been observed. Additional adhesion and invasion studies using *in vitro* models did not reveal any changes in adhesion or invasion behavior of the studied deletion strains on a qualitative or quantitative level. Changes in the strain susceptibility to killing or stimulation of ROS response in contact with macrophages were observed neither. However, macrophages are only one component of the complex immune system and effect of *AUF* genes on its other parts still can not be excluded.

The only experiments that revealed differences between wild-type strain SC5314 and *auf1-8* mutant were competition assays employing co-cultivation of both strains in the same flask. In this assay the *auf1-8* strain was displaying higher fitness compared to the wild-type strain in a co-culture that was re-inoculated every 24 hours, thus the strains underwent lag, exponential and post-exponential phase in 24-

hour cycles. When the co-culture was kept in stationary phase, the advantage of *auf1-8* strain in the expense of wild-type lasted only until day 5. After this time point the strain population started to decrease. From these observations it seems that the *auf1-8* strain has some advantage in exponential and early stationary phase, while in later stationary phase it leads to elimination of this strain from the co-culture. Unfortunately, reversion of the whole 10 kb cluster (*AUF2-5*) into the *auf1-8* strain was not possible, in order to restore the effect of *auf1-8* deletion. The reversion of functional alleles *AUF8* or *AUF1* into the *auf1-8* strain did not lead to restoration of the observed phenotype. However, the effect of deletion of all the genes was shown in two *auf1-8* mutants constructed in parallel. Thus additional genome mutations participating in the studied phenotype are improbable; however, this possibility can not be excluded. When additional strains were employed in the assay, no effect of the individual deletion of *auf1* or *auf8* separately was observed. Thus it seems that this phenotype involves more of the *AUF* genes than one. When the optical density of the strain cultures was monitored for 12 days, no differences in maximal OD_{600} or time of OD_{600} decreasing of was observed, not further supporting the data from competition assay with additional evidence from the cultures. However, the differences in the culture can be only slight and may not be revealed from the optical density.

The *AUF8* transcript increases after 36 hours of cultivation in YPD medium after the diauxic shift (switch from glucose to other carbon sources) and remains elevated (about 4 x), while in minimal YNB medium the increase in expression was observed after 72 hours more after the post-diauxic shift (exhaustion of alternative carbon sources and beginning of starvation) and then decreased again. Moreover, *AUF8* expression was found to be decreasing in the presence of acetate in the medium in a dosis dependent manner. However, the biological relevance of these regulations remains unclear like in case of adhesion.

In order to show any function of the *AUF* genes for the infection *in vivo*, indicated by the induction during *in vitro* infection of tissue equivalents and *AUF2* expression in rabbit kidney lesions (Walker*, et al.*, 2009), mouse systemic models would need to be employed for the further characterization of the strains. There is a possibility that the importance of the *AUF* genes can be revealed only in *in vivo* studies, while the *in vitro* studies offer only transcriptional profiles without proper biological relevance.

Gene families in *C. albicans* are often involved in pathogenesis like in case of adhesive *ALS* proteins (at least 9 members) and SAP proteases (10 members), offering different functions under various conditions. These genes, additionally, are highly variable among themselves and also differing in sequence or gene-copy number between *C. albicans* and the closely related *C. dubliniensis* (Moran*, et al.*, 2004). The members of these families are also often transcriptionally active under different conditions. Moreover, such genes often do not have strong homologues in other fungi. *AUF* genes fulfill easily all these criteria. Additionally, the *C. albicans* genome contains a family of 13 *IFF* genes. All were predicted to contain a GPI anchor with an exception of *IFF10* and *IFF11* and encode for putative cell wall genes. Very little is known about this family. Few of the genes were studied, but none of the genes are essential for the cell. All the members share similarity in N-terminal domain. What resembles *AUF* genes is that there is no family of homologues in *S. cerevisiae* and the amount of genes belonging to this family in the closely related *C. dubliniensis* is reduced. There are two possibilities, either *C. dubliniensis* lost several of the genes during evolution or *C. albicans* amplified these genes from a common ancestor. In either case, such differences might be one of the reasons explaining the difference in virulence levels of these two very close species.

8 LITERATURE

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