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Subcellular Localization of Superoxide Dismutase Sod5 in Opportunistic Human Pathogen *Candida albicans*

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Verfasserin / Verfasser:	Kristina Yatsyk
Matrikel-Nummer:	0202687
Studienrichtung /Studienzweig (lt. Studienblatt):	Molekulare Biologie A490
Betreuerin / Betreuer:	aoProf. Dr. Karl Kuchler

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Zusammenfassung

Candida albicans ist ein weit verbreiteter humaner bedingt-pathogener Krankheitserreger und ist oft auf der Haut, im Genitalbereich und im natürlicherweise Verdauungstrakt zu finden. *Candida albicans* kann sich zu einem pathogenen Pilz entwickeln wenn das Immunsystem geschwächt ist, der Hormonhaushalt oder die Schleimhautbarriere nicht intakt sind. Eine Infektion mit *Candida albicans* bei immunsupprimierten Patienten kann zu lebensbedrohlichen systemischen Infektionen führen. Bei der Infektion spielen vor allem die Zellwand und Zellwandbestandteile des Pilzes eine wichtige Rolle. Die Zellwand ist ein komplexes Netzwerk aus Glucan, Chitin, Mannan und vielen Proteinen. Die Zellwand vermittelt die ersten Wechselwirkungen zwischen dem Pathogen und dem Wirt. Pathogene werden im Organismus von den Zellen des angeborenen Immunsystems phagozytiert und getötet oder Zellen des adaptiven Immunsystems, den T-Zellen, präsentiert, was einen wichtigen Mechanismus in der Abwehr gegen Mikroorganismen darstellt. In den Phagozyten werden unter anderem reaktive Sauerstoffmoleküle (ROS) produziert, die Pathogene töten. Da *Candida albicans* für Patienten mit einem geschwächten Immunsystem eine Lebensbedrohung darstellt, ist es sehr wichtig dessen Virulenzfaktoren zu erforschen.

Die Superoxid Dismutase Sod5 ist ein Protein, das den *Candida albicans* Zellen hilft gegen die Makrophagen zu arbeiten, indem es die reaktiven Sauerstoffradikale neutralisieren kann. *Candida albicans* Stämme ohne Sod5 haben im Vergleich zum Wildtyp in der interaktion mit Immunzellen eine niedrigere Überlebensrate. Außerdem konnten bei einer Interaktion zwischen Makrophagen und dem *sod5*Δ/Δ Deletionsstamm erhöhte ROS Werte gemessen werden.

Wir verwendeten zwei verschiedene genetische Methoden um die genaue Lokalisierung des Proteins in der Zelle zu studieren. Einerseits, sollten spezifische Antikörper gegen das Protein produziert werden mit welchen das Protein immunologisch detektiert werden kann. Andererseits, sollte Sod5 mit einem „Green Fluoreszenz Protein“ fusioniert werden um es in der Zelle mikroskopisch sichtbar zu machen. Es ist uns gelungen zu zeigen, dass das Protein an der *Candida albicans* Zelloberfläche lokalisiert ist.

I. Introduction

I. 1. The Candida species as opportunistic human fungal pathogens

Candida spp. belong to the phylum *Ascomycota*, which includes a variety of different pathogenic fungi. The *Candida* genus consists of different pathogenic and non-pathogenic species. There are two types of pathogenic fungi: fungi as a primary pathogens and opportunistic pathogens. Primary pathogens can cause human disease without any known defects in infected individuals. In contrast, changes in the host are required for disease caused by opportunistic pathogens (Haynes, 2001). Opportunistic pathogens are able to respond very rapidly to environmental changes in the host and can become life-threatening, especially in immunocompromised patients, including individuals after treatment with broad-spectrum of antibiotics, after cancer therapy and HIV-positive individuals. Some members of *Candida* genus are important pathogenic species, the most common pathogen being *Candida albicans* (*C. albicans*). More than 17 different species of *Candida* have been identified as agents of infections (Pfaller and Diekema, 2004). However, 95% of all *Candida* bloodstream infections are caused by *C. albicans, C.glabrata, C.parapsilosis* and *C.tropicalis* and the remaining 5% are caused by 12 to 14 other *Candida* species (Pfaller et al., 2004).

Under normal conditions ***C. albicans*** is a harmless human commensal existing in the mouth, mucosal membranes, in gastrointestinal and genitourinary tracts and also on the skin. Nevertheless, *C. albicans* is the most common *Candida* species causing infections. When the immune system is weakened, *C. albicans* is able to invade host tissue and disseminate. This fungus is a major human pathogen that is especially virulent to people with a compromised immune system such as cancer therapy patients, patients after organ transplantations or coinfected with human immunodeficiency virus, cousing mortalities of up to 40%. *C. albicans* species are the fourth leading cause of nosocomial bloodstream infection in the United States (Pfaller and Diekema, 2007).

This fungus is able to escape the host immune surveilence and in case of invasive Candidiasis to penetrate host tissues, to enter the blood stream and to spread to to different organs (Mavor et al., 2005). Those virulence properties turn the otherwise harmless commensal into an aggressive pathogen. *C. albicans* can grow in three morphological distinct forms: yeast, pseudohyphae and hyphae. The hyphal form is suggested to be the invasive, pathogenic form of the fungus (Ruiz-Herrera et al., 2006) because it is able to penetrate through the host tissue and to escape from the

phagosome. Furthermore, *C. albicans* forms biofilms on catheters and other medical equipments. This may also explain why *C. albicans* is more pathogenic than *Candida* species that are less able to form biofilms (such as *C.glabrata, C.tropicalis* and *C.parapsilosis*) (Haynes, 2001). Other known virulence factors in *C. albicans* are secretion of hydrolytic proteinases and lipases for host tissue damaging and phenotypic switching. Hence, for example the expression of *SAP* proteinase genes plays important role in disease mediation (Schaller et al., 2000).

C.glabrata is the second most important opportunistic pathogen of the *Candida* genus. *C.glabrata* is a haploid yeast unable to form true hyphae. It has been shown that *C.glabrata* is often resistant to fluconazole (Hajjeh et al., 2004) and has the ability to rapidly develop resistance to all azoles. For this reason infections with *C.glabrata* are difficult to treat and cause a high mortality in infected immune compromised patients. (Fidel et al., 1999)

C.dubliniensis is identified as an opportunistic human pathogen that is most closely related to *C. albicans.* Like *C. albicans*, *C.dubliniensis* can grow in three different forms and undergo phenotypic switching (Sullivan et al., 2004). Although, *C.dubliniensis* is less pathogenic, it can causes systemic infections. Studies on *C.dubliniensis* show that it is less able to invade into host epithelial tissues and to form hyphae than *C. albicans* (Stokes et al., 2007) and it doesn't develop resistance to azole treatment. These facts may explain why *C.dubliniensis* is less pathogenic than *C. albicans*.

C.krusei causes about 2 to 3% of all *Candida* bloodstream infections (Pfaller and Diekema, 2004). Like *C.glabrata, C.krusei* is more resistant to fluconazole and therefore the mortality rate due to *C.krusei* is high (Panackal et al., 2006; Malani et al., 2005; Safdar et al., 2001). In general, *C.krusei* is less pathogen than *C.albicans*, it is less anherent to epithelial surfaces, has less proteolytic potential. In tissues infested with *C.krusei* the fungus shows less penetration into the tissue, compared to other *Candida* spp (Samaranayake and Samaranayake, 1994)

I. 2. Cell wall and GPI-anchored proteins

The cell wall plays a very important role in fungal pathogenicity, because it is the structure that first comes into contact with the host and is responsible for the cross-talk between host and pathogen, also mediating adherence to host cells. Therefore, an understanding of the cell wall construction is necessary to understand how it is recognized by the immune system. The cell wall provides a protection to *Candida* species against environmental stress like chemicals and biological aggression. On the other side, a number of specific proteins responsible for virulence-associated processes are located in the cell wall, which also plays important roles in the protection against the host immune defence. Moreover, cell wall proteins in *C. albicans* are also required for virulence.

The *C. albicans* cell wall consists of four to eight different layers depending on the methods used for the analyses (Poulain et al., 1978) (Fig.I.1). About 80 to 90% of the cell wall of *C. albicans* is composed of carbohydrates. The major polysaccharides of the cell wall are branched polymers of glucose consisting of a network of β-1,3 and β-1,6 linked β-glucans (50–60%), unbranched polymers of β-1,4 linked *N*-acetyl-D-glucosamine (GlcNAc) containing chitin (0.6–3%) and polymers of mannose (mannan) which are covalently associated with proteins (mannoproteins) (about 40%) (Chaffin et al., 1998; Kapteyn et al., 1999; Kapteyn et al., 2000). The skeletal components of the cell wall are β-glucans and chitin. The mannoproteins are the main material of the cell wall matrix. In addition, cell walls contain proteins (6- 25%) and minor amounts of lipids (1 - 7%). A very important characteristic of *C. albicans* is the presence of β-1,2-mannans, which are absent in *S.cerevisiae*. This difference is used by macrophages to distinguish between these two species (Fradin, 2000)

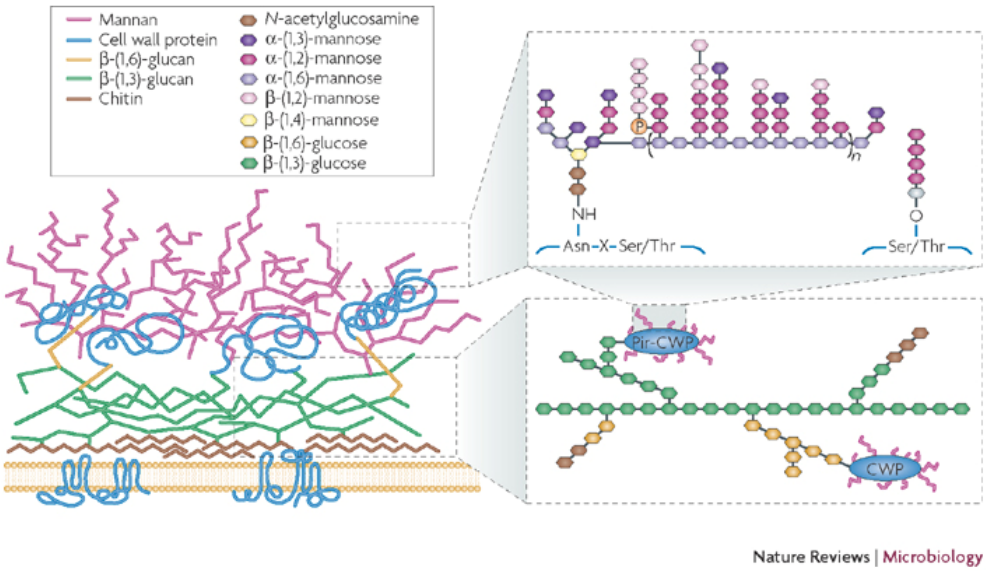


Fig.I.1. The structure of *C. albicans* cell wall. The major components of the cell wall are β-(1,3)-glucan and chitin (poly-β-(1,4)-N-acetylglucosamine) and these are located towards the inside of the cell wall. The outer layer is enriched with cell wall proteins (CWP) that are attached to this skeleton mainly via glycosylphosphatidylinositol remnants to β-(1,6)-glucan or via alkali-sensitive linkages to β-(1,3)-glucan. (Netea et al., 2008)

Cell wall proteins can be separated in two classes. Class I proteins are not covalently linked to the cell wall and can be extracted with detergents. Class II proteins can be extracted only after disruption of polysaccharides structures or by breaking the linkages between proteins and polysaccharides. Class III proteins are true cell wall proteins; they include glycosylphosphatidylinositol (GPI)-anchored proteins bound to β-(1,6) glucans in the cell wall (de Nobel and Lipke, 1994), proteins with internal repeats (Pir), which are attached via alkaline-soluble linkages (Castillo et al., 2003), as well as reducing agents-extractable cell wall proteins (RAE-CWPs) (Moukadiri and Zueco, 2001)

The genome of *C. albicans* encodes more than 100 predicted GPI-anchored proteins (de Groot et al., 2004). GPI-anchored proteins are the most complex post transcriptionaly modified proteins. About 30% of cell wall proteins of *C. albicans* are GPI-anchored proteins (Kapteyn et al., 2000). Furthermore, 88% of β-glucanase-extractable proteins in the cell walls of *C. albicans* are GPI-anchored (Kapteyn *et al*., 2000), GPI anchors are complex glycolipids that covalently anchor cell surface proteins to the plasma membranes or to the cell wall of eukaryotic cells through a highly branched β-(1,6)-glucan linker (Tiede et al., 1999). GPI-anchored proteins are highly glucosylated with mannose-containing polysaccharides. The carbohydratres can reach up to 90% of the molecular mass of the GPI-anchored proteins (Netea et al., 2008). Common features of the precursors of GPI-anchored proteins include an N-terminal hydrophobic signal sequence that is required for trafficking the protein through secretion pathway, and a C-terminal

hydrophobic signal sequence that is required for the attachment of the GPI anchor (Caro et al., 1997; Tiede et al., 1999).

The biosynthesis of GPI anchors occurs in two major steps: preassembly of the donor GPI in the ER membrane and attachment of the GPI to the newly synthesized peptide after the cleavage from the signal sequence. This complex posttranscriptional modification occurs on the C-terminus of the protein. The pathway of GPI assembly and attachment is highly conserved, as is the structure of GPI- anchored proteins. They consist of the N-terminal signal sequence for localization to endoplasmatic reticulum, a C-terminal domain for attachment to the ER membrane and the ω site. The ω site, a cleavage site of the protein for the future ligation to the GPI anchor, is localized 9 to10 amino acids before the C-terminal end. There is a linker region on position ω-1 to ω-11 and a spacer region at position ω+3 to ω+9. These regions are flexible and consist of uncharged amino acids (Richard and Plaine, 2007). In yeast, the amino acids on the C-terminus upstream from the site of GPI anchor addition, functions as a signal sequence for attachment of the protein to the plasma membrane or to the cell wall (Frieman and Cormack, 2003). The protein is connected to the GPI-anchor via an amide linkage from the phosphoethanol-amine group. The GPI anchor consists of a lipid group, myoinositol group, an N-acetylglycosamine group and three mannose residues in mammalian cells. Fungal GPI anchors have one additional mannose group attached to the third mannose group.

The biosyntesis of a GPI-anchor (Fig.I.2.) starts with the generation of GlcNAc-PI on the cytoplasmic side of ER. This step is followed by de-N-acetylation to GlcN-PI that occurs on the cytoplasmic side of the ER membrane. The next reactions occur in the lumen of the ER; therefore, the GlcN-PI must be flipped across the ER membrane. Once inside the lumen, the GlcN-PI is inositol-acylated, inositol-mannosylated and modified by phosphoethanolamine. The C-terminal signal sequence from the newly synthesised protein localized in the ER is cleaved by GPI-transamidase and the GPI precursor is attached (Udenfriend and Kodukula, 1995).

GPI-anchored proteins are transported via a protein secretion pathway to the plasma membrane; some of them become attached to cell wall polysaccharides. As already mentioned above, there are C-terminal signals in the sequence of the protein, which target the proteins either to the plasma membrane or to the cell wall (Mao et al., 2008). If the protein has two basic amino acids in the ω-minus side, this protein will be localized in the plasma membrane, if those amino acids are replaced with hydrophobic amino acids the protein will be inserted into the cell wall. In fungi, the proteins that have to

be transported to the cell wall are cleaved within the GPI anchor after the glucosamine. Afterwards, proteins are linked to β-1,6-glucans, transported to the cell wall and cross-linked to β-1,3-glucans (Pittet and Conzelmann, 2007)

There are also some additional modifications in GPI-proteins which exit the ER to be transported to the plasma membrane or to the cell wall. The inositolacyl chain is removed and replaced though longer lipid chains, that allows the association with membranes. These reactions take place in the ER.

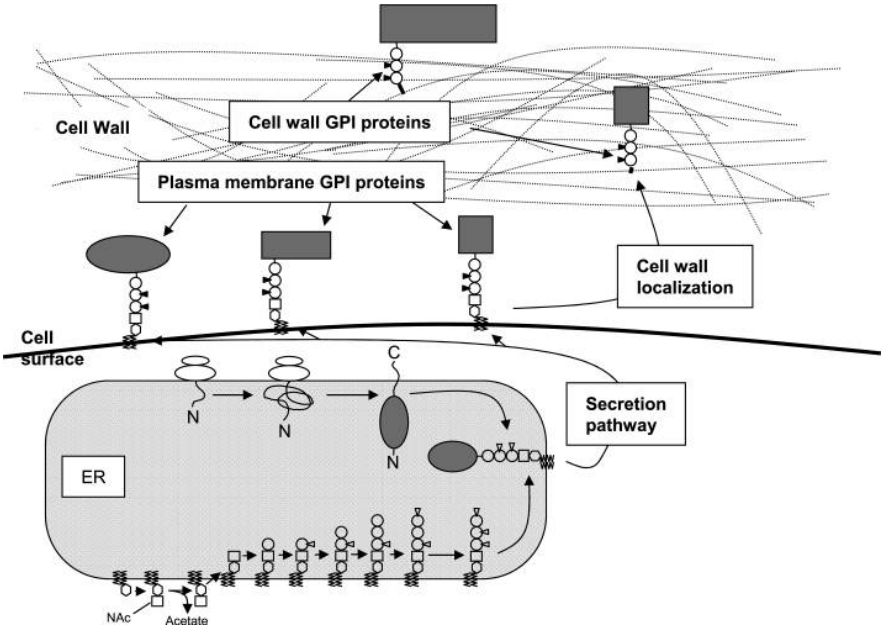


Fig.I.2. The different steps of the synthesis and transport of a GPI-anchored protein in yeasts or fungi.
The first steps of GPI anchor biosynthesis occur in the ER. The attachment of the protein to the GPI anchor takes place in the ER lumen. Then the GPI-anchor proteins follow the secretory pathway to be presented at the cell surface. Some fungi have an additional step in which the GPI anchor is cleaved after the glucosamine and the protein with the remnant part of the anchor is directed to the cell wall and covalently linked to ß-1,6-glucan (Richard and Plaine, 2007)

Many GPI-anchored proteins of *C. albicans* have been implicated in virulence, as their localization at the cell surface is involved in the interaction with the host cells. GPI-anchor proteins are also important for processes like adhesion to mammalian cells, phenotypic switching, biofilm formation, CW biosynthesis, interaction with the host immune system and stress tolerance (Mao et al., 2008).

The number of predicted GPI-proteins in *C. albicans* is 115 (Richard and Plaine, 2007). Most of them have unknown functions (corresponds to 76 genes or 66% of the GPI genes). Many of them are involved in the cell wall biosynthesis or remodelling (Phr1, Phr2, Utr2) (15 genes or 13% of the GPI genes) and adherence or host-

interactions (Hwp1, Als proteins) (13 genes or 11%). Some have enzymatic functions (Sap9, Sap10, Gpi7) and a role in oxidative stress (Superoxide dismutase) (11genes or 9,5% of the GPI gens) (Richard and Plaine, 2007).

I. 3. Immune response to fungal pathogens

The mammalian immune system consists of two branches: the innate and the adaptive immunity. The innate immunity is the major component in response to invading pathogens, because it provides the first response in the recognition as well as elimination of pathogens. The components of innate immune system include physical and chemical barriers, such as epithelia and antimicrobial substances, cells responsible for phagocytosis so called phagocytes or phagocytic cells (neutrophils, macrophages, granulocytes), mast cells, natural killer (NK) cells, members of the complement system and signalling proteins known as cytokines that regulate and coordinate the activities of immune cells. The functions of the innate immune system are recruiting of immune cells to the side of infection, the production of cytokines, identification and removal of foreign substances and activation of the complement system and instructions for the adaptive immune system. The innate immune system can in many instances eliminate or kill invading pathogens and it is also responsible for the future stimulation of the adaptive immune system which has a high specificity for distinct molecules and the ability to memorize and respond more powerfully to repetitive infections (Janeway and Medzhitov, 2002)

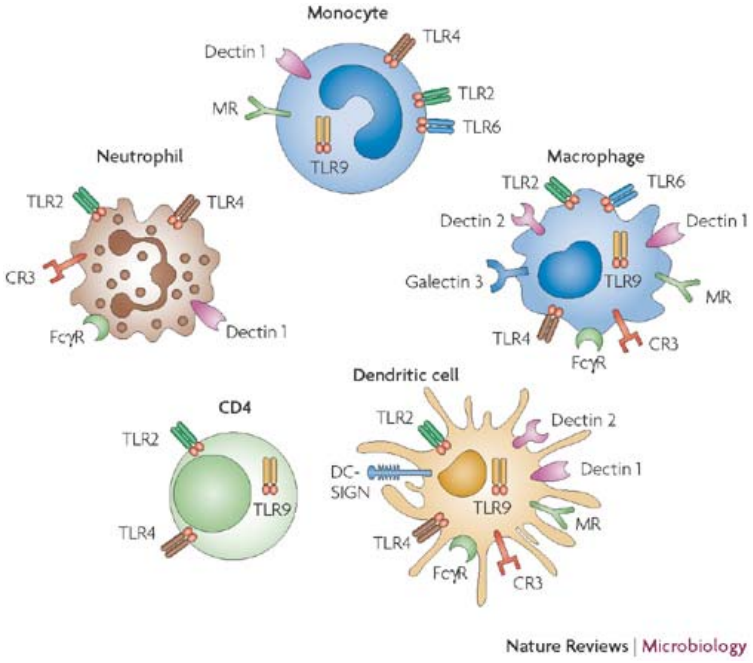


Fig.I.3. The main populations involved in the recognition of *C. albicans* during the innate immune response are the monocytes, neutrophils and macrophages. Dendritic cells are crucial for processing of, and antigen presentation to, T cells, and thus to activate the more specific adaptive immunity (Netea et al., 2008)

The innate immune response is mediated by professional phagocytes such as macrophages, dendritic cells and neutrophils (Mansour and Levitz, 2002) (see Fig.I.3). Phagocytic cells are able to recognize structures characteristic for microbial pathogens

and not present in mammals, so called pathogen-associated molecular patterns (PAMPs). These cells are able to recognize PAMPs via a small number of germline-encoded pattern-recognition receptors (PRRs) on their surface. These innate immune receptors are evolutionary highly conserved (Akira and Takeda, 2004b), non-clonal receptors, which are constitutively expresses in the host on innate immune system cells like dendritic cells and macrophages (Akira et al., 2006). Different classes of pathogens express different PAMPs that are recognized by different PRRs (Akira et al., 2006).

Toll-like receptors (TLRs) are the main class of PRRs, with as yet 12 members of the TLR family identified in mammals. Toll-like receptors are known to activate immune cells in response to different types of microbial components. TLRs are type I integral membrane glycoproteins characterized by the extracellular domains containing varying numbers of leucine-rich-repeats (LRR) and characteristic cysteine-rich motifs at their extracellular regions and a cytoplasmic signaling domain homologous to that in interleukin 1 receptor (TIR domain), which is essential for signalling (Bowie and O'Neill, 2000). The expression of TLRs is modulated rapidly in response to pathogens, where they induce expression of inflammatory proteins. The genes that are expressed through TLR signalling are cytokines and proteins important for microbial killing mechanisms, because the signaling pathways activated through TIR domain lead to activation of proinflammatory transcriptional factors, like NFκB (Bowie and O'Neill, 2000). TLR2 and TLR4 are the best characterized TLRs, and are described as important mediators in innate immunity. Four TLRs have been shown to be important for the immune defence against C. albicans (TLR2, TLR4, TLR6 and TLR9) (Netea et al 2002 Miyazato, 2009)

After the recognition of pathogens, macrophages become activated, resulting in pathogen phagocytosis, and secretion of cytokines leading to inflammation. TLRs use the same signalling pathway that is used for proinflammatory cytokine interleukin-1 receptor (IL-1R) signalling (Akira and Takeda, 2004a). Activation of TLRs with their ligands recruits adaptor proteins containing TIR domain, such as MyD88, this leads to activation of a signalling pathway downstream and production of inflammatory cytokines.

Nod-like receptors (NLRs) are localized in the cytosol. They are composed of an N-terminus effector caspase-recruitment domain (CARD), which is thought to regulate homotypic and heterotypic binding, a nucleotide-binding domain (NBD) and leucine-rich repeats (LRR) at the C-terminus. Ligand stimulation leads to recruitment of a receptor interacting protein 2 (RIP2), which results in the activation of the inhibitor of NF-κB (IκB) kinase complex (Ip et al., 2009).

Scavenger receptors are mainly expressed by myeloid cells and play an important role in the uptake of apoptotic cells and modified host molecules. They are also involved in lipid metabolism and bind low-density lipoproteins (Peiser et al., 2002).

C-type lectin receptors are responsible for binding to the polysaccharides. C-type lectin receptors are also found on the surface of professional antigen-presenting cells like dendritic cells and Langerhans cells where they do not only serve as antigen receptors but also regulate the migration of dendritic cells and their interaction with lymphocytes. (Figdor et al., 2002).

Dectin-1 is a C-type lectin receptor, containing an extracellular C-type lectin domain, a short stalk region, a single transmembrane domain and a short intracellular tail (Ariizumi et al., 2000). This receptor is mainly expressed on macrophages, DCs and neutrophils and is involved in a number of immune responses such as phagocytosis (Gantner et al., 2005; Hernanz-Falcon et al., 2009), neutrophil degranulation and production of cytokines. The role in ROS signalling remains unclear, because the macrophages isolated from dectin$^{-/-}$ mice stimulated with *C.albicans* show no alterations in ROS production (Saijo et al., 2007). Dectin-1 plays an important role in fungal *β*-glucan recognition; receptor recognizes β-1,3 glucans via C-type lectin-like domain (CTLD) (Tsoni and Brown, 2008). The other CLR known to recognize fungi are dectin-2, macrophage mannose receptor (MR) galectin-3, dendritic cell-specific ICAM3-grabbing nonintegrin (DC-SIGN) and mincle (Taylor et al., 2005; Taylor et al., 2007).

The characteristic feature about Dectin-2 is its specificity for high mannose structures and the ability to recognize hyphal forms of C.albicans and of other fungi (McGreal et al., 2006). The recognition of hyphal forms results activation of NFκB, and up-regulation of TNFα and IL-1ra (Sato et al., 2006).

Mannose Receptor (MR) is also a C-type lectin receptor, able to recognize oligosaccahides, fructose and mannose. Receptor mediates phagocytosis of phatogens by macrophages. Elected recognition of highly branched *N*-linked mannosyl chains by MR has been shown (Netea et al., 2006).

I. 4. Fungal recognition and the ability to escape

Both the innate and the adaptive immune systems play an important role in immune response to fungal infections. Several of fungal PAMPs, like β-glucan, located on the surface of the cell are recognized by mammalian TLRs (Netea et al., 2004b) and C-type lectin receptors (especially Dectin-1) (Goodridge et al., 2009) (see also Chapter I.3).

C. albicans appears in two different morphological forms, yeast and hyphae. In the yeast form the cells are round and are separated; in hyphal growth cells bild long filaments, where the single cells are separated by septae (Berman and Sudbery, 2002). Both forms interact with the host in a different way, thereby initiating diverse immune responses. The ability of *C. albicans* to avoid an immune response and live as a commensal may depend on its location in the host and on its morphological state. Dendritic cells are able to discriminate between yeast and hyphal forms of *C. albicans,* and the uptake of opsonized cells occurs through different receptors (d'Ostiani et al., 2000). *C. albicans* mutants lacking the ability to switch from the yeast to hyphae form are avirulent (Lo et al., 1997).

One of the characteristics, allowing *C. albicans* to survive within the host is a specific difference in the cell wall composition in the yeast and hyphal forms. β- glucan is the main component in the cell wall that is recognized by immune cells. The binding of β-glucan to the Dectin-1 receptor on the macrophages leads to phagocytosis of the pathogen (Brown et al., 2002). However, only the yeast form of *C. albicans* triggers Dectin-1 immune response whereas filaments do not (Underhill et al., 2005). This fact can be explained by the down-modulation of β-1,6 glucan expression during yeast-to-hyphae transition. In hyphal form, the filamentous daughter cells also do not separate from each other and the β-glucan layer remains concealed and covered with mannan. Therefore β -glucan masking is another strategy of *C. albicans* to escape the host immune system and explains why filaments are more virulent (Chai et al., 2009).

The loss of TLR-4 mediated proinflammatory signals is the mechanism of *Aspergillus* to escape host immune defence (Netea et al., 2003). It has been also demonstrated that *C. albicans* is using the same mechanism as *Aspergillus* species to suppress the host immune system. TLR4-mediated proinflammatory signals are lost during transition of *Candida* into hyphae (van der Graaf et al., 2005). TLR2 and TLR4 both recognize *C. albicans*, but only TLR2 is able to recognize hyphal forms. The recognition of *C. albicans* by TLR2 induces an anti-inflammatory cytokine production. Phenotypic switching from yeast to hyphae leads to release of IL-10 and induces immune suppression (Netea et al., 2004a; Netea et al., 2004b). *C. albicans* recognition by TLR2 inducing anti-

inflammatory response helps *C. albicans* to escape host immune defence. Hyphal forms are less stimulatory for cytokine production in human monocytes than yeast forms (Torosantucci et al., 2000).

As already mentioned, about 50-60% of the *C. albicans* cell wall are β-glucans. β-glucans are localized below the mannose layer but is also presented on the surface via bud scars (Gantner et al., 2005). They are recognized by following receptors: CR3 and dectin1. Carbohydrate recognition by CR3 is mediated by a lectin domain and is specific for hyphal and yeast forms of *C. albicans*. Dectin-1 is a major receptor on macrophages for the phagocytosis of different fungi. It has been shown, that Dectin-1 is able to interact with other receptors to induce a strong inflammatory response (Gantner et al., 2003), but Dectin-1 is not recognizing the filamentous forms of *C. albicans*.

Chitin is also recognized as a fungal PAMP. The recognition leads to a immune response and release of IL-4 and IL-13. Although, the receptors for chitin recognition have not been described so far. According to their localisation, mannoproteins and mannans on the outer side of the cell wall may be the first components that come into contact with immune cells. There is a number of receptors on the immune cells involved in recognition of mannans and mannoproteins of *C. albicans*. The main receptor on macrophages assigned for mannan recognition is the C-type-lectin mannose receptor, recognizing branched N-bound mannans in *C. albicans*; the recognition of shorter O-bound mannan occurs by TLR4 (Netea et al., 2006). Mannose and mannoproteins can be also recognized by Dectin2, which is a receptor on the surface of myeloid cells and inflammatory monocytes and has the ability to recognize hyphal forms of C.albicans additionally to yeast forms (McGreal et al., 2006). However, glycolsylation is an important factor for the recognition and phagocytosis of *C.albicans* by macrophages. The phagocytosis by the mutans deficient in phasphomannan biosynthesis is significantly reduced (McKenzie et al.).

I. 5. Phagocytosis

A phagocyte has many specific receptors on its surface that bind peptides, proteins and microbial cells. They include receptors for opsonin, mannose and scavenger receptors, toll-like receptors and different G-protein coupled receptors. All those receptors have the task either to recognize pathogens or to promote phagocytosis and kill ingested microbes. It has been shown, that dectin-1 is directly involved in the uptake of fungal pathogen (Ezekowitz, 1990; Romani et al., 2004), while mannose receptors are not professional phagocytic receptors, TLRs may be involved in detecting, but not in the uptake of pathogens (Blander and Medzhitov, 2006). Receptors for phagocytosis (FcR) have a tyrosine-based activation motive (ITAM). Phosphorylation on tyrosine residues occurs on the ITAM consensus sequences and leads to recruitment and phophorylation by Syk kinase (Greenberg et al., 1996). Syk kinase plays an important role in the phagocytic signal transmission (Zhang et al., 1996; Cox et al., 1996).

After phagocytosis, killing of *C. albicans* occurs through oxidative and non-oxidative mechanisms. The oxidative mechanism is also known as the oxidative or respiratory burst and is throught to require dectin-1. Activated macrophages and neutrophils convert molecular oxygen to reactive oxygen species (ROS), which is used to destroy microbes. ROS production occurs after assembly and activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase on the cell surface. NADPH oxidase is an enzyme that catalyses the production of superoxide (O_2^-) from oxygen and NADPH: $NADPH + 2O_2 \rightarrow NADP^+ + H^+ + 2O_2^-$ (Babior, 2004). Other oxidizing agent produced by NADPH oxidase is hydrogen peroxide H_2O_2 generated by dismutation of superoxide. In neutrophils, H_2O_2 is used by the enzyme myeloperoxidase to catalyse hypohalous acids from halide ions (Cl^-, Br^-,I^-). Hypohalous acids are highly toxic for bacteria.

In addition to ROS, macrophages produce reactive nitrogen intermediats (RNS). During the conversion of arginine to citrulline, which is catalyzed by iNOS, nitric oxide gas is released. Further, peroxynitrite radicals are generated through the interaction between ROS and NOS, which kill pathogens inside the phagosome (Fig.I.4.) These reactions are also known as respiratory burst (Schrenzel et al., 1998). However, hydroxy and superoxide radicals are also very reactive to the host cell, since they react with cellular components, resulting in the oxidation of proteins and nucleic acids, that may lead to inactivation of enzymes, mutations in DNA and proteins and cell death. Nevertheless, the respiratory burst is an important antifungal defence mechanism.

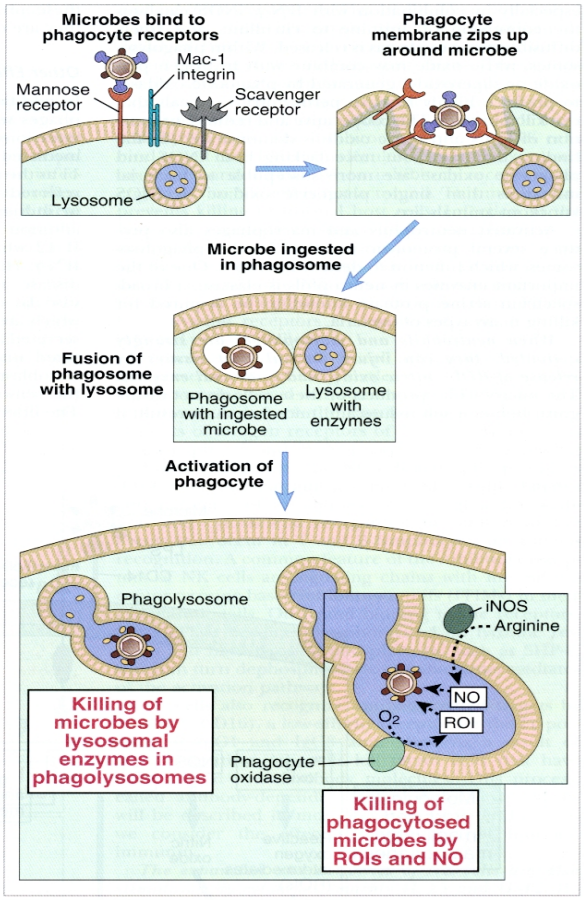


Fig.I.4. Phagocytosis and destruction of microbes. Pathogens are ingested by different membrane receptors on phagocytes. The pathogens are then taken up and fused with lysosomes. In this phagolysosome the microbes are killed by ROS and iNOS (Abul K. Abbas, 2005).

I. 6. Superoxide dismutases in *C. albicans*

Free radicals that are produced in the phagosome during an immune response are usually also produced in small amounts during redox reactions in somatic cells. Since free radicals are very reactive and damage to DNA and proteins, cells have developed several mechanisms to minimize the amount of free radicals. There are enzymes called superoxide dismutase, catalase and glutathione peroxidase responsible for neutralizing these free radicals.

Once inside the phagosome, *C. albicans* has developed strategies to use enzymes for ROS detoxification. This provides *C. albicans* a possibility to escape killing the host immune system.

The Cat1 catalase in *C. albicans* is implicated in counteracting the respiratory burst and protects cells from killing (Chauhan et al., 2006). The important role of another enzyme, superoxide dismutase Sod5, in detoxifying ROS and protection of *C. albicans* has also been shown (Frohner et al., 2009). Mutants lacking Sod5 have defects in virulence (Martchenko et al., 2004), and decreased viability in coculture with macrophages compared when to the wild type (Frohner et al., 2009). SODs convert superoxide radicals into hydrogen peroxide, which is less damaging to the cell. All SODs are depending on metal co-factors such as Cu/Zn, Fe or Mn. The classifications of SOD enzymes into families is according to co-factors. There are two types of superoxide dismutases in eukaryotic cells: cytosolic copper- and zinc- dependent and mitochondrial manganese-dependent SODs (Fridovich, 1995).

SODs protect the cells from the superoxide toxicity and are very important antioxidants in the cell, as shown by knock-out approaches in the mice. Mice with a knock out have a reduced life span and several pathologies (Elchuri et al., 2005). Mice lacking Sod2 die several days after birth from the massive oxidative stress (Lebovitz et al., 1996; Li et al., 1995). SOD knock-outs have been performed in other model organisms as well. In prokaryoties, the loss of SODs leads to attenuated virulence.

Six different *SOD* genes have been identified in *C.albicans. C. albicans* Sod2 and Sod3 need manganese as a cofactor. Sod2 is localized in the mitochondria, Sod3 is a cytoplasmic enzyme. Sod1, Sod4, Sod5 and Sod6 are CuZn dependent enzymes. Sod4, Sod5 and Sod6 are predicted to be localized in the cell wall and to be GPI-anchored proteins (Fradin et al., 2005; Richard and Plaine, 2007).

Sod5 is one of the most studied SODs in *C. albicans*. Sod5 is characterised as an important virulence factor. C.albicans strains lacking *SOD*5 show attenuated virulence in mice *in vivo* (Martchenko et al., 2004) and reduced survival in co-culture with macrophages (Frohner et al., 2009). *SOD*5 is expressed during yeast-to-hypha transitions, also during oxidative or osmotic stress and increased pH (Martchenko et al., 2004) and *SOD*5 is also upregulated during phagocytosis (Fradin et al., 2005).

I. 7 Aims of my diploma thesis

Sod5 is one of six superoxide dismutases in *C. albicans*. Sod5 is a copper-zinc dependent enzyme involved in detoxification of extracellular superoxides produced by macrophages and neutrophils. Cells lacking *SOD*5 have attenuated virulence and reduced survival in coculture with macrophages (Frohner et al., 2009). We were interested to identify the exact cellular localization of this protein. Therefore, we wanted to visualize this protein as a GFP- fusion protein Sod5-GFP. This method enables us to study the localization of this protein under the microscope and on Western blot using anti-GFP antibody.

The main aim of my thesis was the further characterisation of the protein.

Therefore I had to:

1. To create *C. albicans* strains expressing XFPs under constitutive expressed and SOD promoters and to study promoter activity of Sod5. As previous publications show, *SOD*5 promoter is induced under yeast-to-hyphae transition and under osmotic stress. I tried to confirm this using constructs with GFP tagged *SOD*5 promoter.

2. To study the localisation of Sod5 using a strain with GFP-tagged Sod5. Therefore, I had to clone the construct and to test Sod5-GFP for functionality and to detect the localisation using a fluorescent microscope.

3. Another part of my diploma thesis was to produce and characterise specific polyclonal antibodies against Sod4, Sod5 and Sod6 proteins.

II. Materials and Methods

II. 1. *C. albicans* Methods

1. Media for *C. albicans*

1xYPD
20g/l Bacto Peptone (BD Biosciences)
10g/l Yeast extract (BD Biosciences)
2% Glucose (w/v) (Merck)

Yeast extract and bacto peptone are dissolved in 900ml ddH_2O and autoclaved separately for 20min at 121°C. A 20% glucose solution is autoclaved separately and added at final concentration of 2% to 900 ml sterile YP-medium.

2xYPD
40g/l Bacto Peptone (BD Biosciences)
20g/l Yeast extract (BD Biosciences)
4% Glucose (w/v) (Merck)

For YPD plates: Yeast extract and peptone are dissolved in 200ml H_2O and autoclaved for 20min at 121°C. 50ml of 20% glucose solution are added to YP-media as well as 250ml 4% autoclaved bacteriological agar (w/v) (BD Biosciences).

Minimal medium:

16,75 g/l Bacto YNB (w/o AA and $(NH_4)_2SO_4$) (BD Biosciences)
12,5 g/l Ammoniumsulfat (Merck)

Glucose is added to 200ml minimal medium at a final concentration of 2%. For plates 250ml of 4% bacteriological agar are added to the medium (final concentration 2%).

2xSC:
4,5 g/l Bacto YNB w/o AA and $(NH_4)_2SO_4$
3,9 g/l Amino acid Mix (all aminoacids from Sigma)
10 g/l Ammoniumsulfat

Bacto-YNB, $(NH_4)_2SO_4$ and amino acid mix are dissolved in 740ml H_2O and autoclaved at 121°C for 20min. pH is controlled before autoclaving and regulated to pH5,5 – 6.
For liquid medium: 185ml 2xSC, 50ml 20% glucose stock solution, 250ml H_2O, and 5ml of the required amino acids from the 100x stock.
For plates: use instead of H_2O 250ml of autoclaved 4% bacteriological agar (w/v).

For stock solutions:

100x URA : 0,4g of L-uracil in 100ml H_2O
100x HIS: 0,6g of L-histidine in 100ml H_2O
100x LEU: 2,6g of L-leucine in 100ml H_2O

This 3 stocks can be autoclaved at 121°C for 20min.

100x Trp: 0,8g of L-tryptophane in 100ml H_2O

Tryptophane stock has to be sterilfiltered (Millipore 0,22µm filter) and stored in the dark at 4°C.

Amino acids mix (Sigma):

	g per 11.7 g mix
Adenine	0,4
L-arginine	0,2
L-tyrosine	0,3
L-isoleucine	0,3
L-phenylalanine	0,5
L-glutamic acid	1,0
L-aspartic acid	1,0
L-threonine	2,0
L-serine	4,0
L-valine	1,5
L-methionine	0,5

Growth of Candida species

- *C. albicans* strains are grown to saturation overnight in YPD with continuous shaking at 30°C.
- Next morning, the cultures are diluted to 0.2 OD_{600} in 25ml YPD and incubate with continuous shaking at 30°C until the culture reaches an OD_{600} ~1.

2. Colony-PCR

Single yeast colony can be used for a PCR reaction instead of isolated DNA. Pick and dissolve some cells from one colony in PCR-mix 1 or H_2O using a sterile tooth pick. Break the cells by heating in the PCR machine for 10min.

Colony PCR-Mix1: 1X

30 µl H_2O (+cells)
4 µl dNTPs [25mM]
3 µl Primer 1 [10µM]
3 µl Primer 2 [10µM]
→ breack up the cells by heating at 93°C for 10 min
→ put reaction on ice

Colony PCR Mix-2: 1x
5 µl 10x Taq Buffer
4 µl H_2O
1 µl Taq Polymerase

→ add Mix 2 to the reaction

PCR-Programm:
I: preheating 94°C → pause
II: 10min 94°C → PCR on ice+ Master-Mix2
III: 5min 94°C
IV: 30 sec 94°C
30 sec 50°C } x 30-35
xxmin 72°C
III: 10 min 72°C
IV: hold 10°C
Annealing temperature depends on the primer
Elongation time is depending on the size of the amplified fragment.

3. Extraction of *C. albicans* chromosomal DNA

- Grow an overnight culture in 10 ml YPD Medium at 30°C
- Harvest the cells at 2500rpm, 5minin a microfuge
- Resuspend the pellet in 500µl H_2O and pipette cell suspension into screw-cap tube.
- Centrifuge again for 5min, at 2500rpm
- Resuspend cells in 200µl DNA lysis buffer by vortex-mixing, add 1 volume glass beads and 200µl PCI (phenol/chloroform/isoamyl alcohol)
- Put tubes on Vibrax to break up cells by full speed at 4°C for 60min.
- Add 200µl sterile water to the cell suspension.
- Spin down for 5min, 14000rpm
- Transfer aqueous toplayer into new tube, add 1ml ice-cold 100% ethanol.
- Incubate on -20°C for 30min. (or overnight)
- Centrifuge for 15min, 14000rpm.
- Resuspend pellet in 400 µl 1xTE +3µl RNAse A and incubate at 50°C for 50min.
- Repeat PCI step
- Transfer the supernatant into a new tube, add 10µl 3M NaAc and 1ml ice-cold EtOH
- Incubate on -20°C for 30min (or overnight)
- Centrifuge 15min, 14000rpm
- Wash the pellet in 70% ethanol and let it dry at 55°C
- Resuspend the pellet in 30-50µl H_2O or TE

Lysis buffer:
2% Triton X-100 (Sigma)
1% SDS (Applichem)
10mM Tris/Hcl pH8,0 (Amresco)
1mM EDTA (Sigma)

4. Yeast electroporation

Competent yeast cells:

- Grow the culture in YPD overnight, and dilute to an OD_{600}= 0.2 in the morning, and regrow to the logarithmic growth phase until OD_{600} ~ 1.

- Alternatively dilute the culture 1x10^5 in 50ml YPD and grow on 30°C overnight OD_{600} ~1,5
- Transfer culture into 50ml Falcon tubes.
- Harvest cells by centrifugation 5min, 3000rpm.
- Resuspend cells in 8ml H_2O by vortex-mixing.
- Add 1ml 10xTE and 1ml 1 M LiAc
- Incubate by shaking at 150rpm, 30°C, 60min
- Add 250µl DTT. Incubate and shake slowly (30°C, 30min, 150rpm)
- No vortex-mixing from now on
- Add 40ml H_2O
- Harvest the cells by centrifugation (4°C, 5min, 3000rpm) and keep cells on ice from now on!
- Resuspend cells gently in 25ml cold H_2O and centrifuge (4°C, 5min, 3000rpm)
- Resuspend cells gently in 5ml cold 1 M Sorbitol and centrifuge (4°C, 5min, 3000rpm)
- Resuspend cells gently in 550µl cold 1 M Sorbitol

Transformation by electroporation:

- Put 43µl cell solution and 7µl of DNA construct into a clean electroporation cuvette
- Electroporator settings: BioRad gene pulser 200Ω, 1,5kV, 25µF; time constant ~4,6
- Add 950µl YPD and transfer into a 1,5ml tube.
- Incubate and shake slowly (30°C, 4h)
- Plate on selection plates
- Incubate at 30°C for 1-2 days

4. Indirect immunofluorescence of *C. albicans*

- Grow overnight culture and dilute it next morning 1:100 and grow to OD_{600} ~1
- Prepare a 24 well-plate with 12mm∅ round glass slides and coat slides by spotting 200µl of Concanavalin A solution (6mg/ml) on them and incubating for 45min.
- After incubation aspirate the liquid, wash one time with water and air dry slides.
- Add 200µl of a 2x10^6 fungal cell suspension (=4x10^5 cells/well) into each well and let cells settle on the coated glass slides for 30min.
- Aspirate unbound cells carefully from the edge of the wells.
- Wash gently with 0,5ml sterile PBS two times.

- Fix fungal cells with 300µl of 5% buffered parafomaldehyde for 15min on room temperature (RT). Discard paraformaldehyde solution and wash 3 times with sterile PBS.
- Incubate cells in blocking buffer (4% BSA/PBS) for 1h at RT.
- Aspirate blocking buffer and replace with 300µl of primary antibody or dilution (1:100 to 1:1000) in blocking solution. Incubate overnight at 4°C.
- The next morning wash 5 times with ice-cold sterile PBS.
- Add 300µl of secondary antibody dilution (1:700; anti-rabbit Cy5-conjugate) in blocking solution and incubate for 2h at RT in the dark.
- Wash 5 times with ice-cold sterile PBS.
- Carefully invert the cover slips onto a drop of mounting medium for fluorescence on a microscopy slide. Observe slides under the microscope using contrast phase and Cy5 (ex 650nm/em 670nm) filters.

6. Filipin staining of yeast plasma membrane

Filipin is dissolved in DMSO at a concentration of 5mg/ml and stocks are stored in 20µl aliquots. Filipin fluorescence is not stable, it bleaches quickly

Prepare a working solution always fresh and use for staining immediately: 20µl of filipin stock in 10ml PBS (1:500 dilution). For using filipin plus the antibody, you can add filipin (1:100 dilution) in blocking buffer (10% FBS) after incubation with secondary the antibody and incubate for 30 min at 37°C.

- Rinse cells 3x with PBS
- Stain cells with working solution for 30 min at RT
- Wash cells 3x with PBS

Use mounting media for slide preparation

II. 2. Bacteriological Methods

1. Media for E.coli

1xLB	10g/l	Tryptone (Merck)
	5g/l	Yeast extract (DIFCO)
	10g/l	NaCl (Sigma)
2xLB	20g/l	Tryptone (Merck)
	10g/l	Yeast extract (DIFCO)
	20g/l	NaCl (Sigma)

Yeast extract, tryptone and NaCl are dissolved in 1000ml ddH_2O and autoclaved for 20min at 121°C. pH is controlled before autoclaving and regulated to pH 7 – 7,5.
For LB plates 250 ml 4% autoclaved bacteriological agar (w/v) is added to 250 ml autoclaved 2xLB. For LB+AMP medium or plates, ampicillin is added at a final concentration of 0,1 mg/ml. Medium and plates containing ampicillin are stored at 4°C.

2. Preparation of competent *E.coli*

- Grow *E.coli* (DH5α) overnight culture in 500ml SOB medium at 18°C or RT to OD_{600} ~1
- Chill bacterial culture on ice for 20min
- Spin down at 2500rpm and 4°C for 10min (split up in sterile 50ml falcons)
- Resuspend the pellet in 15ml ice-cold TB by swirling the centrifuge breaker gently and put on ice for 10min
- Spin down at 2500rpm and 4°C for 10min
- Resuspend in 35ml ice cold TB (gently)
- Add DMSO (tissue culture grade) to a final concentration of 7% (v/v) and put on ice for 10 min
- Make 500µl aliquot, shock-freeze them in liquid nitrogen and store at -80°C

  SOB medium:

2%	Tryptone (w/v) (BD Biosciences)
0,5%	Yeast extract (BD Biosciences)
10mM	NaCl (Sigma)
2,5mM	KCl (Sigma)
20mM	$MgCl_2$ (Sigma)

Dissolve tryptone, yeast extract and NaCl in H_2O, add KCl, pH to 7,5 with 5M NaOH, adjust to final volume, autoclave and add sterile $MgCl_2$

TB:
10mM Pipes
55mM $MnCl_2$
15mM $CaCl_2$
250mM KCl

Mix all components except $MnCl_2$ in H_2O, adjust to pH 6,7 with KOH, add $MnCl_2$, adjust to final volume and sterilise by filtration through a 0,45µm filter, store at 4°C.

3. Transformation of competent bacteria

- Thaw competent cells on ice
- Gently mix the cells and aliquot 100-200µl competent cells to each DNA sample (0,5- 1µg)
- Incubate cells on ice for 30min
- Heat-shock cells for 45sec at 42°C
- Place them on ice for 2min
- Add S.O.C. medium and shake at 300rpm at 37°C for 1h.

II.3. DNA Methods

1. Agarose gel electrophoresis

- DNA samples (5-50 µl) are mixed with an appropriate amount of 10x loading buffer
- Load samples on a gel containing 0,7-1,5% agarose in TAE buffer and 0.5 µg/ml ethidium bromide, in electrophoresis chamber filled with electrophoresis buffer
- Load 5 µl molecular weight standard on the gel (Bioline Hyperladder I)
- Separate DNA fragments in an electric field with 80-100 Volts
- Visualize nucleic acid fragments under UV-light and compare fragment size to molecular weight standards

TAE (50x stock solution):
2 M Tris-HCl pH8
1 M Acetic acid
50 mM EDTA

Electrophoresis buffer:
1x TAE

Loading buffer:
1,2 ml 10xTAE
0,6 ml 5% Bromphenol blue
0,6 ml 5% Xylene cyanol
4,8 ml 100% Glycerol
4,8 ml H2O
0,45 µm filter-sterilize

2. Polymerase-chain-reaction (PCR)

PCR-Mix (50µl): 1X

5µl	10xPCR buffer
5µl	dNTP's [25mM]
2 µl	fwd-primer [10µM]
2 µl	rev-primer [10µM]
1µl	DNA (20-100ng)
0,5µl	Polymerase Taq [5U/µl]
34,5µl	H_2O

PCR-Programm:

I: 5 min	94°C	
II: 30 sec	94°C	x 30-35
30 sec	50°C	
xxmin	72°C	
III: 10 min	72°C	
IV: pause	10°C	

Annealing temperature depends on the primer melting temperature
Elongation time is depending on the size of the amplified fragment

3. Plasmid mini preparation

- Spin down 2x2ml of an overnight culture at 6000rpm for 2min
- Resuspend the pellet in 500µl STET buffer and put on ice
- Add 20µl of Lysozyme (20mg/ml), mix by inverting several times and incubate on ice for 5 min
- For lysis incubate suspention at 95°C for 70 min, put on ice afterwards
- Centrifuge at 14000rpm for 10min
- Remove the pellet from the eppendorf tube using sterile tooth pick
- Precipitate by adding 500µl isopropanol and mixing several times at room temperature for 15min
- Spin down at 14000rpm at 4°C for 10min
- Wash pellets with 70% EtOH, and centrifuge at 14000rpm for 5 to 10min
- Dry pellet and resuspend in 100µl TE or water
- Add RNaseA to final concentration of 50µl/ml

1xTE:

10mM	Tris (Amresco)
1mM	EDTA (Sigma)

The pH should be adjusted to pH 8,0 with HCl.

STET-buffer (100ml):

8%	Sucrose (Merck)
10%	Triton X-100 (Sigma)
50mM	EDTA (Sigma)
50mM	Tris/HCl pH 8,0 (Amresco)

4. QIAGEN Plasmid midi protocol

- Grow *E.coli* cells containing the desired plasmid in 50ml LB+Antibiotic at 37°C overnight
- Harvest cells by centrifugation at 6000xg for 15min at 4°C in the centrifuge
- Resuspend cell pellet in 4ml Buffer P1 (QIAGEN)
- Add 4ml of Buffer P2 (QIAGEN), mix gently by inverting several times and incubate at RT for 5min.
- Add 4ml of chilled Buffer P3 (QIAGEN), mix thoroughly by inverting several times and incubate 15 min on ice.
- Centrifuge at 20000xg for 30min at 4°C and re-centrifuge supernatant again for 15min.
- Equilibrate a QIAGEN-tip 100 by applying 4ml Buffer QTB (QIAGEN) and allow the column to empty by gravity flow.
- Apply the supernatant from the step before to the QIAGEN-tip and allow it to enter into the resin by gravity flow.
- Wash the tip twice with 10ml Buffer QC (QIAGEN)
- Elute DNA with 5ml Buffer QF (QIAGEN)
- Precipitate DNA by adding 3,5ml isopropanol at RT
- Mix and centrifuge immediately at 15000x g for 30min at 4°C
- Decant supernatant and wash pellet with 2ml 70% ethanol. Centrifuge again at 15000x g for 10min at 4°C.
- Decant supernatant, air-dry the pellet for 15mon and redissolve DNA in 100µl sterile H_2O.

5. Cloning procedures

Plasmid digestion

Use a small digestion-mix for a control digest or big mix for digest during cloning.

Small Mix (30µl) → for control digestion:

2 µl (3-5µg)	DNA-solution
3 µl	Buffer (10x)
0,5µl	Restriction enzyme (10U/µl, end concentration 1U/1µg)
24,5µl	H_2O

Incubate for 2h at 37°C

Big Mix (100µl) → for preparative digestion:

10-20µl (30µg)	DNA
10µl	Buffer (10x)
2-3µl	Restriction enzyme (10U/µl, end concentration 1U/1µg)
x µl	H_2O

Incubate for 3-4h or overnight

Phosphatase treatment

- After restriction digest, add 1 µl alkaline phosphatase (1U/µl) (Roche) to the reaction mix.
- Incubate 45 min at 37°C.

→ DNA can be precipitated with ethanol.

Ligation

Ligation-mix:

5µl	2x ligation buffer
2µl	pGEM-T vector (1:5 dilution)
X µl	PCR product
1µl	T4 DNA ligase
X µl	H_2O
10µl	

Ligation of DNA fragments

Ligation was carried out with T4-Ligase Fermentas , using vector and insert at a molar ration of 1:3.

$$\frac{\text{ng vector x kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

Incubate ligation mix for 3-4h at RT or overnight at 4°C before transforming 5-10µl into competent *E.coli* cells.

6. Gel elution (Peq Lab)

- Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
- Add the same volume of Buffer XP2
- Incubate at 55-65°C for 10 min (or until the slice has completely dissolved). Check that the colour of the mixture is yellow (pH > 7,5) otherwise add 5M sodium acetate till the colour turns into yellow (5-10µl).

- Place a HiBind spin column in a provided 2ml collection tube and apply the sample (max. 750µl) to the column and centrifuge for 1min, at 10.000rpm
- Discard flow-through and place HiBind column back into the same collection tube. Load the sample again if necessary.
- Wash 2 times with 600µl SPW- Buffer, centrifuge (1min, 10.000rpm)
- Discard flow-through. Place the column back into the same tube. Centrifuge for
- Additional 1min to dry the column.
- Place HiBind column in a clean 1,5ml Eppendorf tube
- Elute DNA with 30-50µl elution buffer or H_2O and centrifuge column for 1min, 5000rpm

7. DNA precipitation

- Add 2,5 volumes of EtOH/ 0,1 volume NaAc
- Mix by inverting several times
- Put on -20°C for 20 min or overnight
- Centrifuge (10min, 14.000rpm)
- Wash pellet with 70% EtOH
- Centrifuge again (10min, 14.000rpm)
- Dry pellet (heating block)
- Resuspend in 30-50µl H_2O

8. Southern blotting with DIG High Prime

Agarose gel for electrophoresis of genomic DNA**:**

- Pour a big (500ml) gel containing 0,7% agarose in 1x TBE
- Load genomic DNA samples (precipitated to a volume of 20µl) on the gel and put in electrophoresis chamber filled with 1x TBE
- Load 5µl molecular weight standard (Bioline Hyperladder I) on the gel
- Apply an electric field of 60-80 volts to separate the genomic DNA
- Mark bands of molecular weight standard on the agarose gel with a sterile scalpel

Denaturation and neutralization of the agarose gel**:**

- Wash agarose gel for 1h in 0,5M NAOH, 1,5M NaCl
- Wash agarose gel for 1h in 0,5M Tris/HCl pH 7,5, 1,5M NaCl

Capillary Blotting**:**

- Prepare blotting stock in a glass tray as follows:
  2 pieces of Whatman-paper soaked in 20x SSC
  Agarose gel (upside down)
  Nylone membrane soaked in 20x SSC
  Put pieces of Parafilm at the sides to seal the blot
  1 piece of dry Whatman-paper
  Stack of green towels
  Put a 1kg weight on top
- Blot overnight
- Mark bands of molecular weight marker on the membrane with a pencil
- Cross-link DNA on the membrane using Stragene UV Crosslinker

Labeling of DNA**:**

- Dilute 1µg template DNA in double distillated water to final volume of 16µl.
- Denature the DNA by heating for 10min and quickly chilling in an ice
- Mix DIG-High Prime thoroughly and add to the denatured DNA, mix and centrifuge briefly. Incubate for 1h or overnight at 37° C.
- Stop the reaction by adding 2µl 0,2M EDTA and/or by heating to 65°C for 10 min.

Prehybridisation**:**

- Pre-heat an appropriate volume of DIG Easy Hyb (4°C → Kühlschrank bottle 7) to hybridization temperature of 37-42°C
- Prehybridize filter for 30 min with gentle agitation in an appropriate container.
- Denature DIG-labeled DNA probe by boiling for 5 min and rapidly cooling in ice.
- Add denatured DIG-labeled DNA probe to the pre-heated DIG Easy Hyb and mix well (avoid foaming)
- Pour off prehybridisation solution and add probe mixture to membrane.
- Incubate 4h- overnight with gentle agitation, 42°C.

Stringency washes**:**

- Wash 2x 5min in ample 2x SSC, 0,1% SDS at 15-25° C under constant agitation.
- Wash 2x 15min in 0,5x SSC, 0,1% SDS (prewarmed to wash temperature) at 65-68° C under constant agitation.

Detection**:**

- After hybridization and stringency washes, rince membrane 1-5 min in Washing Buffer → RT
- Incubate for 30min in 10ml Blocking solution.
- Incubate for 30 min in 10ml Antibody solution.
- Wash 2x 15min in 100ml Washing buffer.
- Equilibrate 2-5 min in 20 ml Detection buffer.
- Place membrane with DNA side facing up on a development folder and apply 1ml CSPD ready-to-use (bottle 5). Immediately cover the membrane with the second sheet of a folder to spread the substrate evenly, avoid airbubbles, and incubate for 5min at 20-25 ° C.
- Squeeze out excess liquid and seal the edges of the development folder.
- Incubate the damp membrane for 10min at 37°C to enhance the luminescent reaction.
- Exposure to a appropriate imager for 15-20 min or to X-ray film for 15-25 min at 15-25°C.

Solutions:

Blocking Solution: Blocking solution (bottle 6) diluted 1:10 in maleic acid buffer
Washing buffer: 0,1M Maleic acid, 0,15M NaCl; pH 7,5; 0,3% Tween 20
Maleic acid buffer: 0,1M Maleic acid, 0,15M NaCl, adjust with NaOH to pH 7,5
Detection buffer: 0,1M Tris-HCl, 0,1M NaCl, pH 9,5

II. 4. Protein Methods

1. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were mixed with 2xsample buffer and loaded on the gel. The gels were run at 180V until the bromphenol blue front reached the gel bottom.

7.5% running gel:

5 ml	H_2O
2,5 ml	1.5 M Tris pH 8.8
2,5 ml	Acrylamide (30%)
50 µl	SDS (20%)
10 µl	TEMED
100 µl	APS (10%)

Add TEMED and APS at the end and pour the solution immediately between the glass plates. Allow the gel to polymerize. Then the stacking gel solution can be prepared and poured and the comb has to be plased to create the wells.

10% running gel:

4,2ml	H_2O
2,5ml	1.5 M Tris pH 8.8
3,3 ml	Acrylamide (30%)
50	SDS (20%)
10µl	TEMED
100	APS (10%)

4.5% stacking gel:

6 ml	H_2O
2,5 ml	0.5 M Tris pH 6.8
1,5 ml	Acrylamide (30%
50 µl	SDS (20%)
10 µl	TEMED
100 µl	APS (10%)

Protein sample buffer:

40mM	Tris/HCl pH6.8
8 M	Urea
5%	SDS
0,1mM	EDTA
1%	ß-Mercaptoethanol- freshly added
0,1 g/l	Bromphenolblue- freshly added

Running buffer (1l):

1,5 g	Tris
7,2 g	Glycine
4,0 g	SDS

2. Coomassie-staining of protein gels

- Stain protein gel by shaking for 30 min in Coomassie-staining solution.
- Wash gel in destaining solution until background is completely destained.

- Dry gel

Coomassie staining solution: for 2l

200 ml	Glacial acetic acid
500 ml	Isopropanol
0,67 g	Coomassie blue (G250)
1300 ml	H_2O

Destaining solution: for 1l

250 ml	Methanol
70 ml	Glacial acetic acid
680 ml	H_2O

3. Semi-dry protein transfer for immunoblotting

- Prepare the blotting sandwich as followed:
  On the bottom (=anode electrode) of the semi-dry transfer cell from Biorad (Trans-Blot SD) place:
  1. 6 pieces of Whatman-paper (6x9 cm) soaked with anode-buffer 1
  2. 3 pieces of Whatman-paper (6x9 cm) soaked with anode-buffer 2
  3. Nitrocellulose membrane (6x9 cm, Protran; Schleicher & Schuell) moisturized with anode-buffer 2
  4. Gel
  5. 9 pieces of Whatman-paper (7,5x10 cm) soaked with cathode-buffer
- Cover the sandwich with the lid (= cathode electrode) of the blotting machine.
- Blot for 50min at 25V with Trans-Blot Semidry Transfer Cell from Biorad.
- Detect the transfer quality by Ponceau staining (Fluka Chemika) of proteins on the membrane.
- Destain the membrane by washing in H_2O
- Block unspecific binding by shaking overnight at 4°C in Blotto (5% milk diluted in 1x TBS and 0,1% Tween-20)
- Add the primary antibody diluted in Blotto
- Incubate for 1h by shaking at room temperature or overnight at 4°C
- Wash three times for 15 min with TBS-T (1xTBS, 0,1% Tween-20)
- Add the secondary antibody diluted in TBS-T, incubate for 1h at RT
- Wash three times 15 min with TBS-T
- Detection (see Chapter II.4.4)

- If desired, blots can be stripped by shaking 20 min in 50 ml stripping buffer in a 50°C water bath washed with water, shake for 20 min in blotto and reused again for detection with another antibody

Stripping buffer:

100mM	ß-Mercaptoethanol
2%	SDS
62,5mM	Tris/HCl pH6,7

Anode buffer 1:

0,3M	Tris base
20%	Methanol (v/v)

Anode buffer 2:

25mM	Tris base
20%	Methanol (v/v)

Cathode buffer:

25mM	Tris base
40mM	6-Amino-n-Hexanoic acid

4. Detection of immunocomplexes - Enhanced chemiluminescence (ECL)

- Prepare working solution by mixing equal volumes of the peroxide solution and the luminol/enhancer solution of SuperSignal West Pico Chemiluminescent Substrate (Pierce); gently overlay about 1.5ml of the mixture on the membrane
- Incubate blot with working solution for 5min in the dark, remove detection reagents and wrap membrane into Saran Wrap.
- Expose on X-ray film (Kodak) for various times to get optimal exposure.

5. Western blot analyses by the MiniProteanII Multiscreen Apparatus (BioRad).

- Clean and dry the multiscreen apparatus and gaskets prior to assembly.
- Plase the sealing gasket onto the base plate with the raised surface down, using the guide pins to help align the gasket.
- Lay the blocked blot with the antigen side facing up.
- Place the sample cover on the top of the membrane. Finger tighten the four screws. Use the diagonal crossing partner to insure even pressure on the membrane. The multiscreen apparatus is ready for the sample application.
- Load different antibodies or different dilution of the sera (diluted in 5% milk diluted in 1x TBS and 0,1% Tween-20) into the channels. Pipet the samples carefully and avoid trapping bubbles inside the channel.
- Fill the channel with 600µl solution.

- Incubate 1h by shaking at the room temperature or overnight at 4°C.
- Wash solution can be applied as well as antibody solution with a pipet. Use also 600µl for each wash; wash three times. Antibody and wash solution can be rapidly removed by vacuum aspiration.
- Incubate for 1h at the roon temperature with the secondary antibody diluted in TBS-T.
- Wash three times for 15 min with TBS-T before detection.

6. Protein determination by the Bradford method

- Prepare BSA standard solutions (5 µg, 15 µg, 20 µg, 30 µg, 40 µg) and sample tubes (1 µl). Fill up with water to a total volume of 800 µl.
- Add 200 µl of Bradford color reaction solution (BioRad) to each tube, vortex-mixing immediately and let stand for 10 min at room temperature
- Measure absorbance at 595 nm
- Draw standard curve on and calculate protein concentrations of samples from the graph; the standard curve is linear up to about 30 µg of BSA

7. Preparation of *C. albicans* TCA extracts for immunoblotting

- Harvest cells of 5ml yeast-culture with OD_{600} ~ 0,8 – 1 by spinning 5min at 3000rpm; resuspend cells in 1ml H_2O
- Add 150µl Yex-lysis buffer, vortex-mixing and incubate 10min on ice
- Add 150µl cold 50% TCA (trichloracetic acid) and incubate at least 10min on ice
- Spin 5min at 13000rpm and 4°C, discard supernatant and spin again
- Wash pellet with 1ml ice-cold 3% TCA; spin again 10min at 4000rpm (4°C)
- Take off the rest of the supernatant carefully
- Neutralize by adding 15µl unbuffered 1M Tris and resuspend pellet in sample buffer (20µl / 1OD_{600})
- Incubate 15min at 37°C on shaker
- Spin down cell debris at 13000rpm for 5min and load 10µl (0.5 OD_{600}) of supernatant on SDS-PAGE gels

Yex-lysis buffer:

1,85M NaOH
7,5% ß-mercaptoethanol (v/v) freshly added

Protein sample buffer:

40mM	Tris/HCl pH6.8
8M	Urea
5%	SDS (w/v)
0,1mM	EDTA
1%	ß-mercaptoethanol (v/v) freshly added
0,1g/l	bromphenol blue

8. Protein extracts

- Dilute overnight culture (the overnight culture should have OD_{600} ~13) 1:10 and let them grow to OD_{600} of 0,8
- Induce or not the promoter
  - Induction with 10% FCS (37°C) for 2-3h or 0,3M NaCl, pH8 (30°C) for 1h
  - Not induced samples → 30°C
- Harvest the cells by centrifugation for 5min at 4000rpm
- Wash cells with 20ml cold water, centrifuge again
- Resuspend pellet in 300µl buffer and transfer to 2ml eppendorf tube with screw cap
- Add 250µl glass beads and protease inhibitor cocktail (Roche)
- Break the cells with Fast Prep, 3x15 sec at level 6, on ice in between
- Spin down beads and cells debris for 3 min at 6000rpm 4°C
- Take off supernatant and spin again for 20min at 15000 4°C
- Take off supernatant
- Measure protein concentration 280nm, dilution 1:200

Buffer A:

50mM	HEPES pH8
0,4M	(NH4)2SO4
1mM	EDTA
5%	Glycerol

Proteinase inhibitor (Roche)

Lysis buffer:

50mM	Tris-HCl pH7.4
5mM	EDTA
150mM	NaCl
1mM	PMSF

Proteinase inhibitor (Roche)

9. Cellular fractionation

- Dilute the overnight culture 1:100 (in 10ml medium) and let the cells grow for 4 hours
- Let yeast grow at 30°C to an OD_{600} of 1 induce if necessary (see induction chapter II.4.7) and let grow for the optimal time
- Spin down 5min at 3000rpm
- Wash I: resuspend cells in 1ml PreT buffer, spin again

- Wash II: resuspend cells in PreT buffer, incubate them at 30°C for 20min under gently shaking, spin down → take an aliquot (wash fraction)
- Resuspend the pellet in PreT buffer containing 5mM DTT and incubate for 20 min at 30°C under gently shaking, spin down → take an aliquot (DTT wash fraction)
- Resuspend the cell pellet in 300µl Lys-buffer, add glass beads
- Break the cells with Fast Prep, 3x15 sec at level 6, on ice in between
- Spin down beads and cells debris for 3 min at 6000rpm 4°C → transfer supernatant into new tube → take an aliquot (crude lysate fraction)
- Wash pellets twice with Lys-buffer, (spin 10000rpm, 5min, 4°C) and resuspend in this buffer, → take an aliquot (cell wall fraction)
- Dilute cell wall fraction 1:1 with Digest buffer incubate for 10 min, separate cells from the buffer by centrifugation
- Wash pellet tree times with with H_2O
- Incubate pellet with laminarinase at a final concentration of 0.25 unit/ml twice for 1 h at 37°C

PreT buffer:

100mM sodium acetate pH8.0
5mM EDTA
Protease inhibitor (Roche)

PreT buffer+ DTT:

100mM sodium acetate pH8.0
5mM EDTA
5mM DTT
Protease inhibitor (Roche)

Lys-buffer:

50mM sodium acetate pH5.5
150mM NaCl
5mM EDTA
1mM phenylmethylsulfonyl fluoride (PMSF) (added freshly)
Protease inhibitor (Roche)

Digest buffer:

50mM Tris HCl pH8
100mM EDTA
2% SDS

Laminarinase buffer:

100mM sodium acetate pH5,5
1mM EDTA
+ Laminarinase [0,25u/ml]

10. Purification of GST fusion protein from *E.coli*

Checking for optimal induction time

- First, pick about 3 single *E.coli* clones and check for induction of fusion protein expression.
- Induction in total extracts:
- Dilute overnight culture in fresh LB-Amp to OD_{600} 0,25-0,5
- Make up 12ml liquid culture of OD_{600} 0,25-0,5 equilibrate for 15min at 30° C
- Take a 1,5ml control sample at 0min as the uninduced control
- Induce by adding IPTG to a final concentration of 0,2mM at 30°C
- Take 1,5ml samples at 20, 40, 60, 90, 120 and 180min after induction
- Spin down samples (full speed 5min) in eppendorf tubes and aspirate supernatant with the pipette
- Resuspend pellet in protein sample buffer
- Boil on heat block 95°C for 10min
- Spin down at 13000rpm 15min (transfer supernatant to new eppendorf tube)
- Run 0,5- 1 OD_{600} equivalent of the total extract on SDS-PAGE gels
- Stain with Commassie blue

Determinate if GST fusion-protein is soluble or not:

- Grow culture of OD_{600} 0,5 exactly as describe above and incubate 15min at 30°C → take sample from not induced sample
- Induce with IPTG for the optimal time (80min)
- Spin down cells in Sorvall centrifuge for 10min at 7000rpm and wash once with cold PBS on ice!
- Suspend cells in 2ml cold PBS and lyse cell suspension by sonication→ 8x 10imp.
- Spin homogenate for 10min at 7500rpm in Sorvall centrifuge at 4°C to bring down insoluble inclusion bodies
- Take sample from supernatant (soluble fraction) and add sample buffer
- Resuspend insoluble fraction in sample buffer
- Boil all samples for 10min at 95°C and run 0,5-1 OD_{600} extract equivalents of samples in SDS-PAGE gels
- Stain with Commassie blue

Binding of GST fusion-protein to glutathione sepharose beads:

- Grow *E.coli* strain containing the desired plasmid in 5ml LB+Amp at 37°C overnight
- Dilute preculture in 500ml LB+Amp to OD_{600} 0,2 and grow for 1h at 37°C
- Shift culture to 30°C and grow to OD_{600} 0,5 → take an aliquot
- Induce expression of GST fusion-protein by adding 0,2mM IPTG
- Let grow for 80min at 30°C → take aliquot
- Harvest cells by centrifugation at 4000rpm for 5min in a GS3 rotor in a Sorvall centrifuge
- Resuspend pellet in 7ml TpG buffer and add 1mM DTT and protease inhibitor cocktail (Roche)
- Lyse cells by sonication (3x30sec, 30sec on ice in between) → take aliquot
- Spin down cells debris at 4°C for 10min at 13000rpm in eppendorf tubes
- Equilibrate glutathione sepharose beads in TpG buffer (wash in TpG buffer, spin down 5min 2000rpm)
- Add 400µl 50% glutathione sepharose beads to cell lysate and incubate overnight on rotation mixer → take aliquot next morning
- Centrifuge at 3000rpm for 2min at 4°C and wash beads twice with TpG buffer (keep supernatant)
- Elute GST fusion-protein by shaking beads 20 min at RT with 500µl elution buffer
- Spin down beads 1min at 3000 rpm and repeat elution twice

Elution buffer:

50mM	Tris/HCl pH7,5
5mM	gluthation (sterile filtrated)

TpG buffer:

20mM	Tris/HCl pH8,0
100mM	NaCl
1mM	EDTA
0,1M	$ZnCl_2$
0,5%	NP40(v/v)

11. Preparation of rabbit antiserum

- Leave the fresh blood for several hours at RT to let it coagulate.
- Spin the coagulated blood for 20min at 4000rpm and 4°C.
- Sterile-filter the serum through a 0,45µm filter.
- To avoid phosphatase activity, heat-inactivate the serum at 56°C for 30min in a shaking waterbath.
- Add 0,02% of sodium azide to preserve the serum.
- If antibodies will be used later on for Western blotting or immunofluorescence the serum can be stored frozen in 50% glycerol at -80°C. If serum will be lyophilized, or used for further purification, e.g. IgG fractionation, or for immunoprecipitations freeze them without glyzerol.

12. Affinity purification of polyclonal antibodies

- Run a gel with 100-300µg of GST fusion protein
- Transfer to nitrocellulose membrane
- Stain for ca. 10 min with Ponceau
- Cut the band of interest and cut it in small pieces, put them to an eppendorf tube
- Incubate with ~ 1ml of serum overnight at 4°C
- Keep the supernatant
- Washing : 1x 1ml 10mM Tris pH8,0
  1x 1ml 10mM Tris pH8,0 + 0,5 M NaCl
- Prepare eppendorf tubes with 50µl 1M Tris/HCl pH8,0 to neutralize the elution fraction
- Elution: 3x 200ml 100mM Glycin pH2,5 → 2min incubation by rotation
- Test for neutral pH

II. 5. Mammalian Cell Culture

1. Primary culture of bone marrow-derived macrophages (BMDMs)

All given volumes are calculated for the limbs of one mouse and should be increased according to the number of limbs.

- On day 1, sterile dissect tibias and femurs from a 6-8 week old mouse, quickly rinse bones in 70% ethanol and place in 15ml ice-cold sterile PBS. When all the limbs are collected, transfer them in fresh 15ml ice-cold sterile PBS and keep on ice. (Older mice can be used but the number of bone marrow cells recovered will be smaller.)
- To flush out the bone marrow, separate femur from tibia at the knee joint. Holding the bone with forceps above a sterile dish, cut one extremity of the bone and using a 20-ml syringe with a 27GX3/4 needle, flush DMEM with 10% FCS, 100 U/ml Penicillin and 100µg/ml Streptomycin, into the medullary cavity until no more cells are coming out.
- Collect bone marrow suspension and keep it on ice until all bones have been processed. Bone marrow flushing should be performed under as much as possible sterile conditions as required for cell culture.
- To prepare BMDMs, centrifuge the collected bone marrow at 1000xg for 5min and re-suspend the pellet in 44ml BMDM medium. Distribute the cell suspension equally in four 10x10cm Petri dishes (or seven Ø10cm-Petri dishes); transfer to a 37°C-incubator with a 5% CO_2, 95 % humidity atmosphere. Only BMDMs will adhere to non-tissue culture treated plastic, allowing for their separation from other cell types, the latter being eliminated when media are changed.
- On day 2, add 6-8ml of BMDM medium. Check for cell density every day.
- On day 4-5, when the plate is getting confluent, aspirate the media containing non-adherent cells, collect the cells gently by scrapping the plates with a soft rubber spatula and re-plate at a ratio of 1:2-1:3 in square 10x10cm Petri dish (Alternatively, 4-days BMDMs can be frozen in FCS with 10% DMSO and stored in liquid nitrogen until further need. They should then be grown for at least 6-7 days in BMDM media before use.)
- Culture cells grow for another couple of days, change medium completely every 2-3 days.

After 9-10 days of culture, BMDM cell surface markers should be checked by FACS before performing interaction experiments.

BMDM media:

Dulbecco's modified Eagle's Medium DMEM, high glucose (4.5g/l),
with L-Glutamine, without pyruvate (PAA)
+ 10% FCS
+ 100 U/ml Penicillin
+ 100 µg/ml Streptomycin
+ 20% L-conditioned media

2. Preparation of L-conditioned media

- Divide ten confluent diameter-10cm dishes with CSF-1 producing L929 cells (ATCC# CCL-1) into twenty 175cm^2-flasks with 50ml/flask of high-glucose DMEM supplemented with 10% FCS without antibiotics.
- After 2-3 days, when cells are approximately 70% confluent, aspirate the medium and replace with 100ml/ flask of starving medium (high-glucose DMEM without FCS and antibiotics).
- After 10 days, collect and filter the conditioned media on Steritop 0,22µm GP express PLUS membrane (Millipore, Billerica, MA, USA), to prevent membrane clogging. Store 200ml aliquots at -20°C. Keep a small aliquot at 4°C for testing.

Testing L-conditioned media

- Flush the bones of one mouse as described before, divide the cells into five 100x100 square petri dishes.
- Grow in 11ml of BMDM medium supplemented with 0, 10, 15 and 20% of the fresh batch of L-conditioned medium. As a positive control, also grow two plates in BMDM medium supplemented with the optimal concentration of an old working batch of L-conditioned medium.
- After 2-3 days, add 6ml of new medium.
- After 5-6 days, count the cells at each concentration, split the cells 1:3. Let them grow until confluent and count again. Change the medium every 2-3 days. Deduce from the cell count the optimal L-conditioned medium concentration.

3. End point dilution assay

Day1

- Log phase BMDMs cells (day 11-12) are suspended in culture medium (-L-cond DMEM + Glutam + FCS) at $1x10^6$ cells/ml the day before usage
- Distribute 100-µl aliquots in a 96-well cell culture plate $1x10^5$ /well in every second column

Day2

- Wash *C. albicans* two times with 10 ml PBS,
- Resuspend *C. albicans* in 1 ml DMEM +Glut wo FCS
- Count 1:900 dilution with CASY
- Dilute in DMEM to 2 x 10^6fungi/ml *C. albicans* in DMEM,
- Make ¼ serial dilutions in 150 µl (150+50)
- Add *C. albicans* to the 96well plate +/- BMDMs .

Plate Setup: plate 1-9

	BMDM		BMDM		BMDM		BMDM		BMDM		BMDM	
	1:1	1:1	1:4	1:4	1:16	1:16	1:64	1:64	1:256	1:256	1:1024	1:1024
Strain 1												
Strain 2												
Strain 3												
Strain 4												
Strain 5												
Strain 6												
Strain 7												
Strain 8												

4. Crystal violet staining

- Wash cells 3 times with 200µl PBS
- Add 50µl of 0,2% crystal violet, 20% methanol solution
- Incubate for 20min at room temperature
- Wash 3 times with 200µl PBS
- Air dry plate

5. ROS assay

C. albicans strains:

- Check *C. albicans* overnight culture for contamination under microscope
- Measure OD_{600} (1/50 dilution)

- Dilute *C. albicans* in 25ml YPD to OD_{600}~0,2 and incubate at 30°C
- Harvest cells by centrifugation at 2500rpm, 5min, RT, when growth reaches OD_{600} ~1 (after ~3,5-4h)
- Wash cells with 40 ml PBS, spin again (2500 rpm, 5min, RT)
- Resuspend pellet in 1ml HBSS
- Dilute cells 1:10 in 1,5ml HBSS and measure cell concentration with CASY

Immune-cells:

- Harvest log-phase BMDMs 45min before the assay starts (resuspend cells in HBSS)
- Dilute in HBSS w/o Phenol Red w/o FCS to reach a final dilution of $4x10^5$/ml in about 10ml
- Count with CASY, keep in water bath at 37°C

Interaction:

- Distribute 100µl of immune-cells in 96 well-plate
- Incubate them at 37°C for 10-30 min
- Add 50µl HBSS with or w/o stimulus
- 50µl Reaction-mix (5,5ml HBSS+11µl luminol (0,1M stock)+220µl HRP(2000IU/ml)
- Measure chemiluminescence for at least 120min in 2,5 min intervals in Wallac 1420 VICTOR™ Multilabel counter.
- Luminescence is expressed as relative luciferase units/minute/1000cells

III. Results

III. 1. Promoter activity studies of SOD proteins

1. Expression of green fluorescent protein under the constitutive and gene own promoter

Before localisation studies can start, we had to study the expression of Sod5 protein. In general, to study the promoter activity of *C. albicans* Sod proteins, we designed strains with XFP-tagged *SOD4*, *SOD5* and *SOD6* promoter and XFP-tagged actin promoter as a positive control. Using the strains with XFP-tagged SOD promoters, we were able to detect the promoter activity via the expression of the XFP proteins. During my work I was especially interested on Sod5. The constructs with the fluorescent proteins expressed under the *SOD*4 and *SOD*6 promoters can be used in further studies.

It has been shown that *SOD*5 is induced during yeast to hyphae transition (Nantel et al., 2002). There are different possibilities to induce yeast to hyphal transition in yeast, like serum, increased pH, and increased temperature. The presence of serum in yeast media causes an induction of *SOD*5 gene. This increase is even higher if the temperature is 37°C. According to the literature, *SOD*5 is induced in response to neutrophils (Fradin et al., 2005). *SOD*5 is also up regulated under a number of other conditions like oxidative and osmotic stress (Martchenko et al., 2004) .

The following strains were constructed: CFP and GFP were separately cloned behind the actin promoter and transformed either into the SN152 strain or into SNR34R strain (strains can be found in the attachment, see stains used for this study). To study the promoter activity of *SOD*4, *SOD*5 and *SOD*6*,* we cloned YFP behind *SOD*4 promoter, CFP and GFP behind *SOD*5 and GFP behind *SOD*6 promoters and transformed those constructs into the SN152 strain. Additionally we transformed the P_{SOD5}-CFP construct into SNR34R strain.

The fluorescent protein cassettes were amplified from pMG1646, pMG1656, pMG1801 plasmids kindly provided by J. Berman (plasmid maps can be found in the Appendix). Amplified plasmid cassettes include the coding sequence for one of the fluorescent proteins and the *HIS*1 gene for selection.

PCR amplification on the example of P_{SOD5}-GFP construct: for amplification of P_{SOD5}-GFP sequence we used F1.1CaSod5 and R2CaSod5 primer. With these primers, we introduced the overlapping sequence into the PCR product, enabling us the insertion of the construct via homologous recombination behind the *SOD*5 promoter (Fig.III.1.1).

The amplification was done with a polymerase with proofreading activity, the phusion polymerase. The amplified fragments (Fig.III.1.2) were used to replace one allele of the target gene (Fig.III.1.1).

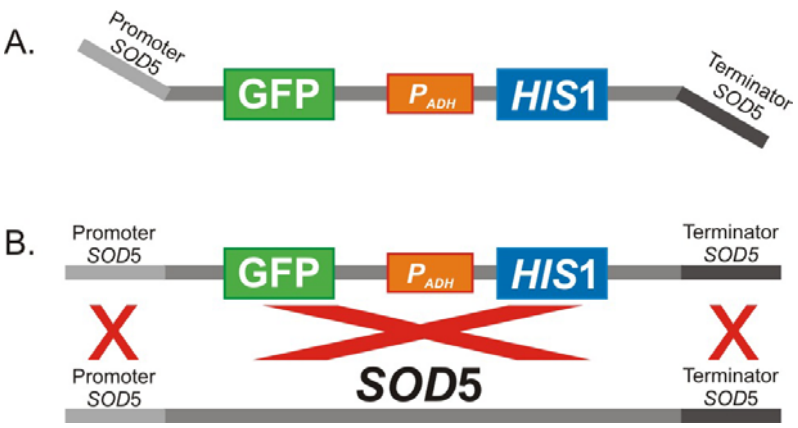


Fig.III.1.1 A schematic overview of Sod5 promoter-tagging.

A. Fragment used for transformation was PCR amplified using the F11CaSod5 and R2CaSod5 primers.

B. The *SOD*5 sequence is replaced by GFP and *HIS*1 via homologous recombination of the flanking reagions.

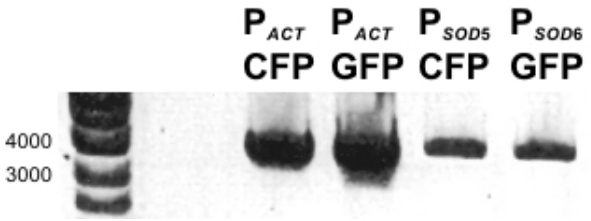


Fig.III.1.2. : PCR Products for transformation

PCR products were amplified using F1.1 CaAct and R2 CaAct or F1.1 CaSod5 and R2 CaSod5 or F1.1 CaSod6 and R2 CaSod6. Primers from pMG1646, pMG1656, pMG1801 plasmids. Plasmids and separated on a 1% agrarose gel to check for amplification efficiency.

We used our construct to investigate the expression of the *SOD5* gene under different conditions (see below). We performed experiments to induce the promoter and to compare the expression rate. The condition inducing the strongest activity was used in further experiments.

The promoter induction with FCS and NaCl, pH8 was done in different ways. For the induction with 10% FCS, we diluted the overnight culture 1:100, grew cells for 1h at 30°C and than added 10% FCS and switched to 37°C for another 3h. For the induction with NaCl and pH, we grew diluted overnight culture for 3h at 30°C to OD_{600} = 0,8 and replaced media by YPD with pH8 (NaOH) and 0,3M NaCl for 1 hour.

We confirmed previous findings that the *SOD*5 promoter is induced with increasing pH in YPD and by adding of NaCl at the final concentration of 0,3M (Martchenko et al., 2004). (Fig.III.1.3). These conditions mimic the environment inside the phagosome where we have both: increased pH and increased salt concentrations. The highest induction of *SOD5* under this condition is explainable through superoxide dismutase activity in defence against phagosome-mediated killing. SOD5 is upregulated during phagocytosis (Fradin et al., 2005).

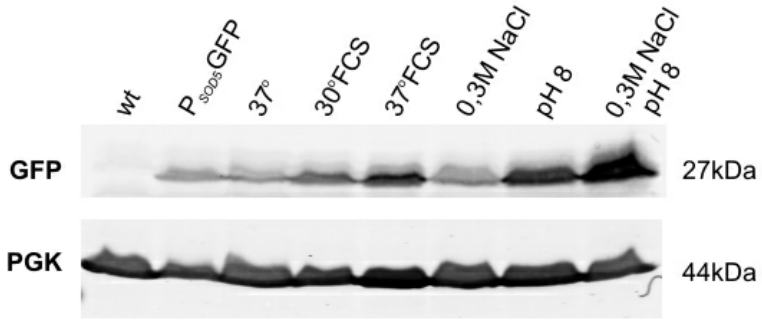


Fig.III.1.3 Comparison of different conditions for *SOD*5 promoter induction

TCA protein extracts were performed and separated on a 10% SDS Page, an equivalent of 0,5 OD_{600} was loaded on each lane, from left to right: (1) wild type, (2) strain with GFP behind the *SOD*5-promoter untreated, (3) P_{SOD5}-GFP grown at 37°, (4) grown at 30° with 10% FCS, (5) grown at 37°C and 10% FCS, (6) grown at 30°C and 0,3M NaCl, (7) at 30°C and pH8, (8) grown at 30°C and pH8 and 0,3M NaCl. GFP fusions were detected with an anti- GFP monoclonal antibody (Roche), to assess equivaltent loading anti-PGK antibodiy was used (kindly provided by Karl Kuchler).

According to these results the *SOD*5 promoter is highly induced under osmotic and pH stress. In our future experiments, we induced *SOD*5 promoter with 0,3M NaCl and pH8.

A Candida strain expressing P_{SOD5}-GFP was used for the detection of the promoter induction under the fluorescent microscope. The promoter was induced with 0,3M NaCl and ph8, and we were able to detect expression of GFP in the P_{SOD5}-GFP strain after the promoter induction. The same strain without induction of *SOD*5 expression delivered no fluorescence signal (Fig.III.1.4).

Taken together, we were able to construct a *C. albicans* strains expressing XFP proteins under constitutive or SOD promoters. Using the strain expressing P_{SOD5}-

GFP, we confirmed previous findings about the *SOD*5 promoter induction under osmotic stress and increased pH.

A.

B.

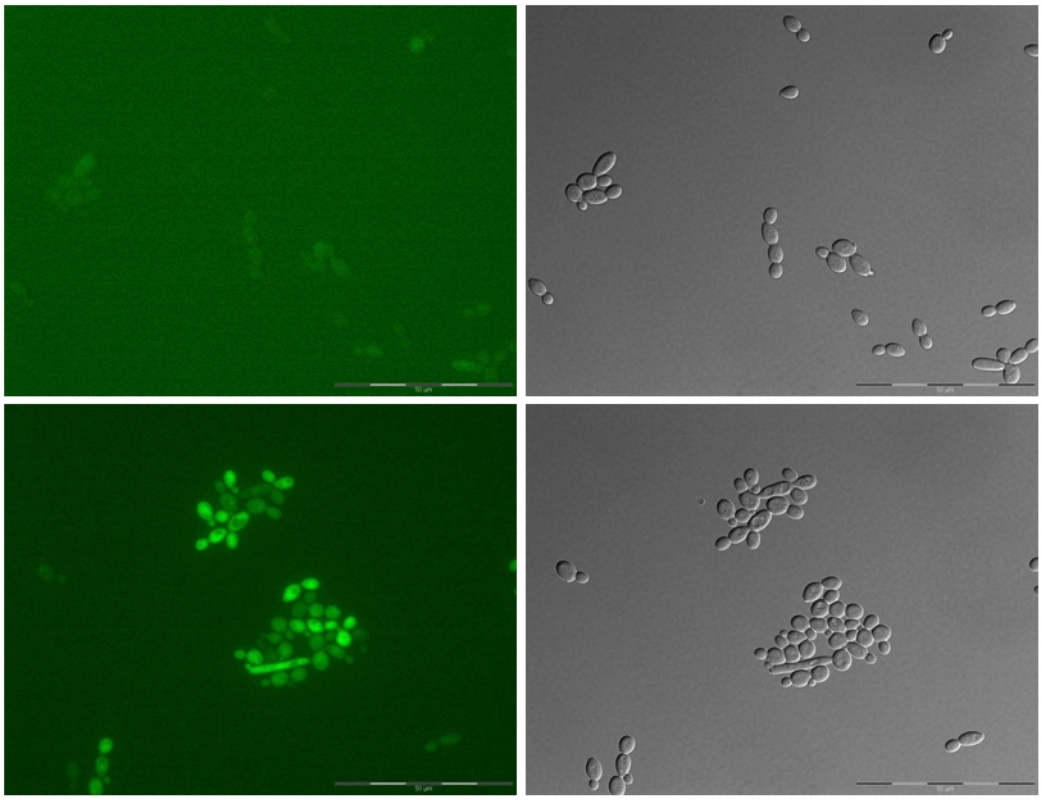


Fig.III.1.4 Microscopy pictures.

A. KY-Ca010 strain (P_{SOD5}-GFP) not induced in eGFP filter and in transmission.

B. KY-Ca010 strain (P_{SOD5}-GFP) induced by yeast media with pH8 and 0,3M NaCl.

Slides were observed in eGFP and in contrast phase filters. Cells were not fixed and observed direct after promoter induction.

III.2. Studies of Sod5 localization

1. Internal tagging of Sod5 with green fluorescent protein (GFP)

Cloning strategy

To determine the subcellular localization of Sod5 in cells, and also for the immunodetection of Sod5 protein on Western blots, we decided to tag Sod5 with the fluorescent protein. We constructed in frame fusion of a green fluorescent protein (GFP) that had been engineered for optimal expression and quantum juld in *C. albicans*.

Sod5 is a GPI-anchored protein; therefore we had to consider the localization signal sequences at the both termini of Sod5. Precursors of GPI-anchored proteins have a classical signal sequence for the import into the ER at their N-terminus and a C-terminal signal for GPI anchor (Fig.III.2.1). Both signals are cleaved after transport and GPI targeting, therefore a classical tagging on either the C- or N-terminus is impossible. GFP was then inserted at the N-terminus of protein after the first 16 amino acids or at the C-terminus before the C-terminal 37 residues.

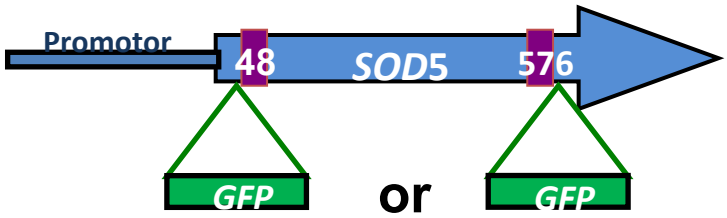


Fig.III.2.1 Schematic overview of Sod5 GFP-tagging.
Two different positions were chosen for tagging of Sod5 with GFP. On the N-terminal site GFP was introduced behind the 48th nucleotide; on the C-terminal site GFP was cloned behind the 576th nucleotide.

For the homologous recombination of Sod5-GFP into the corresponding genomic locus, we used the pSFS2a-Sat1-FLP plasmid (Reuss et al., 2004). The *SAT1*-flipping plasmid pSFS2a-Sat1-FLP contains a dominant nourseothricin resistance marker (*caSAT1*) for the selection of transformants, and a *C. albicans*-adapted *FLP* gene under control of a maltose promoter that allows the subsequent genomic excision of the cassette, because it is flanked by FRT target sequences. FLP-mediated excision of the *SAT1* flipper cassette is achieved by simply growing transformants in a medium containing maltose, activating the maltose promoter P_{MAL2}. This results in expression of the flippase and leads to excision of the nourseothricin resistance marker. As a control for positive transformants, cells were plated on YPD medium and YPD-Nat medium. The flipping was successful in colonies unable to grow on YPD-Nat plates.

We amplified the *SOD5* gene sequence and 500bp promoter from genomic DNA of the SN152 strain using the oligonucleotides 55_caSOD5 and SOD5_ct_Not (sequence can be found in the Table1 p.78-80). For amplification, we used Phusion polymerase to avoid mutations in the coding sequences. We cloned the amplified *SOD*5 PCR product into the pGEMT vector resulting in pGEMT-Sod5ct. The next step was performed to introduce restriction sites into *SOD*5. Since we had two different positions for the GFP insertion, we used either the Sod5-KasI-48s and the Sod5-KasI-48as primers for the KasI restriction side insertion behind the first 16 amino acids or the Sod5-KasI-575s and the Sod5-KasI-575as primers for the restriction side insertion before the last 37 residues. The *GFP* gene was amplified and KasI restriction sites were introduced at the ends. The amplified pGEMT-Sod5ct vector and *GFP* were digested with KasI and ligated. The correct vector : insert ratio was calculated(Fig.III.2.2.a):

$$\frac{\text{ng vector x kb size of insert}}{\text{kb size of vector}} \text{ x insert:vector molar ratio = ng of insert}$$

Fig.III.2.2a. Formula for calculating the optimal vector to insert ratio for ligation.

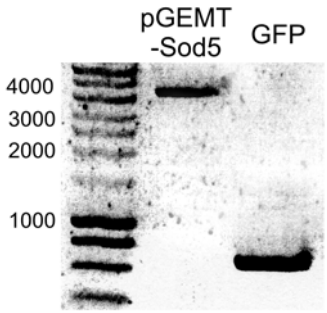


Fig.III.2.2b. Vector and insert.
pGEMT-Sod5 (3713bp) vector digested with KasI and phosphatase treated and GFP (714bp) digested with KasI were separted on a 1% agarose gel.

We transformed the pGEMT-Sod5-GFP vector into competent *E.coli* and checked the constructs via colony PCR and sequencing. The pGEMT-Sod5ct plasmid and the pSFS2a-Sat1-FLP were digested with restriction enzymes NotI and SacI and the 1949bp *SOD*5-GFP fragment was gel purified and ligated with pSFS2a-Sat1-FLP vector (7488bp). Before ligation the pSFS2a-Sat1-FLP vector was treated with phosphatase.

The next step was to bring the 3'*SOD*5 fragment into pSFS2a-Sod5-GFP vector. We amplified 3'*SOD*5 fragment in the first PCR, pSFS2a-Sod5-GFP in the second and fused these two fragments in the third PCR reaction (Fig.III.2.3). For amplification of the 3'*SOD*5 region we used FRT-S5term and 33CaSod5 primers. The pSFS2a-Sod5-GFP vector fragment was amplified with 55CaSod5 and FRT-f.

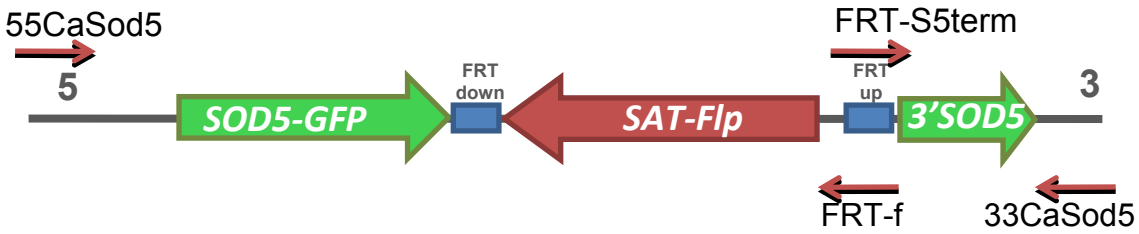


Fig.III.2.3. Schematic image of the end construct that was used for the transformation.
In the final PCR reaction, the *SOD*5-GFP fragment was fused (amplified with 55CaSod5 and FRT-f primers) with the *SOD*5-terminator fragment (amplified with FRT-S5term ans 33CaSod5 primers).

We fused 3'*SOD*5 and pSFS-2a-*SOD5*-GFP fragments in the third the Phusion polymerase PCR reaction with an increased $MgCl_2$ concentration [3mM]. The PCR product was transformed into the competent *sod*5Δ/Δ SN152 and into the wild type control strain. The correct genomic integration into the corresponding locus was verified by colony PCR (Fig.III.2.4). The strains carrying positive constructs were inoculated in maltose to loose the *SAT*1 cassette. Futhermore, strains were also inspected by Southern blot analysis for the correct genomic integration (data not shown).

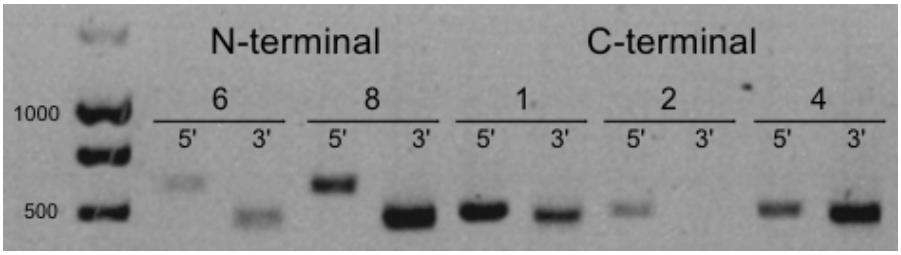


Fig.III.2.4. Colony PCR performed to identify positive transformants.
Using 5CaSod5 and YFP_100as primers for the 5'-prime end and 3CaSod5 and SAT108a primers for the 3'-prime end, we were able to identify two positive transformants for each internal tagging construct. Number 6 + 8 for N-terminal and 1 + 4 for C-terminal tagged construct.

We had two positive construct for the N-terminal tagged Sod5 and two positive for the C-terminal tagged construct.
The constructs with the N-terminal tagged Sod5-GFP (KY-Ca010), which were picked for further studies, were named clone 6.8; clone 6.10; clone 8.8 and clone 8.10.
The constructs with C-terminal tagged Sod5-GFP, which were used for further studies, were clone 1.1; clone 1.15; clone 4.4 and clone 4.13.

2. Determination of Sod5-GFP functionality

Reactive oxygen species (ROS) assay

To determine whether the Sod5-GFP fusion proteins are functional, we performed a ROS assay. As published by our group *C. albicans* Sod5 plays an important role in defence against oxidative stress and is able to convert harmful superoxide from macrophages into H_2O_2 (Frohner et al., 2009). Hydrogenperoxide can be then converted to H_2O and O_2. The ROS assay was established to measure the accumulation of superoxides and other ROS during the innate immune response to *C. albicans*.

For the ROS assay, we used a luminol-dependent chemiluminescence assay. During the infection of macrophages with *C. albicans,* NADPH oxidase is activated, which leads to rapid ROS production (DeLeo et al., 1999). Luminol is a chemical that exhibits chemiluminescence, when mixed with an appropriate oxidizing agent. In other words, the oxidation of luminol by ROS leads to chemiluminescence. Luminol is used to measure total ROS. The chemiluminescence is proportional to the amount of ROS produced.

Zymosan is a cell wall component of the yeast *S.cerevisiae* known to induce ROS. For this reason, zymosan was used as a positive control for ROS assays. The signal can be detected by a luminescence plate reader (Wallac 1420 VICTOR™ Multilabel counter).

The ROS assay was established in our lab to study the role of *C. albicans* superoxide dismutases, especially Sod4, Sod5 and Sod6, in the defence of *C. albicans* against host immune system. We used this assay to prove the functionality of Sod5-GFP protein. The accumulation of superoxides was compared between the wild type strain, *sod5*Δ/Δ strain, *sod5*Δ/Δ*::SOD*5 and *sod5*Δ/Δ*::SOD*5-GFP strains. The experiment was carried out as follows: 100µl from a $4x10^5$ /ml BMDM cell suspension were distributed to each well of 96-well plate. *C. albicans* strains were washed in PBS, diluted in HBSS and distributed to the wells. *C. albicans* induces ROS in BMDMs at an MOI (multiplicities of infection) from 2:1 to 10:1 (fungi to macrophages). For our experiment, we used an MOI 5:1 and 2,5:1. Zymosan (100µg/ml) was used as a positive control. Afterwards, 50µl of HBSS containing 200µM luminol and 4U HRP were added to start the reaction. Chemiluminescence was measured at 2,5min intervals in WallacVictor3 plate reader for at least 90min.

For our experiment, we expected the same ROS levels in *sod5*Δ/Δ:*SOD*5 and *sod5*Δ/Δ:*SOD*5-GFP when Sod5-GFP is functional protein. As expected, we observed no increased ROS accumulation in macrophages co-cultured with *sod5*Δ/Δ:*SOD*5-GFP strain at an MOI 2,5:1 (Fig.III.2.5.A). The ROS accumulation was similar to ROS levels during infection with the wild type strain or *sod5*Δ/Δ:*SOD*5 revertant strain. At this MOI the

functionality of *sod5*Δ/Δ strain was restored by Sod5-GFP protein. Sod5-GFP was still strong enough to detoxify the total ROS produced by BMDMs. This led us to conclusion that Sod5-GFP is functional. However, at an MOI 5:1, the functionality of *sod5*Δ/Δ strain was not completely restored by Sod5-GFP (Fig.III.2.5.B). The accumulation of superoxides in *sod5*Δ/Δ:*SOD5*-GFP was higher than that in the *sod5*Δ/Δ:*SOD5* strain, but lower than in *sod5*Δ/Δ strain.

The higher ROS levels at an MOI 5:1 might be explained due to the fact that the extracellular tag with GFP is increasing ROS, because ROS can be generated by GFP (Goto et al., 2003). We performed a survival assay to confirm the results obtained with the ROS assay.

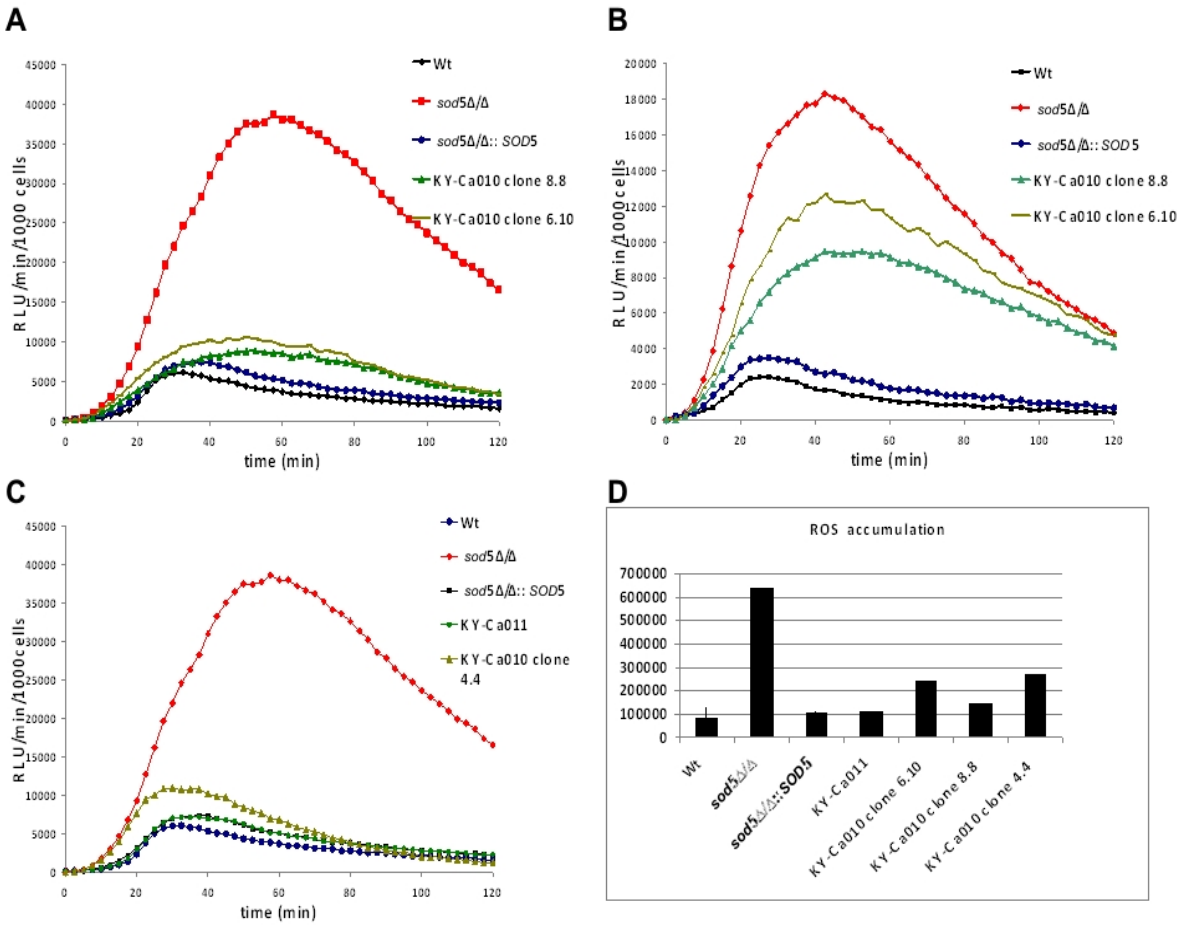


Fig.III.2.5. **ROS accumulation during infection with *C. albicans***.

A-B. ROS measurement by luminal-dependent chemiluninescence at 37°C in 2,5min intervals.

A. ROS accumulation after stimulation of BMDMs with wild type, *sod5*Δ/Δ (CA-IF019), *sod5*Δ/Δ::*SOD5* revertant strain (CA-IF027) and *sod5*Δ/Δ::*SOD5*-GFP (KY-Ca010) strains at an MOI 2,5:1. Similar levels of ROS are observed in wild type, *sod5*Δ/Δ::*SOD5* and in two different clones from *sod5*Δ/Δ::*SOD5*-GFPstrain.

B. The figure shows the ROS accumulation during the infection with the same strains at an MOI 5:1. The ROS levels by *sod5*Δ/Δ::*SOD5*-GFP strain clones are higher compared to wild type strain but still lower compared to the *sod5*Δ/Δ::*SOD5* strain.

C. Stimulation of BMDMs with wild type, *sod5*Δ/Δ (CA-IF019), *sod5*Δ/Δ::*SOD5* revertant strain (CA-IF027) *SOD5*-GFP (KY-CA011) and *sod5*Δ/Δ::*SOD5*-GFP (KY-Ca010 clone 4.4) at an MOI 2,5:1.

D. Quantification of the total ROS release between 10 and 50 min by calculating the area under the curve (moi 2,5:1). The highest accumulation was detected by *sod5*Δ/Δ (CA-IF019) deletion strain, the strains with *SOD5*-GFP showed small differences in ROS accumulation compared to the wild or revertant strain.

End point dilution assay

In addition to the ROS assay, we also used the end point dilution assay to prove the functionality of the Sod5-GFP protein. Sod5 is important for the detoxification of superoxide anions produced by macrophages during the phagocytosis. If Sod5 is functional it helps *C. albicans* to survive in the interaction with macrophages. Therefore, this protein has a direct role in the protection of *C. albicans* against oxidative stress.

To analyze the survival rate of *C. albicans* cells exposed to macrophages, we used an end-point dilution survival assay. This survival assay is based on the simple counting of viable *C. albicans* colonies after the interaction with macrophages. Colonies are visible two days after infection of BMDM with *C. albicans* stains in wells with the highest dilution of *C. albicans*. They can be counted and compared between the different stains used for assay.

The experiment was carried out as follows: The overnight culture of *C. albicans* grown in YPD was washed twice in PBS, resuspended in DMEM + 10% FCS and diluted to $2x10^6$ cells/ml. *C. albicans* suspension was diluted 4-fold in serial dilution steps. Fifty microliters of the suspension were added to the next well containing 150µl medium only or medium with macrophages. Afterwards plates were incubated for 48 h at 37°C in 5% CO_2 atmosphere. To visualize colonies, we stained them with crystal violet, using a 0,2% solution. Colonies were counted and the amount of colonies in the wells with and with out macrophages were compared.

For the experiment we used the wild type SN152 strain, the revertant strain, the deletion strain *sod5*Δ/Δ as controls and *sod5*Δ/Δ::*SOD5*-GFP strain. Three independent survival assays were performed. The data obtained from previous experiments showed that 60-70% of wild type cells are able to survive in the presence of macrophages. The survival rate by *sod5*Δ/Δ mutant is about 25-30%. The revertant strains have almost the same viability like the wild type. To test the functionality of Sod5-GFP, we compared the survival rate between wild type, *sod5*Δ/Δ, revertant strain and *sod5*Δ/Δ*::SOD5*-GFP strain. We expected similar viabilities of our Sod5-GFP and the wild type or to the revertant strain.

If Sod5-GFP is a functional protein, it should restore the viability of a *C. albicans sod5*Δ/Δ deletion strain. In our assay, we could not observe any significant differences between the wild type, the revertant and the Sod5-GFP strains. This led us to the conclusion, that Sod5-GFP is a functional protein. The viability of Sod5-GFP strains was similar to the wild type and revertant strains.

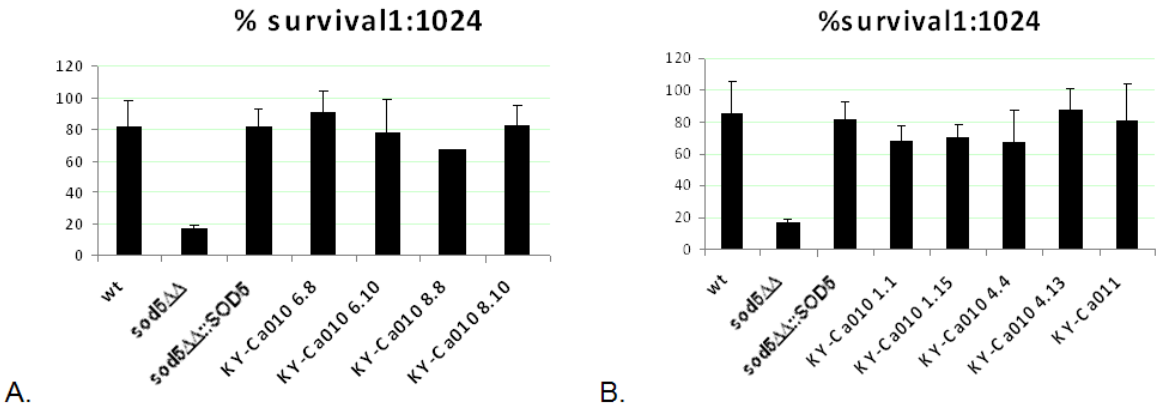
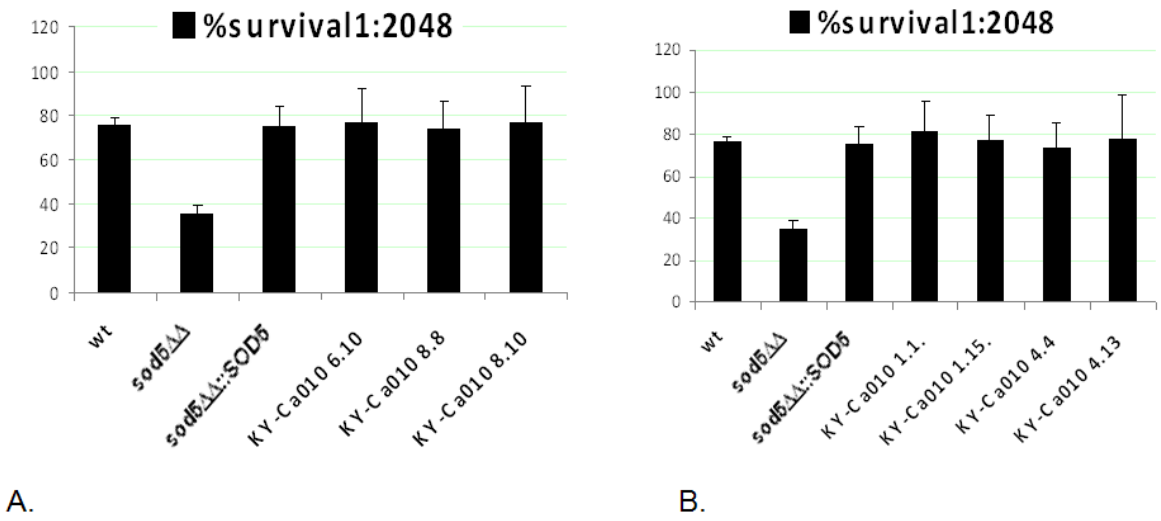


Fig.III.2.6. **End-point dilution assay**. Co-incubation of wild type macrophages with different *C. albicans* strains. Strains with re-integrated Sod5-GFP were tested for their viability compared to wild-type and to the *sod5*Δ/Δ::*SOD*5 strain. We used wild type and *sod5*Δ/Δ strains as a control. The viability of the strains was defined through counting and comparing of the equivalent dilution from the viable colonies and colonies survived after the incubation with macrophages. The viability of wild type *C. albicans* exposed to macrophages is about 80%. The Sod5-GFP strains show similar viability of about 70-80%.

A. Figure shows strains tagged on the N-terminus (KY-Ca010 clones 6 and 8)

B. Figure shows strains tagged on the C-terminus of the protein (KY-Ca010 clones 1 and 4). We tested a wild type strain heterozygote Sod5-GFP allele (KY-Ca011). The behaviour of this strain was similar to Sod5-GFP strains in *sod5*Δ/Δ background.



A. B.

Fig.III.2.7. **End-point dilution assay**. The experiment was repeated using the higher dilution of *C. albicans* strains for counting. We co-incubated wild type macrophages with different *C. albicans* strains. Strains with re-integrated Sod5-GFP were tested for their viability compared to wild type and to the *sod5*Δ/Δ::*SOD*5 strain. We used wild-type and *sod5*Δ/Δ strains as a control. The viability of the strains was defined through counting and comparing of the equivalent dilution from the viable colonies and colonies survived after the incubation with macrophages.

A.Figure shows strains tagged on the N-terminus (KY-Ca010 clones 6 and 8)

B.Figure shows strains tagged on the C-terminus of the protein (KY-Ca010 clones 1 and 4).

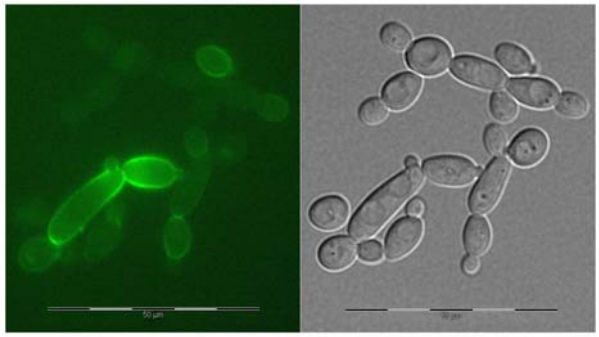
End-point dilution assay, performed to test different Sod5-GFP strains for the functionality of Sod5, showed that the viability of these strains is similar when compared to the wild-type or to *sod5*Δ/Δ::*SOD*5 strain. The viability of these strains is an important criterion for the functionality of Sod5 as we know that the survival rate of *C. albicans* strains lacking Sod5 is about 30% reduced in the presence of macrophages (Frohner et al., 2009). The viability of *sod*5Δ/Δ strain was restored by integration of Sod5-GFP and similar to the wild type. No significant differences between the two different positions of the GFP tag were detected. The viability of all strains was about 70%.

3. Detection of protein localisation under the fluorescence microscope

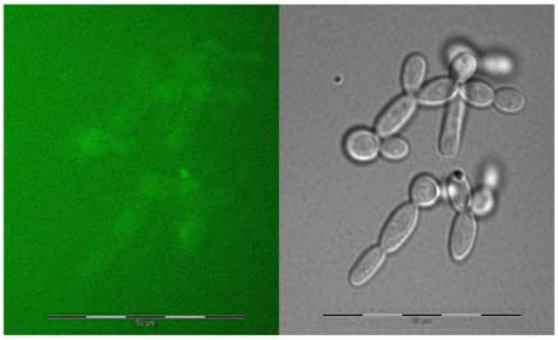
For detection of Sod5-GFP in the fluorescence microscope in the living cells, an overnight culture, was diluted and induced with YPD + 0,3M NaCl and pH8. Cells were examined under the fluorescent microscope with out any fixation with 490nm excitation and 525nm emission filters. Cells expressing the Sod5-GFP protein were fluorescent at the cell periphery as expected for GPI-anchored proteins targeted to the cell wall or to the plasma membrane. We were able to detect fluorescence at / or close to the cell surface. Cells in which Sod5 promoter was not induced, did not show fluorescence.

Unfortunately, the resolution of the fluorescence microscope (Fig.III.2.8) did not allow us to identify the exactly localization of the protein; to differ between the plasma membrane and cell wall.

A.



C.



B.

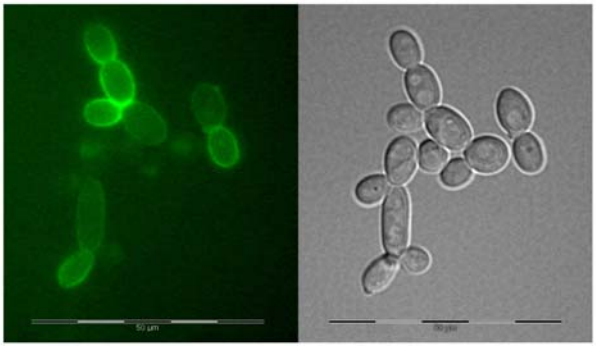


Fig.III.2.8. Microscopy pictures show the localisation of protein on the cell surface. Images were taken using Olympus cellR imaging fluorescence microscope.

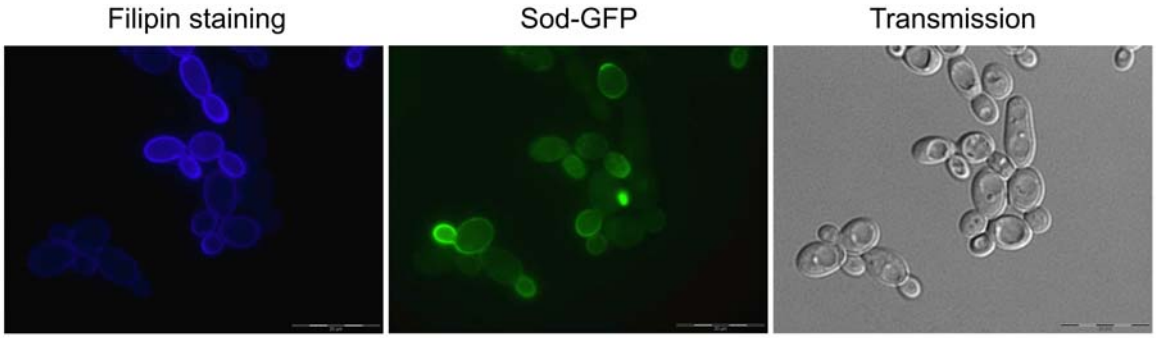
A. *sod*5Δ/Δ::*SOD*5-GFP strain (KY-Ca010 clone 6.8). The Sod5-GFP protein expression was induced by adding YPD media with 0,3M NaCl and pH8.

B. *sod*5Δ/Δ::*SOD*5-GFP strain (KY-Ca010 clone 8.8). The Sod5-GFP protein expression was induced by adding YPD media with 0,3M NaCl and pH8.

C. *sod*5Δ/Δ::*SOD*5-GFP strain (KY-Ca010 clone 6.8). The expression of protein was not induced.

To check whether the protein is expressed at the plasma membrane or in the cell wall, we used filipin to stain the plasma membrane of *C. albicans* and to compare the signal from the plasma membrane with the signal from the Sod5-GFP protein. Filipin is a chemical that stains specifically the cholesterol in the plasma membrane. Filipin staining was performed as described in Materials and Methods.

Using the Olympus cellR imaging fluorescence microscope, we were unable to detect the differences between signals coming from the filipin-stained plasma membrane and the Sod5-GFP signal. The resolution from this microscope did not allow us to draw any conclusions about the localization of the protein. Detecting the filipin under the confocal microscope was difficult because of the fluorescent instability of filipin (Fig.III.2.9)



B. Fig.III.2.9 **Filipin staining**. *sod5*Δ/Δ::*SOD5*-GFP strain (KY-Ca010 clone 6.8) where the Sod5-GFP protein expression was induced by adding YPD media with 0,3M NaCl and pH8. The overlay of images from the Filipin stained plasma membrane and images taken in eGFP channel using Olympus cellR imaging fluorescence microscope showed no additional evidence about the localization of the protein.

We were able to detect Sod5-GFP protein either in the plasma membrane or close to the cell surface, but filipin staining, as a the attempt to get additional evidence about the localization of the protein failed. The images taken from the filipin-stained plasma membrane and overlay with the images taken in the eGFP channel showed no conclusive results.

4. Detection of protein localization using cell fractination

First, we performed experiments to prove if we are able to detect Sod5-GFP protein on the Western blots. Therefore, we used TCA protein extracts from our stain, which were either induced or not. Detection of protein occurred by all four clones with Sod5 protein tagged on the N-terminus with GFP (KY-Ca010, clones 6.8; 6.10; 8.8; 8.10) after induction of promoted with 0,3M NaCl and pH8. P_{SOD5}-GFP strain was used as a positive control for induction (Fig.III.2.10).

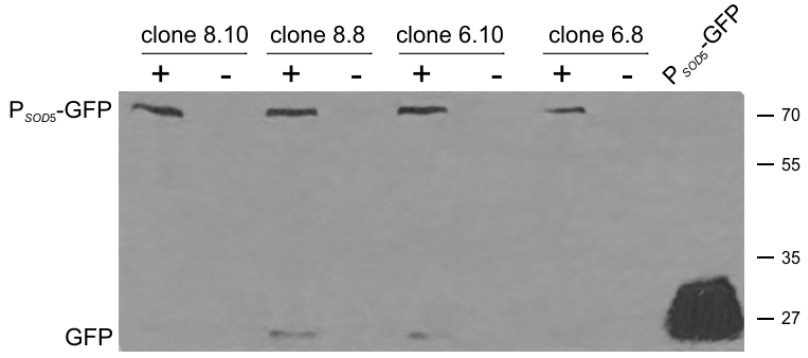


Fig.III.2.10. **TCA extracts from *C. albicans* N-terminal constructs for immunoblotting**. Lane1: KY-Ca010 clone 8.10 induced; lane2: KY-Ca010 clone 8.10 not induced; lane3: KY-Ca010 clone 8.8 induced; lane4: KY-Ca010 clone 8.8 not induced; lane5: KY-Ca010 clone 6.10 induced; lane6: KY-Ca010 clone 6.10 not induced; lane7: KY-Ca010 clone 6.8 induced; lane8: KY-Ca010 clone 6.8 not induced; lane9: P_{SOD5}-GFP induced. Induction occurred with pH8 and 0,3M NaCl.

Many yeast GPI-anchored proteins are cell wall-localized, but there are also few in the plasma membrane (Richard and Plaine, 2007). Among the proteins with enzymatic activities in *C. albicans* both are found, GPI-anchor targeted to the cell wall and non-covalent secreted proteins. In order to determine the exact localisation of Sod5, we used a subcellular fractionation protocol. Using the monoclonal anti-GFP (Roche) antibody, we detected Sod5-GFP by Western blotting. This protocol enabled us to isolate in different steps proteins from the cell wall and the plasma membrane. Depending on the fraction that contains the protein we were able to identify the localization. Crude cell lysats were prepared after breaking cells without a centrifugation step, this fraction contains all proteins. Proteins not covalently linked to the cell wall were extracted in an SDS fraction. For the extraction of proteins linked to the β-glucans in the cell wall, we used digestion with laminarinase and zymolyase. Both enzymes are β-glucanases that are able to digest β-glucan linkages. GPI-anchored proteins are linked to β-1-6 glucans in the cell wall of *C. albicans*. Digestion with β-glucanases is a good way to determine if the protein is targeted to the cell wall or to the plasma membrane. Protein extracts were than separated on 12% SDS-PAGE gel. For the immunodetection the monoclonal anti-GFP antibody was used (Fig.III.2.11).

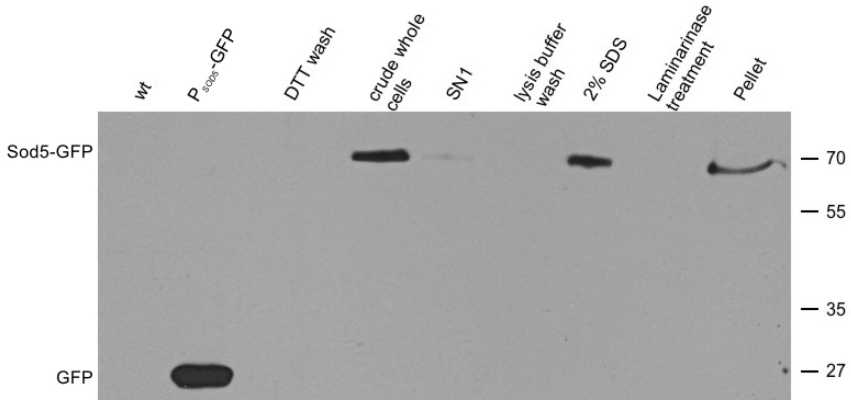


Fig.III.2.11. **Cellular fractionation protein extraction**. In the first lane whole cell protein extract from the wild type, in the second lane whole cell protein extract from the P_{SOD5}-GFP strain, in the following lanes the fractionation from Sod5-GFP strain after the wash with buffer containing 5mM DTT, the crude whole cell protein extract in the lane 4, lane 5 – supernatant obtained after centrifugation from the whole cell protein extractes, lane 6 – supernatant obtained after washing pellet in lysis buffer, lane 7 – pellet boiled with 2%SDS, lane 8 – supernatant obtained after incubation of pellet with laminarinase, lane 9 – pellet boiled with sample buffer.

We were able to detect the protein in crude cell fraction and a small amount in the supernatant from this fraction. The largest amount of protein was isolated after boiling all pellets with 2%SDS, whereas we were unable to detect protein after digestion with laminarinase. These results suggest that Sod5 is localised in the cell wall and is either

non-covalently bound to the cell wall or belongs to the small number of cell wall proteins that can be extracted from the glucan mesh simply by boiling with SDS.

5. Tunicamycin treatment

A common modification of plasma membrane proteins is glycosylation. GPI-anchored proteins are often glycosylated as well as mannosylated. To check Sod5 for glycosylations, we treated it with tunicamycin. Tunicamycin is an inhibitor of N-glycosylation that works by inhibition of N-acetylglycosamine-1-phosphate transfer to dolichol monophosphate (Kuo and Lampen, 1974). It prevents attachment of N-glycosidic carbohydrate side chains to the proteins and it also inhibits GPI-anchor glycosylation/formation. On our immunoblots, we expected to get a slower migrating form of protein by cells with out tunicamycin treatment and another form of protein that is not glucosylated after treatment and is able to migrate easier through the gel. The expected molecular mass of the Sod5-GFP fusion protein is about 51kDa, formed of the predicted Sod5 molecular weight of 23,6kDa and GFP molecular weight of 27kDa.

As expected, the results showed two isoforms of protein. Protein from the cells without tunicamycin treatment migrated at about 70kDa, whereas protein from the cells treated with tynicamycin showed isoform of a protein that was about 55kDa (Fig.III.2.12). This result confirm the suggestion of Sod5 to be a highly glycosylated protein.

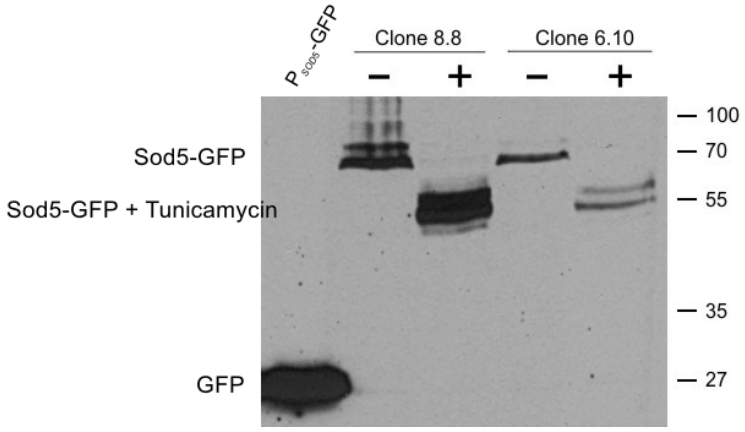


Fig.III.2.12. Tunicamycin from Streptomyces sp. (1µg/ml) treated Sod5-GFP protein extracts. Lane1: P_{SOD5}-GFP; lane2: KY-Ca010 clone8.8 not treated; lane3: KY-Ca010 clone8.8 treated; lane4: KY-Ca010 clone 6.10 not treated; lane5: KY-Ca010 clone 6.10 treated; Proteins treated with tunicamycin showed a shift in mobility, the mass of proteins was about 55kDa.

III.3. Production of anti-Sod4, anti-Sod5 and anti-Sod6 antibodies

1. Cloning of antigens, expression of GST-Sod fusion protein in E.coli

To have more biochemical tools to study *C. albicans* extracelluar SODs, we wanted produce polyclonal antibodies against Sod4, Sod5 and Sod6 proteins, therefore we constructed GST-Sod fusion proteins for antibody production of our three proteins of interest, Sod4, Sod5 and Sod6 from *C. albicans.* We compared the sequence of those proteins and studied the folding of proteins and secondary structures using diverse antigenic prediction programs. We used area of proteins, predicted to be on the surface and thus have antigenic properties. Since all three proteins are homologous and show high sequence similarities we used a certain domain of each protein as antigen source. We were restricted to the areas, which are different between these proteins to avoid cross reactions of anti-sera.

For expression of Sod4, Sod5 and Sod6 proteins as GST fusions in bacteria, we chose 56-59 amino acids from each protein fused in frame to the C-terminus of GST. The fragments were generated by PCR using the primers S5_2TBamHIs and S5_2TXEcoRIas (corresponding names for Sod4 and Sod6). We introduced two restriction sites into this fragment BamHI on N-terminus and EcoRI on C-terminus of fragment. We used the pGEX-5x2 plasmid (see Appendix) and fused the SOD peptide behind GST. Using this vector with GST-Sod-fusion protein localized behind lac-operon, we were able to induce the expression of protein and purify large amounts of this protein.

Fig.III.3.1. Schematic image of the construct that was used for expression and purification of GST-Sod-fusion protein. Expression of GST-Sod protein was induced by adding of IPTG to the media.

Afterwards the fragments were ligated into pGEMT-easy vector. The ligation was performed according to the protocol in Materials and Methods and products were transformed into competent DH5α *E.coli*. Selection occurred on LB-Amp plates containing IPTG and X-Gal. IPTG-inducible promoter in pGEMT-easy plasmid allows the blue-white selection of transformants. We isolated the plasmid DNA from positive colonies, digested it with BamHI and EcoRI enzymes. The fragment was cloned into pGEX5x-2 vector that was also digested with the same enzymes (data not shown). The new ligation reaction was transformed into competent *E.coli*. Obtained colonies were tested on next day by colony-PCR. The constructs were sequenced and those without any mutations used further.

The constructs were used for the expression of GST-fusion protein expression. GST-fused peptide is localized behind the IPTG inducible promoter. The induction of promoter leads to expression of protein. First, to determine the optimal induction time, samples were taken after different time points of induction (0', 20', 40', 60', 90', 120' and 180 min). Proteins were separated in 12% SDS-PAGE gels (Fig.III.3.1).

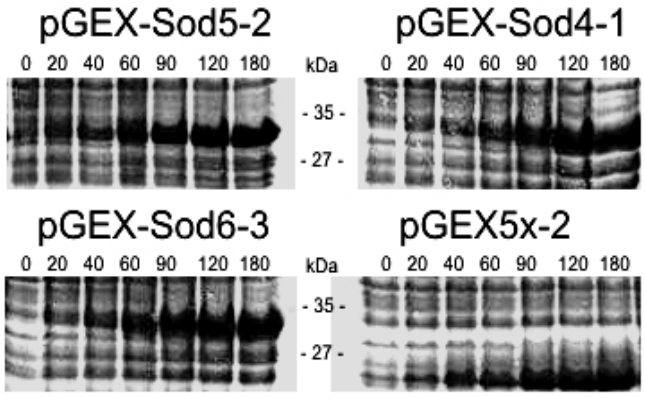


Fig.III.3.1. **The expression of GST-fusion proteins after the induction of promoter**. Sod5-GST on the first gel, Sod4-GST on second and Sod6-GST on the third gel and pGEX5x-2 vector allow on the fourth gel. Samples were taken at time point 0 and then after 20, 40, 60, 90, 120 and 180 min. Expression of protein increased with the time.

To determine if GST fusion proteins are soluble or not, cells were broken after the induction of protein expression and aliquots from pellet and supernatant were compared. Proteins were again separated on a 12% SDS-PAGE gels.

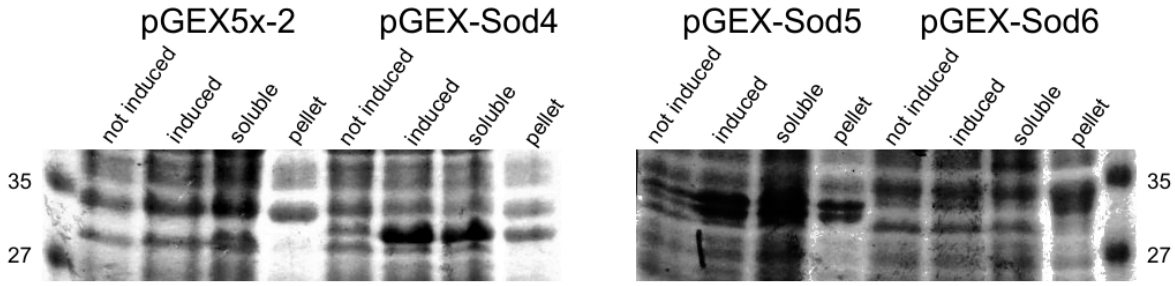


Fig.III.3.2. After comparison of soluble phase and pellet we determined Sod4 and Sod5 as soluble and Sod6 as not soluble proteins. The main amount of Sod4 and Sod5 proteins could be found in the soluble fraction.

Comparison of pellet and supernatant fractions in Commassie-stained SDS-PAGE gels showed that most of the fusion proteins Sod4 and Sod5 were soluble. Sod6 was insoluble; therefore an alternative method for purification was used (inclusion body purification).

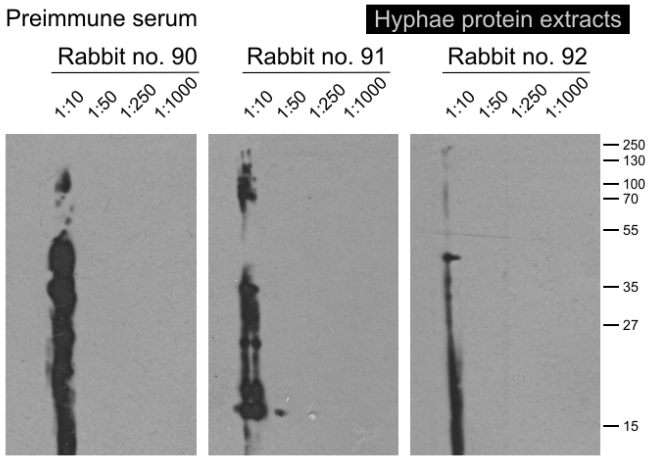
We purified Sod4 and Sod5 fusion proteins with glutathion sepharose beads from 500ml bacterial culture. The expression of GST-fusion protein was induced by adding of 0,2mM IPTG for 80min. Cells were broken by sonication in TpG and DTT. The supernatant was incubated with glutathion sepharose beads overnight. Next morning

protein bond to sepharose beads could be eluted after incubation with the elution buffer by room temperature. After exchanging the buffer to PBS, the purified proteins were used for rabbit immunisation.

2. Immunisation procedure of rabbits

Before immunization, pre-immune sera of the rabbits were tested by immunoblotting for pre-existing antibodies against *C. albicans* proteins. TCA-extracts of SN152 were separated in a 10% SDS-gel and immunoblotting was carried out using different dilutions of pre-immune serum (1:10, 1:50, 1:250, and 1:1000 in blocking milk) (Fig.III.3.3).

A.



B.

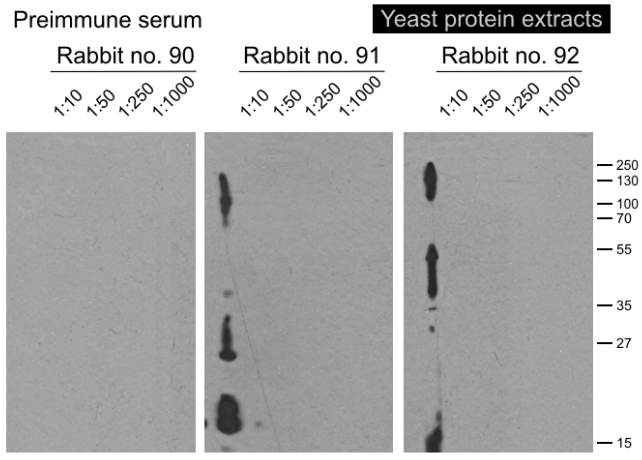


Fig.III.3.3

A. Preimmune serum from rabbits tested on hyphae protein extracts in different dilutions 1:10, 1:50, 1:250 and 1:1000. A signal was only obtained at the 1:10 dilution by all three rabbits. The higher dilutions showed no recognition of protein extracts by preimmune sera.

B. Preimmune serum from rabbits tested on yeast protein extracts in different dilutions 1:10, 1:50, 1:250 and 1:1000. A signal was only obtained at the 1:10 dilution by two of three rabbits. The higher dilutions showed no recognition of protein extracts by preimmune sera.

The rabbits showed cross-reaction with fungal proteins only at the lowest dilution (1:10). No cross-reactions were detected in all other dilution; therefore the rabbits were used for immunization with the GST-Sod fusion proteins.

For the primary injection, we used 500µg of the purified GST-fusion protein mixed with an incomplete Freund's adjuvant (IFA). Boosting was carried out every 3-4 weeks. See schedule of the immunization protocol. After the second boost, the first sera were tested for the presence of anti-Sod antibodies.

Injection/ boosts	Date	Amount injected	Days	Bleeds	Date
Primary injection	29.08.08	500µg			
			18		
1st boost	16.09.08	250µg			
			29		
2nd boost	15.10.08	125µg			
			28	1st	31.10.08
3rd boost	13.11.08	125µg			
			34	2nd	1.12.08
4th boost	18.12.08	130µg			
			21	3rd	8.01.09
5th boost	8.01.09	100µg			
			32	4th	10.02.09
6th boost	10.02.08	50µg			
			21	5th	5.03.09
7th boost	03.03.09	20µg IV			
				final	30.03.09

Table III.3.1: **Immunization schedule**

Figure shows the date of every immunisation/ boosting, and the amount of antigen used for injection mmunisation. The amount of protein was determined using Bradfort method. We used a decreasing amount of antigen for every next boosting.

Figure III.3.4 shows anti-sera of the first bleed tested for the antigen recognitions. Antigen (GST-fusion with one of the SOD proteins) was separated on the 10% SDS-PAGE gel and incubated with different dilution (1:200; 1:500, 1:1000) of the serum from the rabbits. For the Western blot analysis we used MiniProtean II Multiscreen Apparatus (Bio-Rad). The purified antigen was loaded into the gel. Blotting was carried out as usual and the incubation with the sera occurred in the channels of the Multiscreen Apparatus, which allowed us to use economical amaonts of the sera and test different dilution on one membrane. We detected a signal against the antigen in the first sera taken from the rabbits. Futhermore, the recognition signal was depending on the dilution.

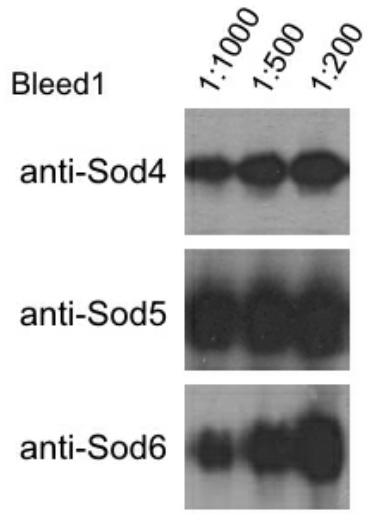


Fig.III.3.4 For the Western blot analyses we used MiniProtean II Multiscreen Apparatus (Bio-Rad) (described in Materials and Methods). 800ng of each purified fusion protein have been loaded in the 10% SDS-PAGE gel. After blotting, membranes were uncubated with the different appropriate dilutions (1:200, 1:500 and 1:1000) of bleed 1 taken from rabbits immunized with Sod4- Sod5- or Sod6-GST fusion proteins. Sera were tested for the recognition of antigen.

To proof the stability of the antibodies, we tried different freezing conditions for the antibodies. The sera were frozen at -20°C, at -80°C and dry frozen and used afterwards for Western blot analysis. We detected no differences between the sera frozen under the different conditions. The antibodies in the sera were stable (Fig.III.3.5).

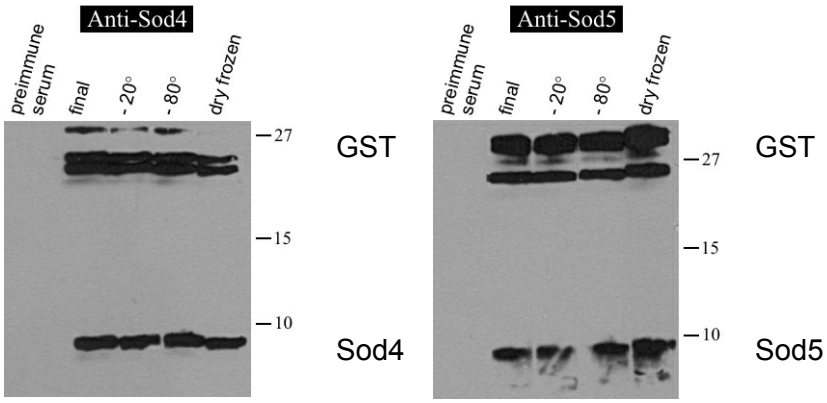


Fig.III.3.5 Sera were frozen under the different condition and used for detection in order to determine the quality of sera after freezing. Aliquots were frozen at -20°, -80° or dry frozen and then used for detection of antigen. 1µg of recombinant proteins digested with FactorXA is loaded into the gel. No differences between aliquots handled under different conditions were detected.

Sera from each bleed were tested for the antibodies against Sod proteins on TCA or whole cell protein extracts. The proteins extracted from the wild-type and from the *sod*4/*sod*5/*sod*6Δ/Δ deletion strains were separated on the 12% SDS-PAGE gel and incubated with the polyclonal sera diluted 1:1000. We were unable to detect a specific recognition of Sod proteins. All three sera recognized unspecific proteins on protein extracts in the size between 40 and 70kDa in wild-type and in knock-out strain (Fig.III.3.6).

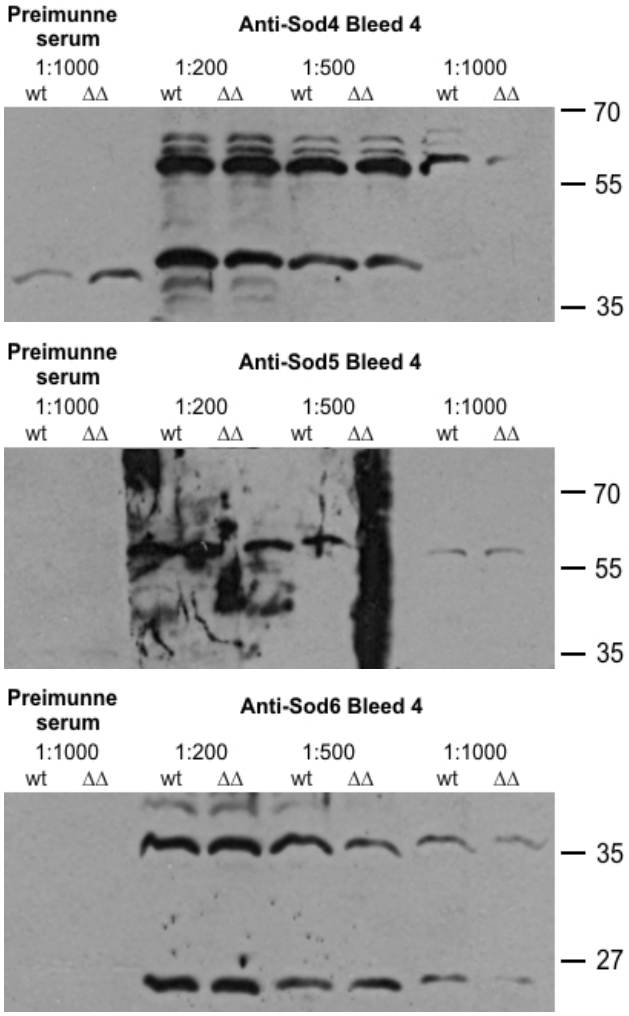


Fig. III.3.6. Sera from bleed IV, used for immunobloting with protein extracts. Sera were tested in different dilutions. There are wild-type and *sod*4/*sod*5/*sod*6Δ/Δ strain protein extracts loaded in alternation. Using the sera we detected signals in wild type and in deletion strains suggesting the unspecificity of the sera.

The seventh boost was carried out by the intravenous injection of 20µg of the protein. After the seventh boost we decided to end the immunisation of rabbits. The titers of antibodies did not significant increase anymore and we still did not obtain specific signals.

3. Indirect immunofluorescence of *C. albicans*

The anti-Sod5 antibodies were also tested for their ability to recognize Sod5 protein via indirect immunofluorescence. Therefore, *C. albicans* wild type and *sod*4/*sod*5/*sod*6Δ/Δ mutant strains were immobilized on concanavalinA coated glass slides, fixed with paraformaldehyde and stained with the polyclonal rabbit anti-Sod5 antibodies followed by anti-rabbit Cy5-conjugated secondary antibodies. We used two different dilutions of rabbit sera (1:100; 1:200) for the immunofluorescence. Moreover, we

performed additional calcoflour white-staining to visualize chitin in the cell wall (Molano et al., 1980; Roncero and Duran, 1985). We detected a signal from the anti-Sod5 antibody stained-cells in wild type and in deletion strain (Fig.III.3.7). Anti-Sod5 antibodies recognized the whole cell wall of C. albicans.

Unfortunately, we did not detect any specific signals with the three polyclonal sera (data not shown). In the case of a specific recognition, we would expect detection of proteins in the wild type but no recognition on deletion strains. No staining was observed with the pre-immune sera (data not shown). All three polyclonal antibodies were unspecific and recognized proteins in the cell wall *C. albicans sod4/sod5/sod*6Δ/Δ deletion strain. We saw no difference between the recognition of both strains by the antibodies.

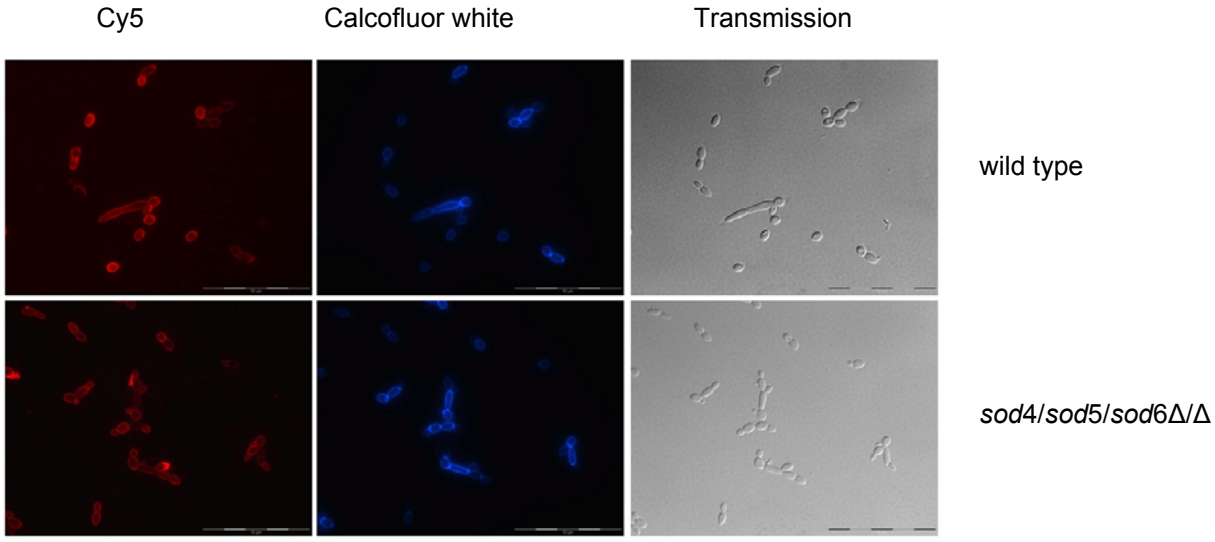


Fig.III.3.7. **Indirect immunofluorescence of *C. albicans***. Indirect immunofluorescence staining was performed as described in Materials and Methods. Sera were diluted 1:100. Wild type and deletion strains were used for immunofluorescence. Additional to antibody cells were also stained with Calcofluor white. Lane 1: wild type cell; lane2: *sod4*/*sod5*/*sod6*Δ/Δ deletion strains. Slides were observed in Cy5, DAPI and contrast phase filters.

4. Affinity purification of anti-Sod4 and anti-Sod5 sera

Affinity purification is a procedure that is often used to purify antibodies from the serum. After the incubation of our sera with protein extracts we obtained several signals that were unspecific recognition. We performed affinity purification to select the antibodies that recognize specifically the GST-fusion protein.

Therefore, GST-fusion proteins were separated in the 12% gel. After the transfer to the nitrocellulose membrane the band of interest was cut out and used for the incubation with the serum. In this step, only the antibodies that specifically recognize

antigen should bind. The antibodies were eluted from the membrane using 100mM Glycin pH2,5 and neutralized immediately by adding 1M Tris/HCl pH8.0.

The purified antibodies were tested for the quality on a Western blot and used for immune fluorescence microscopy. The results for anti-Sod5 affinity purification are shown in Figure III.3.8. We were able to purify anti-Sod5 antibodies. All three eluates, obtained during elution of antibodies bound to the antigen, were tested fore their concentration and fore their ability to recognize the recombinant antigene. In all eluates, the purified antibodies were still able to recognize the antigen and the concentration of antibodies was high enough to use them for Western blot and immunofluorescence experiments.

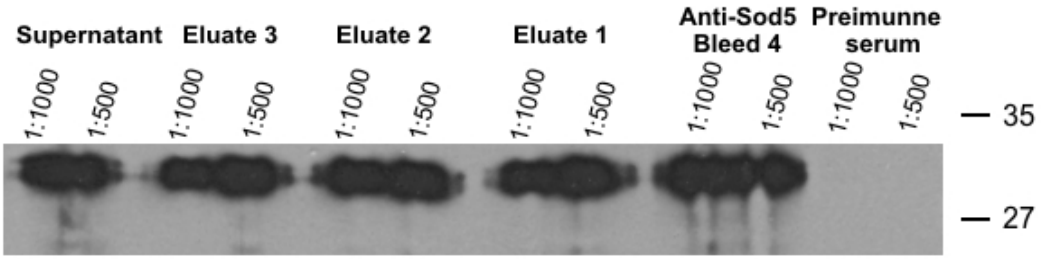


Fig.III.3.8 **Affinity purification of anti-Sod5 antibody**. Recognition of GST-Sod5 fusion protein (1µg protein loaded into the gel) by the anti-Sod5 (bleed4), by the obtained during purification eluates and by the supernatant remaining afterwards. In contrast no recognition of the antigen by preimmune serum was detected. Two different concentration 1:1000 and 1:500 were used, the signal by the higher concentration of the antibody is stronger. For the Western blot analyses we used MiniProtean II Multiscreen Apparatus (Bio-Rad).

Affinity purified anti-Sod5 antibodies were used for immunofluorescence. Even with affinity purified antibodies, we were unable to detect specific signals. Antibodies recognized the whole *C. albicans* cell surface. Moreover, the signals detected on the wild type *C. albicans* and on *sod*4*/sod*5*/sod*6Δ/Δ strains were of comparable intensity. Hence, the signal obtained with anti-Sod5 antibodies were not a cross-reaction with one of other Sod proteins located on the surface of the *C. albicans* cell (Fig.III.3.9).

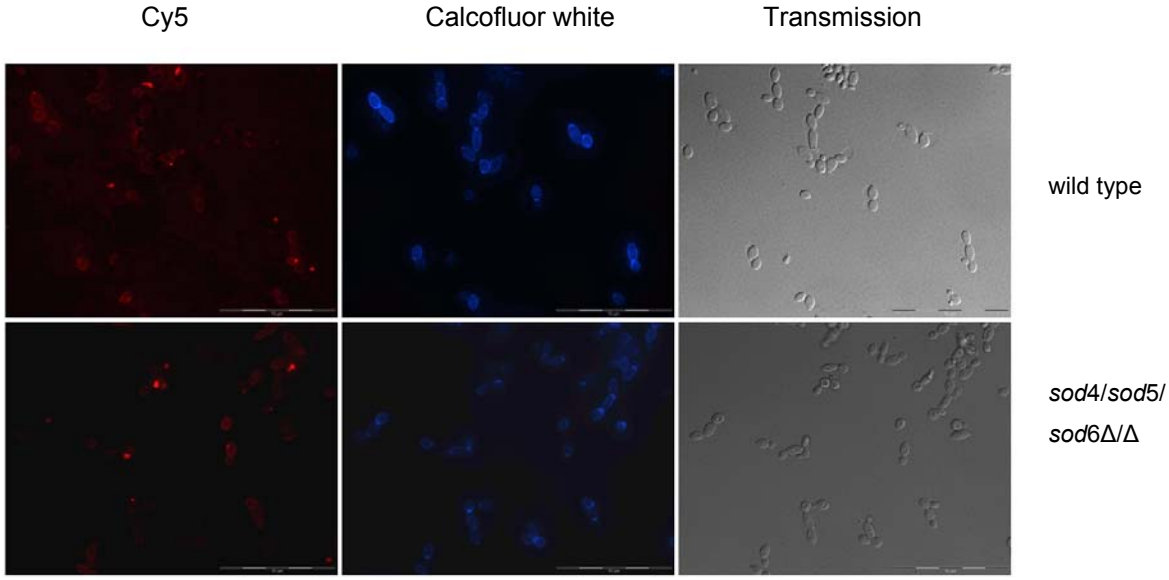


Fig.III.3.9. **Indirect immunofluorescence of *C. albicans*.** Indirect immunofluorescence staining was performed as described in Materials and Methods. Sera were diluted 1:100. Wild-type and deletion strains were used for immunofluorescence. Additional to antibody cells (secondary antibody anti-rabbit Cy5-conjugate) were also stained with Calcofluor white. Lane 1: wild type cell; lane2: *sod4*/*sod5*/*sod6*Δ/Δ deletion strains. Slides were observed in Cy5, DAPI and contrast phase filters.

5. Immunization procedure of mice. Characterisation of mice antibodies

C. albicans is known to be a commensal in human and some animals. For example, *C. albicans* is a commensal in rabbits but not in mice. Considering this, we decided to use the same antigen, GST-fused Sod peptide to immunize mice. We expected the mouse immune response to be more specific for the antigen. Four mice were used for immunization, two for anti-Sod4 antibody production and two for anti-Sod5 (see Fig.III.310 immunization schedule).

Immunisation	Date	Amount	Days	Bleeds	Date
Immunisation	9.07.09	50µg			
			11		
1st boost	20.07.09	50µg			
			10	1. bleed	23.07.09
2nd boost	30.07.09	50µg			
				2.bleed	3.08.09

Fig.III.3.10. **Immunization schedule of the mice**. Mice were immunized each time with 50µg of the antigen. The amount of the antigen was determined by Bradford method (described in Materials and Methods).

After the immunization and the first boost, the first bleed was collected from the immunized animals. The polyclonal sera were tested for the presence of specific antibodies. First, we performed a Western blot and tested mouse sera for the antigen recognition. However, we were unable to detect any antigen recognition with the first bleed (Fig.III.3.11).

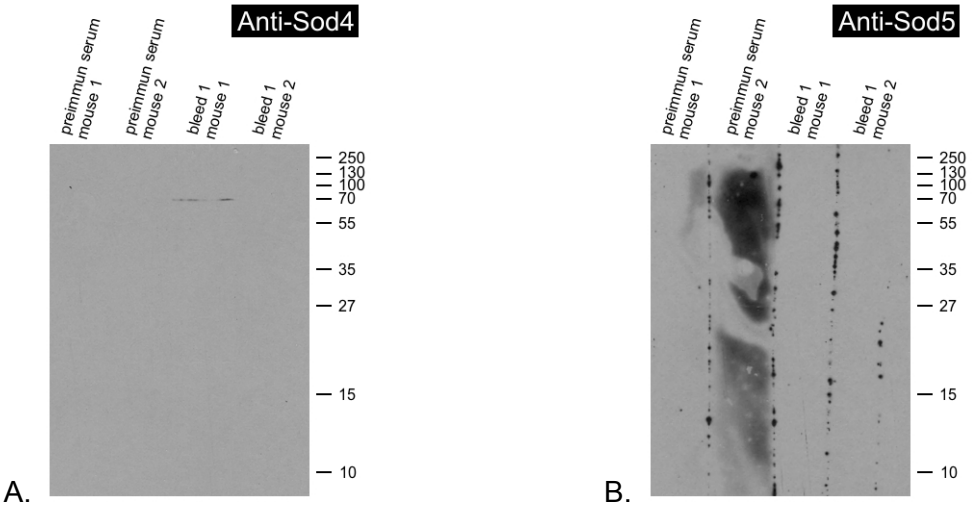


Fig.III.3.11. **Mouse bleed1 tested for the antigen recognition**

A. GST-Sod4 fusion protein digested with Factor Xa protease (1µg protein loaded into the gel) was tested for the recognition by anti-Sod4 antibody. Lane1: preimmune serum from mouse No.1; lane 2: preimmune serum from the mouse No.2; lane 3: first bleed mouse No.1; lane 4: first bleed mouse No.2. No recognition of antigen was detected.

B. GST-Sod5 fusion protein digested with Factor Xa protease (1µg protein loaded into the gel) was tested for the recognition by anti-Sod5 antibody. Lane1: preimmune serum from mouse No.1; lane 2: preimmune serum from the mouse No.2; lane 3: first bleed from the mouse No.1; lane 4: first bleed mouse from the mice No.2 immunized with Sod5-GST fusion protein. Dirt on the right side of the figure on the blot incubated with preimmune serum is probably coming because of bad quality of the serum which was already coagulated.

For this reason, we boosted animals and then tested the next bleed. In our second bleed we had already antibodies inside against the antigen. We detected a clear and strong recognition of GST-fusion protein by the immune serum (data not shown). Since we had an immune response, we tested the sera obtained from immunized mice on protein extracts. Again no specific recognition was seen. We obtained the same signals on protein extracts from the wild type and from the triple deletion strain (for details see chapter below). Sera obtained from mice immunized with GST-Sod4 or GST-Sod5 fusion proteins were unspecific.

The second serum was also used for indirect immunofluorescence, but we were unable to detect any specific immunodecoration of fungal cells (data not shown). Antibodies recognized the *C. albicans* surface unspecifically. The signal obtained in wild type and *sod*4/*sod*5/*sod*6Δ/Δ deletion strains was similar. The result was the same as already obtained on Western blot. Antibodies recognized several unspecific proteins. Based on these results, we decided to stop the immunization procedure. Compared to the second bleed, the final (third) bleed contained more antibodies. The signals on Western blots were stronger (by the same dilution 1:1000), but again only unspecific bands (Fig.III.3.12). TCA extracts from the wild type and the *sod*4/*sod*5/*sod*6Δ/Δ deletion strain were loaded into the SDS-PAGE gel in alteration (Fig.III.3.12) and incubated with the final mouse bleed from the four mice used for immunization. We detected only unspecific recognition of proteins from both strains. The immune response was different between the mice immunized with the same antigen; so the mouse #2 immunized with Sod5-GST fusion protein showed more unspecific immune response compared to the mouse #1.

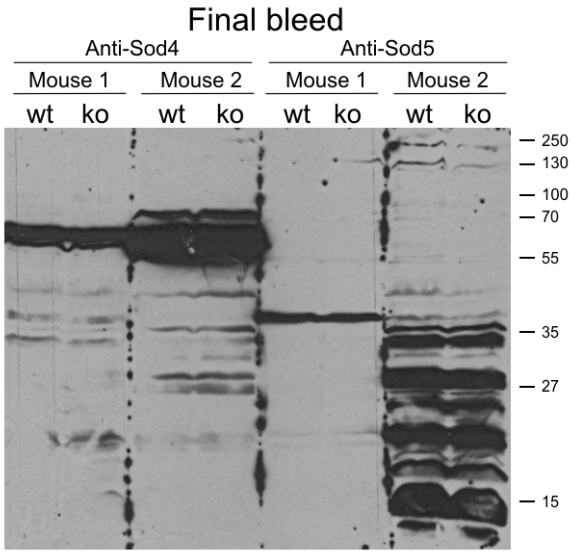


Fig.III.3.12. **Final mouse bleed on protein TCA extracts from the wild type and *sod*4/*sod*5/*sod*6Δ/Δ deletion strain**. TCA protein extracts from the wild type and *sod*4/*sod*5/*sod*6Δ/Δ deletion strain are loaded in alternation. Protein extracts from both strains were incubated with the final bleed with anti-Sod4 and anti-Sod5 antibodies.

No specific signals were detected. The sera from the immunized animals seam to recognize a lot of different proteins. But we obtained no signal by the wild type protein extracts which is not detectable in deletion strain protein extracts. The results obtained from the mice immunization were similar to those obtained from the immunized rabbits.

IV. Discussion

The recognition of microbial pathogens by cells of the innate immune system leads to phagocytosis of pathogens and increased ROS production (Dinauer, 1993) Somehow, *C. albicans* is able to survive and to destroy phagocytes through filament formation inside the phagosome. Sod5 plays important role in protection of *C. albicans* against extracellular stress (Fradin et al., 2005; Frohner et al., 2009). *C. albicans* strains lacking *SOD5* are attenuated in their virulence and have a decreased survival rate in coculture with BMDMs (Frohner et al., 2009). Sod5 is an important enzyme in *C. albicans* involved in degradation of extracellular ROS produced by macrophages. We are interested in showing the cellular localisation of this protein by applying two different methods: tagging of Sod5 with GFP and production of antibodies against this protein. We proved the induction of *SOD5* promoter under different conditions using P_{SOD5}-GFP construct. During my diploma thesis, I was able to successfully tag Sod5 with a GFP. Furthermore, a strain with internal tagged Sod5 was used for localisation studies.

1. Promoter activity studies

The promoter, a sequence located upstream of the open reading frame, is responsible for the transcription of the target genes. Studies about the regulation and activity of a promoter give us evidence about the expression of a target gene. Reporter genes are mainly used to study the promoter regulation. The reporter gene is fused to the promoter of interest and promoter activation results in expression of a reporter gene. This is a very sensitive technique and is widely used for the studies of transcriptional regulation (Xue et al., 2007). Fluorescent and luminescent proteins are often used as reporter genes. Common reporter genes are Green Fluorescent Protein (GFP), Luciferase, β-Glucuronidase, β-Galactosidase.

For our experiments we decided to use Fluorescent proteins such as GFP, YFP and CFP from pMG1646, pMG1656 and pMG1801 plasmids (kindly provided by J. Berman). We used strains with constitutive expressed GFP and GFP expressed under *SOD5* promoter and in this way are able to compare the activity of both promoters and to study the induction conditions of *SOD5* promoter.

We tested different condition for the promoter induction. We are able to show the increased *SOD5* transcription at 37°C in the presence of serum, suggesting that serum and temperature are factors that regulate the expression of *SOD5*. This means that *C.albicans* enzyme Sod5 is active during yeast to hyphae transition, which is a an important switch for the virulence of the fungus, since the yeast forms are less virulent (Lo et at.,1997). Our results confirm the data of previous publications. Martchenko et al showed the up regulation of SOD5 promoter during yeast-to-hyphae transition using Northern blot method (Martchenko et al., 2004).

We show highest up-regulation of *SOD5* under osmotic stress condition. Using yeast media with pH8 and 0,3M NaCl, we are able to induce the expression of *SOD5* gene and detect the highest signal on Western blot using an anti-GFP antibody for our reporter gene. *SOD5* seams to be one of more than 100 other genes (Enjalbert et al., 2003) that are induced in *C.albicans* in response to oxidative and hyperosmotic stress. During osmotic stress conditions the activation of promoter is even higher than during yeast to hyphae transition. *SOD*5 is expressed in phagocytosed cells (Fradin et al., 2005). During the phagocytosis, a pH is changing, after the fusion of phagosome with a lysosome a pH decrease and then increase to pH 7,8. The pH change in the media may mimic the phagosomal environment and so give a rise for a *SOD*5 expression. This may explain a high *SOD*5 expression under the osmotic and pH stress conditions.

2. Studies of Sod5 localization

The functional Sod5-GFP is used to study the cellular localization of the protein. First, we detected this protein under the fluorescent microscope. Further, we used Sod5-GFP strain stained with filipin for the microscopy. Filipin is an antibiotic able to form a complex with the free 3-β-hydroxy sterols in the plasma membrane. Using this method, we tried to determine the localisation of the Sod5-GFP comparing this signal to the filipin-stained plasma membrane. There are some problems in the work with filipin. Filipin is unstable; the solution for filipin staining should be prepared fresh. Filipin bleached very fast, therefore the microscopy picture have to be taken very fast. Filipin can also deform membranes, because of the toxicity to the cell (Alvarez et al., 2007). However, filipin is a common method to stain the plasma membrane.

We stained *C. albicans* cells and visualized them by fluorescence microscopy. We detected signals from the filipin-stained plasma membrane and from the Sod5-GFP protein. But we are unable to distinguish whether the Sod5-GFP signal is coming from the cell wall or plasma membrane. Confocal microscopy would be a way to distinguish

between those two signals; we used a confocal microscope for our experiment. However, the filipin staining was very weak and breached before we were able to take a picture. Thus, we are able to visualize Sod5 protein by fusion with GFP and we are able to detected Sod5-GFP protein in the fluorescent microscope after the induction of protein expression, but we are unable to conclude the exactly localization of the protein.

For the detection of protein by Western blot analysis, we use a protein isolation protocol for *C. albicans* that allows us to isolate proteins in different steps (in different fractions) to determine the localization (Mao et al., 2003). Proteins that localise to the plasma membrane should be extracted after breaking the cells and go into the supernatant. First of all, proteins that are not covalently bound to the cell wall, but also some GPI anchored proteins can be isolated in this way. Proteins that are localised in the cell wall are extracted either with 2% SDS and boiling by 95°C or by digestion with ß-glucanase.

We are able to detect the biggest amount of Sod5-GFP protein in the SDS-extractable fraction, after the pellets of broken cells are boiled with buffer containing 2% SDS. Under these conditions the proteins that are not covalently linked to the cell wall can be extracted. There is also evidence from *S. cerevisiae* that some cell wall proteins can be extracted with SDS (Terashima et al., 2002) and also some GPI-anchored cell wall proteins can be extracted under these conditions (Shankarnarayan et al., 2008). This leads us to conclusion that *C. albicans* Sod5 is located in the cell wall and is probably not covalently linked to it.

Tunicamycin treatment shows that Sod5 is a highly glucosylated protein. By blocking of glycosylation by tunicamycin we detect Sod5-GFP protein on Western blot migrating at about 55kDa. This corresponds to the predicted weight of Sod5 protein, which is about 24kDa and the weight of GFP protein (27kDa). Sod5-GFP protein isolated from the cells with out tunicamycin treatment migrates at about 70kDa.

3. Production of antibodies

During my work, I tried to produce specific antibodies against the three *C. albicans* proteins Sod4, Sod5 and Sod6. Since all SODs are highly conserved and have a big similarity, the raising specific antibodies is a difficult task. For the production of antibodies we had to identify regions with antigenic properties in these proteins. Therefore we use antigenic prediction programs and compare the proteins for homology to exclude the cross reactions. For expression of proteins in *E.coli* and purification, we use an established lab protocol, which worked fine.

Unfortunately, the antibodies are not specific. On Western blot the antibodies recognize antigens, but they show cross reactions to each other and no recognition of the SODs in protein extracts. We are able to detect an increase of immune response after each next boost, but we have no specific recognition for anyone of the three Sod proteins.

Since in the rabbit sera we have the antibodies that are able to recognize the antigen, we performed affinity purification to select specific antibodies. Using the purified sera we are unable to detect any proteins on Western blot; used for indirect immunofluorescence in *C. albicans* we obtain again signal from the unspecific recognition in wild type cells and in deletion strain. So, even after affinity purification antibodies are not specific.

For the following immunisation constructs it may be useful to consider this and try to use peptides or other protein regions (maybe whole protein) for immunisation. Animals can be immunized with native proteins, peptides, or any other compound of fungal origin. A recombinant protein can be expressed in *E.coli* in high levels, but it may be improperly folded and not resemble the native protein with post-translational modifications. Therefore it may be important to switch to eukaryotic expression vector system. GST part of fusion protein is also known to trigger a strong immune response, in this case it may also be useful to digest GST from the peptide and use only the peptide for immunisation. Since in some cases the high doses of antigen may lead to immune tolerance instead of immune response, the right amount of antigen for immunization is very important. Therefore, we should work on improving the immunization procedure and maybe try to start the immunization with smaller amount of antigen.

V. Appendix

1. Primers, strains and plasmids used for this study

Name	5'-3'
M5s	ccgctgctaggcgcgccgtgACCAGTGTGATGGATATCTGC
M3as	gcagggatgcggccgctgacAGCTCGGATCCACTAGTAACG
55_caSOD4s	CAGCATAAACCAAATAACATTACTC
53_caSOD4as	cacggcgcgcctagcagcggCATAGTAATAGTGTGTGTGATTAAAAATC
35_caSOD4s	gtcagcggccgcatccctgcTAGATAGAGAATAACTAGAACAATCAAATG
33_caSOD4as	CTTGAAAAAATATCATTAAGTGAACG
55_caSOD5s	CACGGCTGAGAGGTCACTAC
53_caSOD5as	cacggcgcgcctagcagcggCATGATGAATGGTAAGTTAGATTG
35_caSOD5s	gtcagcggccgcatccctgcAGATGAGCCATTTTACTTATTGTG
33_caSOD5as	CATGTCTGTATAGGATAATGAAAGTG
55_caSOD6s	GCTTGGTAGTGGTGGACTAGAG
53_caSOD6as	cacggcgcgcctagcagcggCATCTTGCTGAGACGTTTAGTG
35_caSOD6s	gtcagcggccgcatccctgcTAGTTGAACATAAATACTCTCACCC
33_caSOD6as	CGATTCAGAGCTTGAGATTGAG
5_SOD4s	AACCTCCTAAACGCAACTGC
3_SOD4as	GAACCAAGGAAGCATTGCC
5_SOD5s	CGGCAATTGATTACGACAAG
3_SOD5as	CTCACGTTTGCTTCTCGC
5_SOD6s	GAGGCATCTGTTGCTTCCAC
3_SOD6as	CGGTAGACTATTTGTCATTGGTG
pSFS2a vector primer	
SAT7s	GAAGTGTTCGATGTGCACCTATCC
SAT108as	CTCCATCACCCAGTTTAGTTGTACC
SAT101as	Ggtacaactaaactgggtgatggag
SAT101s	CTCAAGTCTCGAACGAAACAG
internal primer	
SOD6_1s	Atgatctttattcccattatcat
SOD6_2s	GATCTTTATTCCCATTATCATATTAATC
Sod6_232s	CATATACATGAACGGTCTGTTC
RTSod6_339s	CTACTGTCAAGTGGGCGACTTGC
Sod6_644s	CATCGGAAATTTACCATTCTG
Sod6_166as	CTTCTTTAGCACTAAACACCAC
RTSod6_806as	GTTCTGGTCGCTGGGCCTAG
Sod6_951as	CTAAACAAATATTCCTGCAGC
Sod5_1s	ATGAAGTATTTGTCCATTTTCTTAC
Sod5_49s	GATGCACCAATCTCAACTG
Sod5_631s	GTAGTAAATGGTCTCTTGGC
Sod5_65as	GTTGAGATTGGTGCATCAC

Sod5_676as	CAGCAATGACACCAACTAC
Sod5_687as	TTAAATCAAGGCAGCAATGAC
SOD4_1s	ATGAAATACTTGTCTATTATTTCAATTG
Sod4_9s	CTTGTCTATTATTTCAATTGTTGC
Sod4_699as	CTAAATTAAAGCAGCAACAACAC
Sod4_286as	GATTAAAGTGCATACCTGTAGC
Sod4_551s	CTTCTTCTTCTTCGTCATCATC
CA_ACT1	
ACT1_s	ATGGACGGTGGTATGTTTTAGT
ACT1_as	CAGAAGATTGAGAAGAAGTTTGC
5_CaAct1s	GTTGTTGTTGTGGGTGTGTG
3_CaAct1as	GGAATGAATGGGATGAATCATC
YFP_100as	TCACCTTCACCGGAGACAG
His_794s	GGACTGGGTTGCCATCTC
Ca Actin tagging	
F1.1 CaActs	Actcctggttttctttctttcttagaaacattatctcgatattaatattaaaaaaatataatcattcaaaatg**T CTAAAGGTGAAGAATTATTCAC**
R2 CaActas	ATATATGTAATAACAAAAAGAAGAATAACAAGAATACAAAACCAGATT TCCAGATTTCCAGAATTTCACT**GAATTCCGGAATATTTATGAGAAAC**
F1.1 CaSod5s	Aattgtccttatatctcaactctacactcttgttccacttaaattatatcaatctaacttaccattcatcatg**T CTAAAGGTGAAGAATTATTCAC**
R2 CaSod5as	AGCCACAGAGTAATCTTGTACCTGTTTATCAAACACAATAAGTAAAAT GGCTCATCTATTTTATTTTTCT**GAATTCCGGAATATTTATGAGAAAC**
F1.1 CaSod4s	Ttcatctatgccttatttccttccttttattcataaaacattagatttttaatcacacacactattactatg**TC TAAAGGTGAAGAATTATTC**
R2 CaSod4as	TTTAATGAAAAATTGAATAACAACGTTTAATTAAATAGTGTATACATTT GATTGTTCTAGTTATTCTCTA**GAATTCCGGAATATTTATGAGAAAC**
F1.1 CaSod6s	Tttaaaaatcgtcataataatcttgatcttcatatttttgtgaagatttatcactaaacgtctcagcaagatg **TCTAAAGGTGAAGAATTATTCAC**
R2 CaSod6as	AAACATATATTGTGTTTTTTTTTTTCCCGCAATGGAAGATTACGTCCA TAGGGTGAGAGTATTTATGTTC**GAATTCCGGAATATTTATGAGAAAC**
Antigene cloning	
S4_2TBamHIs	ACGggatccGGTTCCACTACTGCCAAAACAC
S4_XBamHIs	ACGggatccATGGTTCCACTACTGCCAAAAC
S4_2TXEcoRIas	ACGgaattcGGAATGAACAACAATAGACAACCC
S5_2tBamHIs	ACGggatccGGAACCGTTAGAGCTGCAAC
S5_XBamHIs	ACGggatccATGGAACCGTTAGAGCTGC
S5_2TXEcoRIas	ACGgaattcGGCGTGAATTACAATTGATAAAC
S6_2TBamHIs	ACGggatccGCTAGTCCAGTTTGCGATGAAC
S6_XBamHIs	ACGggatccATGCTAGTCCAGTTTGCGATG
S6_2TXEcoRIas	ACGgaattcATAATGGAACACCACCGACTTTC
pGST_seq1	CAATGTGCCTGGATGCG
pGST_seq2	TTGTATGACGCTCTTGATGTTG

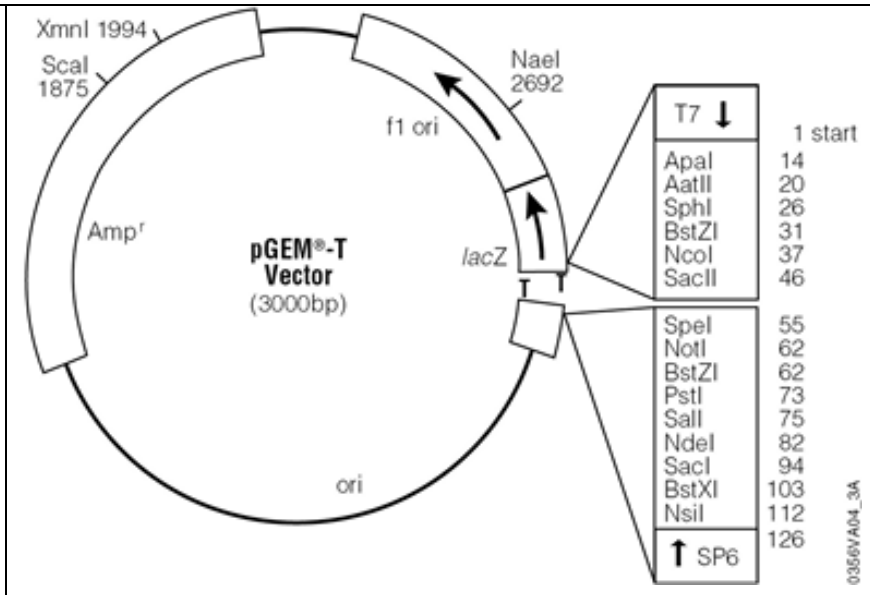
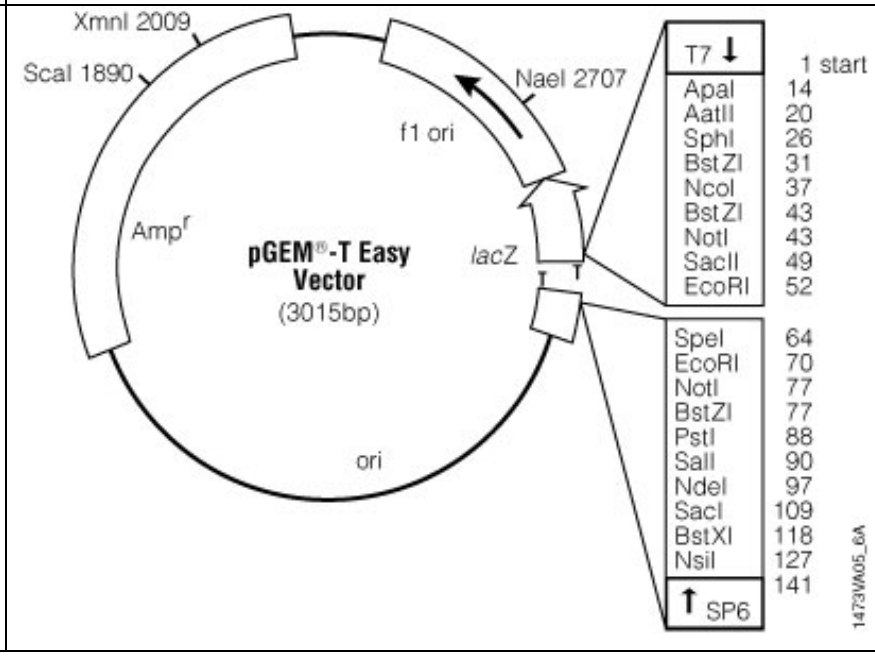
Sod5 internal tagging	
GFP-4s-Kas1	ACGGGCGCCTCTAAAGGTGAAGAATTATTCACTG
GFP-727as-Kas1	ACGGGCGCCTTTGTACAATTCATCCATACCATG
Sod5-Kas1-48as	AGCGGCGCCACCAGCCAAAGCAAAAGTAG
Sod5-Kas1-48s	AGCGGCGCCGATGCACCAATCTCAACTGAC
Sod5-Kas1-575as	AGCGGCGCCGACTGCACTTTGAGATG
Sod5-Kas1-575s	AGCGGCGCCAACACTTCTTCTAGTATGGCTTCTAC
55_caSOD5s	CACGGCTGAGAGGTCACTAC
SOD5_ct_Not as	GAgcggccgcATTTTATTTTTCTTTTTTAAATCAAGGC
FRT-S5term s	ccggtacccaattcgccctatagtgagtcgtattacgcgcagatgagccattttacttattgtg
33_caSOD5as	CATGTCTGTATAGGATAATGAAAGTG
FRT-f s	gcgcgtaatacgactcactataggg
pGEMT primer	
T7prom s	Taatacgactcactataggg
Sp6prom as	Tatttaggtgacactatag

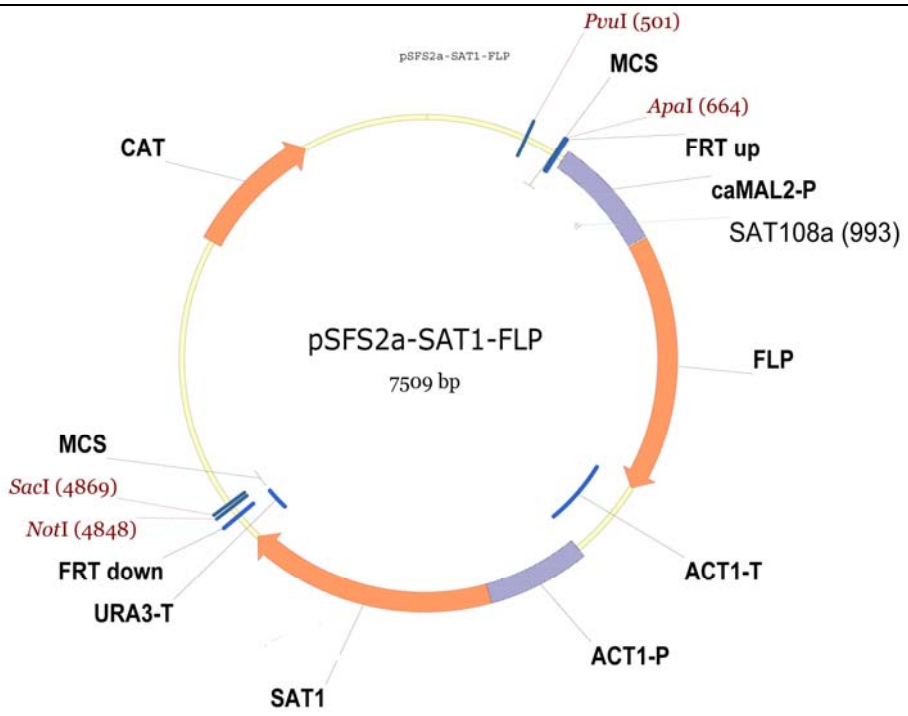
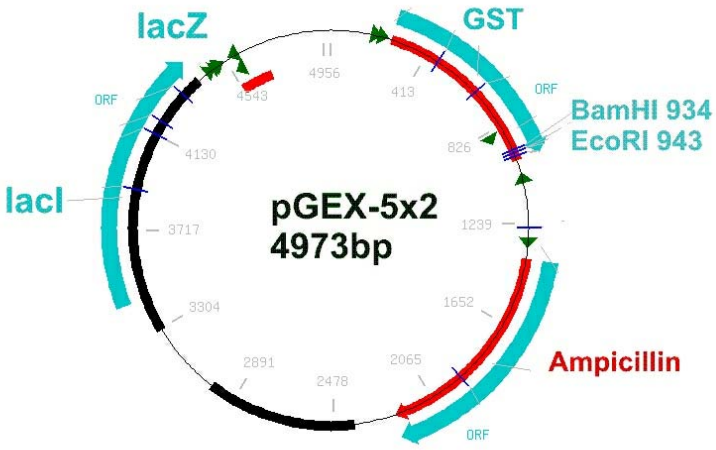
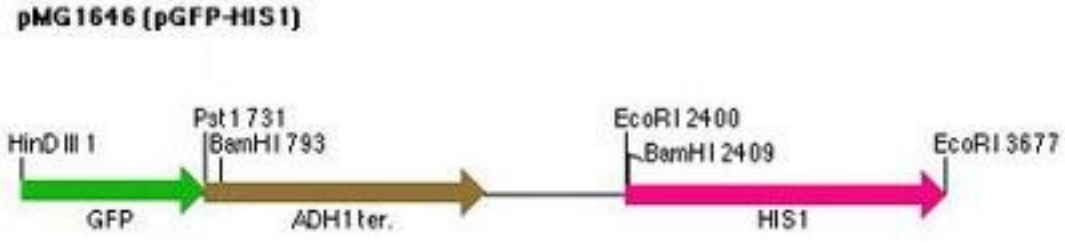
TableV.1.1 Primer list

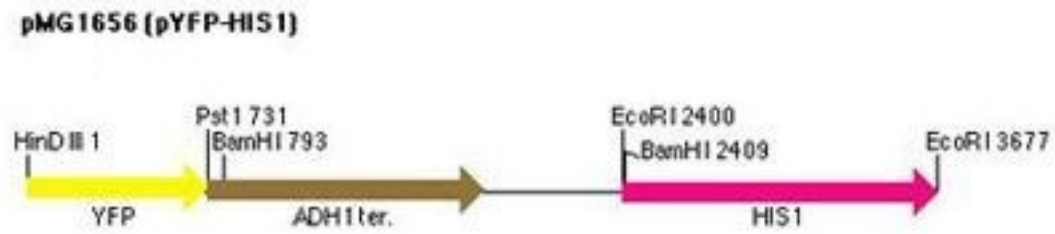
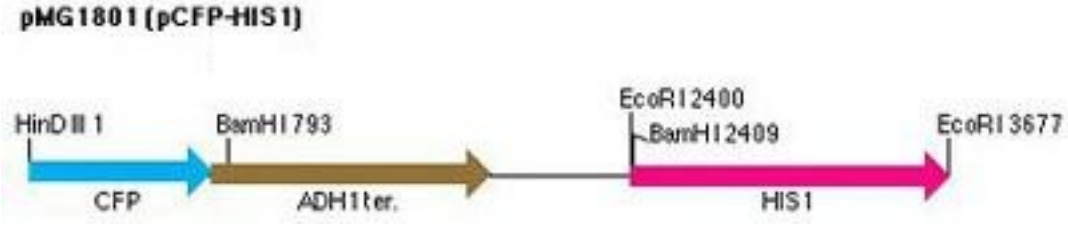
Strains	Short Names	Genotypes	References
SN152	*arg*4Δ/Δ *leu*2Δ/Δ *his*1Δ/Δ	*arg*4Δ/*arg*4Δ, *leu*2Δ/*leu*2Δ, *his*Δ1/*his*1Δ, *URA*3/*ura*3Δ	Noble, Johnson, 2005
SNR34R	*arg*4Δ/Δ *leu*2Δ/Δ *his*1Δ/Δ	MTLa/a ,*arg*4Δ/*arg*4Δ, *leu*2Δ/*leu*2Δ, *his*Δ1/*his*1Δ,*ura*3Δ-*iro*1Δ::imm434/*URA*3-*IRO*1	
CA-IF100	*arg*4Δ/Δ *LEU*2 *HIS*1	*arg*4Δ/*arg*4Δ, *leu*2Δ/*leu*2Δ::cm*LEU*2, *his*Δ1/*his*1Δ::cd*HIS*1, *URA*3/*ura*3Δ	Frohner, 2009
CA-IF019	*sod*5Δ/Δ	SN152, *sod*5Δ::cm*LEU*2/*sod*5Δ::cd*HIS*1	Frohner,2009
CA-IF027	*sod*5Δ/Δ *SOD*5	SN152, *sod*5Δ::cm*LEU*2/*sod*5Δ::cd*HIS*1::*SOD*5-FRT	Frohner, 2009
KY-Ca001	P_{ACT}-GFP	SN152, *ACT*1/*act*1Δ::GFP-HIS	This study
KY-Ca002	P_{ACT}-CFP	SN152, *ACT*1/*act*1Δ::CFP-HIS	This study
KY-Ca003	P_{SOD4}-YFP	SN152, *SOD*4/*sod*4Δ::YFP-HIS	This study
KY-Ca004	P_{SOD6}-GFP	SN152, *SOD*6/*sod*6Δ::GFP-HIS	This study
KY-Ca005	P_{SOD5}-CFP	SN152, *SOD*5/*sod*5Δ::CFP-HIS	This study
KY-Ca006	P_{SOD5}-GFP	SN152, *SOD*5/*sod*5Δ::GFP-HIS	This study
KY-Ca007	P_{ACT}-GFP	SNR34R, *ACT*1/*act*1Δ::GFP-HIS	This study
KY-Ca008	P_{ACT}-CFP	SNR34R, *ACT*1/*act*1Δ::CFP-HIS	This study
KY-Ca009	P_{SOD5}-CFP	SNR34R, *SOD*5/*sod*5Δ::CFP-HIS	This study
KY-Ca010	Sod5-GFP (N-terminal) Clone 6.6; Clone 6.10; Clone 8.8; Clone 8.10	CA-IF019, *sod*5Δ::*SOD*5-GFP/*sod*5Δ::cd*HIS*1	This study

KY-Ca010	Sod5-GFP (C-terminal) Clone 1.4; Clone 1.15; Clone 4.4; Clone 4.13	CA-IF019, *sod*5Δ::*SOD*5-GFP/*sod*5Δ::cd*HIS*1	This study
KY-Ca011	Sod5-GFP	CA-IF100, *SOD*5/*SOD*5::*SOD*5-GFP	This study

TableV.1.2. Fungal strains used in this study

pGEMT	
pGEMTeasy	

pSFS2a	
pGEX5x2 pGEX-5x2 vector used for protein expression	
pMG1646 Berman plasmid	

pMG1656 Berman plasmid	
pMG1801 Berman plasmid	

TableV.1. 3. Plasmids used for this study

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Anhang

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Yatsyk Kristina
Gerlgasse,23/3
1030 Wien
Tel. 0676 9335262
Email: kristina.sl@gmail.com

Lebenslauf

Persönliche Daten:

Geburtstag:	17.06.1984
Geburtsort:	Kalusch, Ukraine
Familienstand:	verheiratet, keine Kinder

Schulausbildung:

1991-1999 allgemeinbildende höhere Schule Kalusch,
Gebiet Iwano-Frankiwsk, Ukraine

1999-2002 Bundes-Oberstufenrealgymnasium Wien 1, Hegelgasse 14
2002 Matura mit gutem Erfolg bestanden.

Universität:

2002-2003 Medizinstudium an der Universität Wien
Ab 2003 Studium der molekularen Biologie

Praxis:

Alle Übungen und Praktika wurden erfolgreich abgeschlossen.

Berufserfahrung:

2004 – 2006 tätig in einer Zahnarztpraxis.
2007 – 2008 tätig im Labor Karl Kuchler als technischer Assistent
Juli, 2007 tätig als „Studenthelper“ beim 32. FEBS Kongress FEBS2007 in Wien, Österreich
März, 2008 Mitarbeit beim ABC2008 – 2nd FEBS Special Meeting on ABC Proteins in Innsbruck, Österreich
Seit Dezember, 2009 tätig bei AFFiRiS AG als technischer Assistent

Sprachkenntnisse:

Deutsch: Fließend
English: Gut
Russisch: Fließend
Ukrainisch : Muttersprache

EDV: fundierte Kenntnisse in
Word,
Excel,
Photoshop
Windows XP

Fähigkeiten: ich bin lernfähig, teamfähig, sorgfältig, belastbar.

Hobbies: Tanzen, Fitness, Ausflüge.

Publikationen:

Candida albicans cell surface superoxide dismutases degrade host-derived reactive oxygen species to escape innate immune surveillance.
Frohner IE, Bourgeois C, Yatsyk K, Majer O, Kuchler K.