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Table of Contents

1. Introduction .............................................................................................................................. 5

1.1. Aim of this work .................................................................................................................. 33

2. Results ................................................................................................................................... 35

2.1. Candida glabrata environmental stress response involves Saccharomyces cerevisiae Msn2/4 orthologues transcription factors ................................................................. 37

2.2. Autophagy supports Candida glabrata survival during phagocytosis .............................. 63

2.3. CgSkn7 and CgYap1 mediate key responses to oxidative stress in Candida glabrata .... 118

2.4. Major results obtained ........................................................................................................... 170

3. General discussion .................................................................................................................. 171

4. Appendix ................................................................................................................................ 193

4.1. Summary ................................................................................................................................ 194

4.2. Zusammenfassung .................................................................................................................. 195

4.3. Danksagung ........................................................................................................................... 196

4.4. Curriculum vitae ..................................................................................................................... 197
1. Introduction:

Yeasts belong to the large and diverse fungal kingdom. This ubiquitous microorganism appears in various shapes and at different environments. Fungi in general are heterotrophic organisms, which possess a chitinous cell wall and sterol-containing cell membranes. Several species are pathogens of humans or other animals. Single celled yeasts belong to the division Ascomycota, the largest taxonomic group within this kingdom. Within this division we find the class Saccharomycotina consisting of the most prominent genera *Saccharomyces* and *Candida*.

The species *Saccharomyces cerevisiae* is one of the most studied model organisms in molecular and cell biology. It is also known as baker’s (or brewer’s) yeast due to its common use in baking and brewing. The majority of the species among the genus *Candida* belong to the group of opportunistic human fungal pathogens. The most prevalent members are *Candida albicans* and *Candida glabrata* [1]. One exception of this taxonomy is the encapsulated yeast *Cryptococcus neoformans*, which belongs to the division Basidiomycota. In general, *Candida* species live as commensals of the skin and the gastrointestinal and genitourinary tracts in mammalian hosts. As part of the human flora, they can enter the bloodstream occasionally and cause infections (candidemia or candidiasis). This can be life-threatening for severely immunocompromised persons, such as cancer, transplant or AIDS patients. Therefore, candidiasis is referred as opportunistic disease.

In the following I will concentrate partly on *C. glabrata* including its successful adaptation to environmental changes inside and outside the host. How do *C. glabrata* cells adapt to changing environments? Which specific responses are crucial for prolonged survival? As part of its pathogenic lifestyle, *C. glabrata* has to get along with drugs and a hostile environment. Cells have to counteract defence mechanisms of the immune system. In general, fungal cells get phagocytosed, when they start to disseminate throughout the entire mammalian host. I tried to find out more about how *C. glabrata* cells react to changing environments, such as internalization by phagocytic cells.

From a phylogenetic point of view, *C. glabrata* is closely related to *Saccharomyces spp*. Its particular positioning among the clade of yeasts will be discussed first.
**Candida glabrata – a S. cerevisiae-like pathogen?**

Among the phylogenetic clade of *Candida* species, *Candida glabrata* has a unique position, closely related to *S. cerevisiae* and distinct from other *Candida* species (Figure 1) [2, 3]. During evolution, a whole-genome duplication (WGD) occurred prior to the divergence of *S. cerevisiae* and *C. glabrata* [4, 5] from other Ascomycetes yeasts. The genome of *C. glabrata* has been sequenced five years ago [4]. It consists of 12.3Mb and 13 chromosomes and has a G+C content of 38.8%, which is close to that of *Saccharomyces cerevisiae* (38.3%). About 5000 putative open reading frames were predicted. The genomes of *S. cerevisiae* and *C. glabrata* display a high degree of synteny. Moreover, the overwhelming majority of *S. cerevisiae* genes have obvious *C. glabrata* orthologues [4].

![Figure 1](image-url)
Compared to S. cerevisiae, C. glabrata has lost many metabolism-associated genes again (e.g. for galactose or sucrose assimilation), probably because of its close relation to the mammalian host [6]. In addition, C. glabrata is an auxotroph for three vitamins: thiamine, niacin and pyridoxine [7, 8]. So far all clinical isolates of C. glabrata were haploid, but nevertheless this yeast owns components of a mating machinery similar to S. cerevisiae [9]. Moreover, mating type switching occurs during infection in clinical isolates, demonstrating a striking variation of phenotypes from different anatomical sites in patients [10]. Interestingly, an uneven mating type distribution was found in isolates from clinical samples: 80% of the strains are MATa [11]. The genome encodes for three mating type loci (MTL1, MTL2 and MTL3) and in addition, encodes the HO endonuclease, which initiates mating type switching in S. cerevisiae [12, 13]. Although C. glabrata is classified as asexual yeast, its mating type machinery per se seems to be intact [9] and much more similar to S. cerevisiae than to C. albicans.

Similar to C. albicans, C. glabrata can undergo phenotypic switching [14, 15] an adaptation required for successful establishing in the human host. In C. albicans, white cells are ovoid, budding yeast cells that form white, domed colonies, while opaque cells are large and elongated and form gray, flat colonies; these cells have different infectious properties and different transcriptional profiles [16]. Phenotypic switching of the white to the opaque form significantly increases mating [17]. Additionally, the ability of C. albicans to switch from cells that make white colonies to cells that make opaque colonies correlates with survival in different host tissues [18, 19]. Although white cells are more suited for bloodstream infections, opaque cells are better at colonizing skin surfaces in mice [20]. C. glabrata switches spontaneously, reversibly and at high frequency among four phenotypes distinguishable by graded colony coloration on agar containing 1mM CuSO₄: white (Wh), light brown (LB), dark brown (DB) and very dark brown (vDB) [10, 14]. These phenotypes in turn can switch to the irregular wrinkle (IWr) phenotype [14], which displays a highly wrinkled colony morphology. Interestingly, DB has an advantage over other switch phenotypes in colonizing two major target organs in a mouse model, the spleen and liver [10, 21]. For C. albicans, the formation of hyphae is one important contribution to virulence [22, 23]. In contrast, C. glabrata does not undergo similar morphological switches inside the host. Similar to S. cerevisiae, it has been shown that C. glabrata can form pseudohyphae in response to nitrogen starvation [24]. Additionally, components of the mitogen-activated protein (MAP) kinase pathway, which induces pseudohyphae in S. cerevisiae, were also found in C. glabrata. Ste12, a conserved transcriptional regulator, is essential for nitrogen starvation-induced formation of pseudohyphae. Furthermore, the ste12Δ mutant shows attenuated virulence in a mouse model [25]. However, pseudohyphal forms of C. glabrata have
never been found in clinical specimens [2]. The formation of hyphae by *C. albicans* is also essential for escaping engulfment by cytotoxic cells like macrophages. *C. glabrata* pseudohyphae seem to be dispensable or even absent during infection of the human body. Therefore, *C. glabrata* seems to have developed other strategies than *C. albicans* to successfully colonize its niche inside the mammalian host.

Despite the fact that *C. glabrata* and *S. cerevisiae* share many similarities, it is more likely that the discrepancies characterize the pathogenicity of *C. glabrata*. For example, the *EPA* (Epithelial Adhesins) gene family, encoding GPI-anchored cell wall proteins, which mediate adherence to epithelial or cells surfaces, is not present in *S. cerevisiae* [2]. *EPA* gene products are involved in adherence and biofilm formation but affects virulence of *C. glabrata* [26-29]. *C. glabrata* is able to adhere to mammalian cells, an interaction that depends on the CgEpa1 lectin. CgEPA1 is a member of a larger family of highly related genes encoded in subtelomeric clusters [30]. Due to their subtelomeric localization, *EPA* genes are subject to transcriptional silencing. In *S. cerevisiae*, silencing of subtelomeric genes is dependent on several trans-acting factors, such as Sir2, Sir3 and Sir4 [31, 32]. It has been shown recently, that *C. glabrata* cells with mutations in these silencing factors are hyperadherent to cultured epithelial cells [27, 28]. In addition, hyperadherent *C. glabrata sir3Δ* mutants displayed better colonization efficiency in kidneys of mice [27]. Interestingly, lack of nicotinic acid triggers *EPA* gene expression. Since *C. glabrata* is an auxothroph for this vitamin, cells are dependent on extracellular sources. In the absence of nicotinic acid, derepression of *EPA* genes occurs, resulting in better adherence [28].

Taken together, although *EPA* genes are absent in *S. cerevisiae*, *C. glabrata* shares more similarities with *S. cerevisiae* than with *C. albicans*. Nevertheless, adaptation to mammalian hosts led to *C. glabrata*-specific, yet mostly unknown, mechanisms to sustain survival in this niche.

**Environmental stress response mechanisms in fungi.**

Investigation of the response of *C. glabrata* and other pathogens to environmental conditions might provide additional information about the specific host-pathogen interactions. In the following, I will give an overview of the state-of-the-art knowledge about important stress responses of non-pathogenic and pathogenic yeasts. Ascomycota occupy diverse niches in nature. The majority of these niches does not stay constant, but changes quickly and continuously. Adaptation of gene expression through transcriptional regulation is a key mechanism in fungal response to fluctuating environmental conditions. Environmental stress causes activation of a
variety of signaling mechanisms each responding to a particular situation, such as oxidative stress or nutrimental starvation and in parallel induces a stereotypic general response. In *S. cerevisiae*, this response was first described and is referred to as general stress response or environmental stress response (ESR) [33, 34]. Stress responses might be divided into acute stress response, repair and starvation responses. Acute stress is for example exposure to high temperature. Repair processes are also constantly required as for example reactive oxygen species (ROS) are generated continuously in metabolizing cells. Finally, starvation response has also a component of the contribution of internal stores and the adaptation of metabolism. For example, glucose is the favored carbon source of yeasts and its shortage induces a broad spectrum of activators and genes. In case of continuous starvation, one important strategy is to recycle internal resources by autophagy [35]. Its importance for non-pathogenic and pathogenic microbes will be discussed below.

Furthermore, pathogenic fungi have to cope with antifungal drugs in hospitals. The selective pressure favors the occurrence of drug resistant strains, which are able to spread to other hosts e.g. in a clinical environment. Cells can develop resistance due to changes in the cell wall composition, which lead to impaired drug uptake. In addition, mutations in drug targets, such as lanosterol 14α-demethylase (Erg11), or their changing expression pattern can abolish susceptibility [36]. Furthermore, it is known that ATP-binding cassette (ABC) transporters also play a crucial role in the development of multidrug resistance (MDR) [37, 38]. MDR is mainly based on the overexpression of membrane transporters, which efflux chemically different compounds out of the cell. Finally, pathogenic yeasts encounter serious threats in the host such as patrolling phagocytic cells. Engagement by these causes a rapid and dramatic change of their close environment, requiring a rapid adaptation to sustain dissemination. In the following part, the most prevalent aspects of stress responses, which can affect survival and dissemination of potentially pathogenic yeasts, are discussed. This includes the general stress response, responses to oxidative stress and carbon starvation, inducible autophagy and the response to application of antifungal drugs.

I. General Environmental Stress Response:

In *S. cerevisiae*, the ESR was originally described as a set of ~900 genes. One third gets induced, whereas about 600 genes are repressed upon induction of various types of stress, such as osmotic stress, heat shock, oxidative stress, nutrient starvation or extreme pH [33, 34]. Magnitude and kinetics of the ESR are dependent on the
severity of applied stresses. Comparable ESR patterns have been characterized in *Schizosaccharomyces pombe* and to a certain extent in *C. albicans* [39, 40]. For *S. cerevisiae*, *C. albicans* and *S. pombe* one major mechanism for controlling general stress responses are p38-type SAP kinases (stress-activated mitogen-activated protein kinases). The SAPKs, Hog1 (*S. cerevisiae*, see Figure 2), Sty1 (*S. pombe*) and CaHog1 (*C. albicans*) are all activated by hyperosmolarity, oxidative stress and to a varying degree by heavy metals. Notably, the HOG pathway of *C. glabrata* functions in a very similar manner to *S. cerevisiae*, in being activated by osmotic stress and additionally by weak acids [41].

![Figure 2](image_url). The Hog1–Msn2/4 pathways in *S. cerevisiae*. (adapted from Gasch AP, 2007).

In *S. cerevisiae* a second general stress mediating mechanism based on the transcription factor Msn2 and its parologue Msn4 exists (Figure 2) [34, 42]. These are activated by a variety of stress conditions and changing nutrient supply situations such as glucose exhaustion [33, 34, 43]. Together, these factors affect almost 90% of the genes induced in the ESR. They also have a role in both chronological and replicative ageing [44, 45]. During high nutrient supply Msn2 is hyperphosphorylated and
inactivated by the PKA and TOR (target of rapamycin) pathways [43, 46, 47]. Activation of Msn2 and Msn4 causes their rapid accumulation in the nucleus and recruitment to chromatin. Msn2 has separate functional domains for nuclear import (NLS), nuclear export (NES) and DNA binding. The $C_2H_2$ Zn finger DNA binding domain at the C-terminus recognizes the stress response element (STRE). Stress signaling requires a region in the N-terminal part of Msn2 which includes its NES and a short stretch of high similarity to Msn4 designated homology domain 1 (HD1) [43, 46-48].

The *C. glabrata* orthologues CgMsn2 and CgMsn4 contain this conserved motif referred to as HD1, also present in Msn2 orthologues from fungi closely related to *S. cerevisiae* [49]. Transcript profiles of *C. glabrata msn2Δmsn4Δ* mutants show that they regulate part of the CgESR and are described in [Results]. In contrast, Msn2-like factors do not appear to play a role in regulating stress response in *C. albicans* and *S. pombe*. The *C. albicans* Msn2-like transcription factor designated CaMsn4 is not involved in the environmental stress response [50]. In addition, the Hog1 Map kinase orthologues play a more general role in stress response in *C. albicans* and *S. pombe*. These differences point to distinct strategies for regulating the stress response in fungi. Interestingly, in both *S. cerevisiae* and *S. pombe*, the ESR is suggested to be involved in cross-protection. Cross protection is defined as acquired resistance to a severe dose of the same or a different stress following the exposure to a non-lethal dose of a particular stress type [51-54]. Nevertheless, recent findings indicate that the primary function of adapted gene expression is to provide protection against impending stress rather than a protective role against the original stimulus [55]. Stress resistance is not acquired by plain activation of the ESR, but seems to be determined by condition specific responses, since cross-stress protection includes different regulatory pathways and therefore varies between different stresses. Msn2 and Msn4 play distinct roles in response to different stresses, which might be due to a different phosphorylation status in response to a particular condition [56].

II. Oxidative stress response:

Oxidative stress is of outstanding interest for fungi. All organisms, growing in an aerobic environment, have to deal with reactive oxygen species (ROS). In general, those species are occasionally produced during metabolic processes. In the special case of phagocytosis ROS is produced actively through macrophages and neutrophils. ROS, such as hydrogen peroxide ($H_2O_2$) or superoxide and hydroxyl radicals ($O_2^-$, $OH^+$) can harm the cell by reacting with cellular structures, including proteins and DNA.
One essential pathway to generate ATP used by aerobic living organisms is the electron transport chain located in the mitochondrial membranes. Normally, molecular oxygen is reduced to produce water. However, occasionally, oxygen is incompletely reduced, resulting in superoxide radicals. Another metabolic process producing ROS is the β-oxidation of lipids. In contrast to higher eukaryotes that also have mitochondrial participation, fatty acid β-oxidation in yeast is restricted to peroxisomes [57, 58]. β-oxidation products are exported out of the peroxisome to mitochondria for full oxidation to CO₂ and H₂O. During the first step of decomposing fatty acids, FADH₂ is produced, which in turn gets reoxidized to FAD producing H₂O₂, which is decomposed by catalase.

In the phagolysosome of phagocytic cells, the NADPH oxidase complex consists of plasma membrane-associated enzymes [59]. It catalyzes the production of superoxide (O₂⁻), serving as initial source for the production of several ROS to mediate oxidative destruction of engulfed microorganisms.

Cells counteract oxidative stress, using various, often redundant antioxidant systems (Figure 3) [60, 61]. Most important cellular redox balance and efficient removal of ROS are two small proteins: glutaredoxins (GRX) and thioredoxins (TRX) [62, 63]. Both act as antioxidants, but are reduced differently.

![Figure 3](https://example.com/figure3.png)

**Figure 3.** Enzymatic systems involved in ROS detoxification and in control of the redox state in *S. cerevisiae*, including their interrelationships (taken from Herrero *et al*, 2008).
The ubiquitous enzymes thioredoxins are kept in the reduced state by thioredoxin reductases (TRR) [64, 65]. Thioredoxins also act as electron donors to peroxidases (TSA, Thiol Specific Antioxidant) [66, 67]. Related glutaredoxins share many of the functions of thioredoxins, but are reduced by glutathione (GSH), a tripeptide, which acts a cofactor. Glutathione is predominantly kept in its reduced form, by activity of the glutathione reductase (GLR), the primary enzyme that controls the redox state of GSH, which is directly regulated by ROS [68]. GSH acts also as cofactor for glutathione peroxidases (GPX), which therefore reduce lipid hydroperoxides to alcohols and further reduce free H$_2$O$_2$ to water [62]. Glutathione tranferases (GST) conjugate GSH to a variety of exogenous and endogenous compounds, including products of oxidative stress [69, 70]. GSH-conjugated molecules are then excreted or transported into vacuoles, resulting in detoxification. Omega-class GSTs, a specific group of glutathione transferases (GTO genes, Glutathione Transferase Omega-like) have a different active site, but show similar oxidoreductase activities [69, 71]. Those ubiquitous transferases appear to be involved in various redox reactions. The *S. cerevisiae* Gto1 is important for biosynthesis of sulfur amino acids [72]. In addition, cells can induce superoxide dismutases (SOD), which convert superoxides to H$_2$O$_2$ and oxygen. Further, the catalase, an enzyme present in nearly all living organisms, can decompose H$_2$O$_2$ to water and oxygen. In case of DNA, reactive oxygen species can lead to oxidation or hydrolysis (e.g. deamination, the conversion of C to U) of bases. If one of the two strands is defect, the other strand can act as template for repair [73]. Damaged or misfolded proteins as a consequence of ROS are rapidly degraded by the proteasome [74]. In conclusion, several enzymes cooperate for the destruction of ROS, supporting the overall importance of constant defence and repair.

Genes coding for ROS defence factors are often under control of specific transcription factors and are induced by oxidative stress. As part of the ESR, genes involved in oxidative stress response have been found to be activated by Msn2/4 in *S. cerevisiae* in response to diverse stresses [34]. Additionally, these genes are under control of other transcription factors in yeast. For *S. cerevisiae*, *C. albicans* and *S. pombe* the most prominent factor is a bZIP DNA-binding protein of the AP-1 family [75]. Yap1, Cap1 and Pap1 are known to induce a similar regulon upon oxidative stress induction [76-78]. Skn7 is a second factor involved in oxidative stress response in *S. cerevisiae* and to a lesser extent in *C. albicans* [79, 80]. Skn7 encodes a transcription factor, which is also implicated in the regulation of cell wall biosynthesis and the cell cycle [81, 82]. In *S. cerevisiae*, Skn7 and Yap1 control the vast majority of oxidative stress-inducible genes [83, 84].
Introduction

What is the contribution of oxidative stress response to virulence and pathogen defense? Within the phagosome of phagocytic cells, engulfed yeasts seem to experience mild oxidative stress [85]. The exact role of transcription regulators is still unclear. Cap1 dependent genes are up-regulated in *C. albicans* in a mouse model [85]. In *C. glabrata* skn7Δ and yap1Δ mutants are less resistant to oxidative stress [86]. In contrast to *C. glabrata*, the sole catalase of *C. albicans* CaCtt1 has an impact on virulence in mice [87]. Other oxidative stress genes have been found to affect virulence in *C. albicans* and *C. neoformans* [88-91]. The exact impact of Skn7 and Yap1 remains to be elucidated and their role in *C. glabrata* is described in [Results].

III. Carbon starvation response:

The nutritional environment determines cellular behavior in various ways. Growth and proliferation are strictly dependent on available carbon sources. For microorganisms, extracellular compounds serve as both nutrients and signals. These signals must be transduced into a cellular response by key regulators, many of which are protein kinases (Figure 4) [92, 93]. Beside mannose and fructose, glucose is the preferred carbon source of *S. cerevisiae*; its presence represses numerous genes involved in alternative carbon catabolism [94]. *S. cerevisiae* is the best investigated yeast regarding glucose signaling. Several partially overlapping systems are known to participate in glucose signaling in yeast, such as Snf1, Ras/PKA, Gpr1/Gpa2, or Snf3/Rgt2, which controls the expression of glucose carriers [95, 96]. Several genes, such as the *HXT* family, encode different glucose transporters with varying affinities for the substrates [97, 98]. Snf3 and Rgt2 proteins are glucose carrier homologues but are unable to carry out active glucose transport; instead, Snf3 and Rgt2 mediate regulation of active glucose carriers by low and high glucose concentrations (see below) [99, 100].

Snf1, the yeast homolog of mammalian AMP-activated protein kinase, is a central component of glucose repression. The Snf1p kinase complex belongs to a highly conserved family of serine/threonine protein kinases, and homologues to each of the subunits (Snf1, Snf4, Sip1, Sip2, and Gal83) have been found in all eukaryotes, including plants and mammals [101-105]. Snf1 influences gene expression upon glucose depletion through activation of the transcriptional activators Cat8 and Adr1 and inactivation of the Mig1-containing repressor complex [106, 107]. Growth of cells in the presence of alternative carbon sources, such as glycerol or ethanol, activates Snf1 through phosphorylation by one of three upstream kinases [108-110]. In the presence
of glucose, the Reg1/Glc7 protein phosphatase 1 complex dephosphorylates and inactivates Snf1 [111]. Comparisons of transcriptional changes following glucose shortage revealed that up to 500 genes are regulated either directly or indirectly by Snf1 [112]. Upon activation, Snf1 also phosphorylates Sip4, a transcriptional activator involved in the positive regulation of gluconeogenesis, to allow expression of glucose-repressed genes [113].

Figure 4. A simplified overview about three well-characterized glucose-sensing pathways: the Snf1 repression pathway, the Rag2/Snf3 glucose carrier pathway and the cAMP/PKA pathway (including Gpr1/Gpa2) represent the most prominent glucose responses (adapted from Geladé et al, 2003).

Further, in the absence of glucose, transcription factor Rgt1 acts in a complex with the two glucose-specific regulators Std1 and Mth1 as a transcriptional repressor of the HXT1-HXT4 genes [99]. When glucose is present, the transcription factor Rgt1 is inactivated through the ubiquitin-ligase complex SCF-Grr1 which initiates degradation of the regulator proteins Mth1 and Std1 [114, 115]. This results in a rapid dissociation of Rgt1 from the HXT promoters. Snf3 triggers the induction of HXT1-HXT4 in response to low glucose concentrations. High glucose concentrations enhance HXT1 expression through Rgt2 in a process that converts Rgt1 into a transcriptional activator.

The Ras/PKA pathway plays the central role in responding to changes in glucose concentration. Glucose addition to cells increases the level of GTP-bound Ras, which in turn elevates intracellular cAMP and subsequently activates PKA [116]. Gpr1, a membrane G protein coupled receptor, and Gpa2, the alpha subunit of this G protein,
comprise a nutrient sensing pathway that works in parallel with Ras to activate PKA [117, 118]. High glucose concentrations activate cAMP synthesis by the adenylate cyclase Cyr1 through the Gpr1/Gpa2 G-protein-coupled receptor system in a glucose-phosphorylation-dependent manner. Nevertheless, it seems to play only a minor role in the transcriptional response to glucose [95]. Notably, the same pathway is present in C. albicans [119].

In yeast, the main downstream target for cAMP is the cAMP-dependent protein kinase A (PKA). PKA negatively regulates the general stress transcription factor Msn2 in S. cerevisiae. During high nutrient supply Msn2 is phosphorylated and inactivated by PKA. PKA phosphorylation sites in its NLS are phosphorylated PKA when glucose is available and rapidly dephosphorylated and activated by glucose starvation [46, 120]. This contributes to rapid nuclear import of the transcription factor. Additionally, the TOR pathway is involved in nutritional control in S. cerevisiae as well as in S. pombe [121, 122]. Further, starvation of amino acids, glucose and purines leads to the activation of Gcn2, a protein kinase that phosphorylates the translation initiation factor eIF2, which in turn leads to a general decrease of initiation of protein synthesis [123]. Unlike S. cerevisiae or C. albicans, C. glabrata does not ferment or assimilate other sugar such as sucrose, maltose or galactose [6, 124-127]. Trehalose, a glucose dimer, is the only sugar other than glucose that is assimilated by a majority of C. glabrata strains. Notably, disruption of the homologue CgSNF1 in C. glabrata resulted in the loss of the ability to utilize trehalose [6]. Further, in C. glabrata, the localization of the orthologue CgMig1 is glucose dependent, which is described in [Results].

Carbon utilization is essential for proliferation of all living cells. Although different yeasts have specific carbon source preferences, transduction signals and subsequent carbon uptake systems are highly conserved [6, 127-129].

IV. Autophagic processes in response to continuous starvation: A very important mechanism for overcoming starvation conditions is autophagy [130]. Autophagy is a process whereby almost all constituents of the cell get continuously recycled to help organisms to sustain crucial cellular functions and to overcome periods of nutrient starvation. Two types of cargo selection by autophagy are known: selective and non-selective autophagy (Figure 5). During the non-selective process bulk cytosol and other cytoplasmic components are randomly sequestered into autophagosomes. This process can also selectively eliminate specific proteins, protein complexes and organelles, and is under control of the TOR pathway, which is necessary for sensing and transducing nutrient availability [131-133]. When nutrients
are limiting, haploid yeast cells exit the mitotic cell cycle and enter a stationary phase, which is characterized by changes in transcription pattern and reduced protein synthesis [134]. *S. cerevisiae* and other yeasts harbor two homologous *TOR* genes, *TOR1* and *TOR2*, each encoding a serine/threonine kinase [135]. These kinases are involved in mediating translation, ribosome biogenesis, transport of nutrients and autophagy in response to nutrient availability.

However, the best characterized type of selective autophagy is the cytoplasm-to-vacuole targeting (Cvt) pathway, which is only found in *S. cerevisiae*; two hydrolases are directly transported from the cytoplasm into the vacuole where they are enzymatically processed into their mature form [136]. In *S. cerevisiae*, most of the organelles and macromolecular complexes are degraded by macro-autophagy, which involves the formation of double-membrane autophagosomes sequestering the structures that have to be degraded [137]. Autophagosomes fuse with the lysosome/vacuole, delivering the cargo. Additionally, a related process called micro-autophagy exists, in which the vacuole limiting membrane directly engulfs cytosolic material [138].

![Figure 6](image.png)

**Figure 6.** Selective and non-selective of types of autophagy in *S. cerevisiae* (adapted from Kraft *et al.*, 2009).
There exist several subtypes of autophagy. Selective mitophagy, pexophagy, reticulophagy and ribophagy are important for a fast adaptation to regularly changing surroundings [139-144]. Furthermore, in S. cerevisiae, a process called piecemeal micro-autophagy of the nucleus (PMN) leads to the pinching off and degradation of non-essential portions of the nucleus into the vacuole in response to starvation conditions [145, 146]. More than 30 autophagy-related (Atg) proteins involved in autophagy have been identified in yeast [147]. On the other hand, certain subtypes of autophagy require factors, which are not involved in other autophagic processes. For example, Atg11 is involved in the Cvt pathway and pexophagy but is not required for non-selective autophagy in S. cerevisiae [148]. Interestingly, ubiquitin is involved selective cargo recognition as a signaling molecule [149]. The cargo destined for degradation is marked with ubiquitin, which is then recognized by an adaptor component to mediate the inclusion of the cargo into an autophagosome.

Autophagy could also be an issue for pathogenic yeasts as described in [Results]. Cells engulfed in macrophages are isolated from the environment and thus have to resort to endogenous resources. Notably, the encapsulated yeast C. neoformans requires autophagy during infection [150]. ATG8 deficient strains displayed a significantly attenuated virulence in a mouse model. In addition, in C. neoformans, autophagy related genes are up-regulated upon during infection [151]. In contrast, C. albicans cells lacking ATG9 were unable to induce autophagy, but showed no diminished survival rate [152]. Cells were able to kill macrophages through hyphae formation and escaped apparent depletion of nutrients.

Autophagy is an essential process to overcome common situations of nutrient deprivation. Importance of autophagy for phagocytosed fungal cells depends on morphological properties and strategies to escape from the phagosome [153].

V. Antifungal Drug Response:

Available antifungal agents can be divided into groups according to their modes of action. Antifungals work by exploiting differences between mammalian and fungal cells. Most of them impair membrane integrity (polyenes), ergosterol biosynthesis (azoles) or inhibit macromolecule synthesis (flucytosine). Resistance to flucytosine is common and usually arises from the loss or mutation of cytosine permeases (involved in its uptake), or other relevant enzymes [154]. Toxic compounds in the environment require an immediate cellular adaptation. In S. cerevisiae it has been shown that this rapid adaptation also includes induction of the general stress response [34].
Continuous application of antifungal drugs favored the development of multidrug resistance (MDR) pathways, which confer resistance to a broad spectrum of unrelated chemicals. In general, MDR is based on the overexpression of membrane transporters, which export chemically different compounds by either gene amplification or change of transcription [38]. In *S. cerevisiae*, MDR is referred to as pleiotropic drug response (PDR) [155]. At least 10 transcription factors are regulating about 70 genes [156]. Among those, Pdr1 is involved in induction of the largest set of genes (about 50). Pdr1 and its functional homologue Pdr3 can confer resistance to a broad variety of unrelated drugs through inducing expression of several ATP-binding cassette transporters, such as Pdr5 and Pdr15 [157-159]. Pdr1 seems to be active under normal growth conditions, since one of its target genes, the ABC transporter Pdr5, is highly expressed [160]. In *C. albicans*, two functional homologues of Pdr5, Cdr1 and Cdr2 (for Candida drug resistance), have been identified, which were involved in drug resistance [161, 162]. However, the regulation seems to work differently in *C. albicans*, since the responsible transcription factor, Tac1, induces only five genes [163]. In *C. glabrata* however, the exact mechanisms involved in drug resistance are not completely understood. It has been shown that increased expression of *CgCDR1* confers resistance to azole treatment in AIDS patients [164]. Deletion of *CgCDR1* in *C. glabrata* led to an increased susceptibility against azole derivatives. Further, a second, *CgCdr2*, and a third ABC transporter, *CgSnq2*, contribute to elevated azole resistance [165, 166]. Accordingly, the disruption of *CgPDR1* ceased *CgSNQ2* expression and decreased azole resistance. Beside the beneficial effect for elder or immunocompromised persons of applying antifungal drugs, the functional immune system of mammalian hosts is able to counteract fungal infections via innate and adapted immunity. This will be discussed below. However, multidrug resistance pathways induced by various applied chemicals seems to be conserved among opportunistic pathogenic yeasts.

**How does the immune system of the human host counteract Candida infections?**

Immunocompetence is essential to withstand infections of opportunistic fungal pathogens. *C. albicans* and *C. glabrata* infections induce a similar but not identical immune system response [167]. The presence of hyphae in tissues of *C. albicans*-infected mice causes a significant inflammatory response, characterized by an early neutrophilic infiltrate, followed by a mononuclear cell infiltrate composed mainly of macrophages. In contrast, the cell infiltrate into tissues of *C. glabrata*-infected mice was composed primarily of macrophages [167, 168]. Further, a dose dependent rapid induction of anti-inflammatory cytokines in response to *C. albicans* was found [169].
Similarly, a fast induction of cytokines, including TNF-α, IL-12 and IFN-γ, and lack of induction of IL-10 does occur upon systemic *C. glabrata* infection. TNF-α seems to play a key role in innate resistance to systemic *C. albicans* and *C. glabrata* infections, as neutralization of endogenous TNF-α activity alone results in significant increase in growth in infected tissues [167, 170-172]. TNF-α facilitates phagocytic cell activation, which results in altered cell functional responses including increased both adherence and phagocytosis [173]. Interestingly, IL-10 impairs development of a protective immune response to *C. albicans*, due to downregulation of phagocytic cell effector mechanisms [174, 175].

Helper T lymphocytes, which are activated through antigen presenting cells, secrete cytokines to mobilize macrophages and to initiate proliferation and differentiation of T and B lymphocytes. T helper cells can be divided into T<sub>h</sub>1 and T<sub>h</sub>2 cells. They differ in cytokine secretion and subsequent activation of phagocytic cells. The T<sub>h</sub>1 differentiation pathway is a response to engulfed or intracellular microbes. IFNγ is produced and macrophages become activated. T<sub>h</sub>2 differentiation is linked to allergens or helminthes. Antibodies stimulated by T<sub>h</sub>2 cytokines do not promote phagocytosis. Concerning *Candida* infections, it is thought that there is a dynamic balance of T<sub>h</sub>1 and T<sub>h</sub>2 responses [176]. For example, IL-12 production is required for the development of T<sub>h</sub>1-cell responses that are maintained in the presence of physiological levels of IL-4 and IL-10 [177, 178]. Accordingly, a regulated balance of directive cytokines, such as IL-4, IL-10, and IL-12, rather than the relative absence of opposing cytokines, appears to be required for development and maintenance of T<sub>h</sub>1 reactivity in mice. Taken together, T<sub>h</sub>1 response conferring a phagocyte-dependent immunity is critical for opposing infectivity of the commensals and clearing the yeast from infected tissue [176, 179, 180].

Interestingly, in contrast to the observations described above, B cell knockout mice are resistant to mucosal and systemic candidiasis of endogenous origin but are susceptible to experimental systemic candidiasis [181]. Further, in liver transplant patients, their serum antibody to CgHsp90 was associated with survival from disseminated disease with *C. albicans* [182]. Further, liposome-based mannan vaccines protect against experimental infection in mice, demonstrating an induced antibody-mediated immunity for protection against systemic candidiasis [183, 184].

The more we find out about the situation in the bloodstream and in tissues during ongoing dissemination, the more exactly we can determine the important roles of cell-mediated and humoral immunity. However, cell-mediated immunity is based on the activation of phagocytic cells. Upon internalization, engulfed cells have to adapt to
a new microenvironment. Investigating the strategies of microbes to sustain phagocytosis is essential to reveal their routes for dissemination.

**In vivo situation during phagocytosis.**

Phagocytosis is a specific form of endocytosis involving the vesicular internalization of microbes. Engulfment of foreign intruders is carried out by professional phagocytic cells, such as neutrophils, macrophages, dendritic cells and mast cells. The phagocytic cell sends out membrane projections (pseudopodia) that make contact with extracellular particles. Multiple Receptor ligand interactions occur between the phagocytic cell and the microbe that will be ingested. This results in the formation of an intracellular vesicle. This so-called phagosome is a prison-like, hostile environment. After internalization of pathogenic cells, these organelles undergo a maturation process, resulting in an acidic phagolysosome with pH 4.5-5.5 equipped with mature hydrolytic enzymes [185, 186]. Inhibition of phagolysosomal acidification and associated lysosomal fusion reduced germ tube formation of *C. albicans* [187].

In addition, the NADPH oxidase complex mediates oxidative destruction of internalized microorganisms through catalysing ROS [59]. It was found that engulfed *Candida* cells are exposed to ROS when they come into contact with phagocytic cells in the bloodstream, but that oxidative killing is no longer a significant threat once an infection has been established in the kidney of mice. Investigation of the oxidative stress response of these cells indicated a mild stress situation [85]. Quantification of fluorescence levels of induced reporter proteins suggested that they were exposed to an oxidative stress that is equivalent to about 0.4mM H$_2$O$_2$ for early and late infection time points. The involvement of oxidative stress as defence strategy is not restricted to fungal infections. Invading bacteria, such as *Staphylococcus aureus*, or the malaria parasites *Plasmodium sp.*, also face ROS stress upon engulfment [188, 189].

Besides being exposed to oxidative stress, cells engulfed in macrophages are sealed from the environment and thus have to rely on endogenous resources or those acquired from the phagocytic cell. *C. albicans* engulfed within macrophages induces genes involved in non-fermentative carbon metabolism [190, 191]. In detail, ingestion of *C. albicans* cells by macrophages enforced two reprogramming events: the early and the late response. Early response is based on the reconfiguration of carbon metabolism: cells shift from glycolysis, the metabolic pathway to convert glucose, to gluconeogenesis, the generation of glucose from non-carbohydrate carbon substrates
Transcript profiles also showed a clear induction of genes required to convert fatty acids to glucose. Further, this shift is accompanied by a massive downregulation of genes involved in translation. The late response is a resumption of glycolysis and restoration of the translation machinery, and is induced as *C. albicans* escapes through formation of hyphae [191]. Moreover, the glyoxylate cycle, required to channel fatty acid derived carbon into catabolism, is a virulence determinant for *C. albicans* [194]. Similar to engulfed *S. cerevisiae* cells, phagocytosis also up-regulates the most important enzymes of the glyoxylate cycle, isocitrate lyase (*ICL1*) and malate synthase (*MLS1*) in *C. albicans*. *C. albicans* mutants lacking *ICL1* are markedly less virulent in mice than the wild type.

In addition, other nutrient restrictions might be crucial as well. Phagocytes use iron-binding proteins to reduce iron availability. It has recently been shown, that lactoferrin restrains infection of the filamentous fungal pathogen *Aspergillus fumigatus* [195]. Lactoferrin is a multifunctional protein, which can be found mainly at mucosal areas as part of the innate defence. Its antimicrobial activity is based on scavenging free iron and its specific interaction with microbial components, thereby interrupting the attachment to cell membranes of the host [196, 197]. Human lactoferrin is also able to kill *C. albicans* in a dose- and time-dependent manner [198].

However, in case of *C. glabrata*, the *in vivo* situation is still unclear. *C. glabrata* experiences mild oxidative stress and in parallel sustained carbon starvation stress inducing autophagy, as described in [Results]. Obviously, when the immune system is compromised, the opportunistic pathogen *C. glabrata* has the ability to counteract engulfment and propagate dissemination.

Besides overcoming the oxidative burst and nutrient shortage, cells might actively try to sabotage maturation of the phagosome. Bacteria have developed effective strategies to survive engulfment. For example, it is known that intracellular *Mycobacterium* species can inhibit phagosomal maturation, including inhibition of acidification of the phagosome and inhibition of phagosome-lysosome fusion [199, 200]. Infantile mice are more susceptible to bacterial infection due to lack of acidification of the lysosomes of macrophages [201]. Strikingly, pretreatment with IFN-γ could rescue acidification. There is evidence that there exists a direct link between cytokine effects on the host cell and phagosome maturation in the macrophage [202]. If this is crucial to repress bacterial infection, this might be important for infections by yeasts as well.

Taken together, fungal cells have to overcome a multiple stress situation during prolonged engulfment. The strategy for escaping from phagocytic cells is still unclear for *C. glabrata.*
References


Introduction


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Introduction


Introduction

Aim of this work:

Although *Candida glabrata* shares the same genus name with *Candida albicans*, it is much closer related to *Saccharomyces cerevisiae*. Therefore it was the aim to find out more about striking similarities and discrepancies between *C. glabrata* and *S. cerevisiae*. Despite their close relatedness, only *C. glabrata* evolved into a commensal/pathogen.

The following study can be divided in three parts:

1) Characterization of the environmental stress response (ESR) in *C. glabrata*.

*S. cerevisiae*, *S. pombe* and *C. albicans* have a qualitatively similar ESR. It was the aim to define the genome wide environmental stress response expression profile of *C. glabrata*. Therefore cells were exposed to different stress such as heat stress, glucose starvation, osmotic and oxidative stress. Further, in *S. cerevisiae*, Msn2 and Msn4 are important transcription factors of the ESR. Comparing several putative Msn2-like sequences, a related group of fungi among the class Saccharomycotina possessing Msn2 orthologues was identified. In *C. glabrata*, orthologues for Msn2 and Msn4 were found. Their role in the ESR and their contribution to virulence was investigated.

2) Analysis of the in vivo situation upon engulfment through primary mouse macrophages.

To erase microbes, phagocytic cells try to catch and engulf them. Captured intruders have evolved different strategies to resist engulfment. For example, *C. albicans* is able to escape phagocytosis through hyphae formation. To investigate the response of *C. glabrata* to engulfment, fluorescent reporter proteins were employed. Microscopy revealed a transient acute oxidative stress and lasting glucose starvation inside the phagosome of macrophages. During prolonged phagocytosis, a decline of peroxisome numbers due to pexophagy was observed. CgAtg11 and CgAtg17 were found to be crucial factors to mediate this response. Both selective and non-selective autophagic processes were shown to contribute to survival.

3) Investigation of the role of the two oxidative stress response transcription factors CgSkn7 and CgYap1 in *C. glabrata* in vitro and in vivo.

In *S. cerevisiae*, two transcription factors, Skn7 and Yap1, are responsible for rapid transcription upon oxidative stress. In *C. glabrata*, the orthologous transcription factors CgSkn7 and CgYap1 induce the majority of crucial genes of the oxidative stress regulon. Their contribution to virulence was investigated in an ex vivo model.
Additionally, analysis of the binding capacities of CgSkn7 and CgYap1 revealed a strict dependence on each other to induce expression of set of target genes. Finally, during glucose starvation, numerous genes of the oxidative stress regulon get induced, which may mimic the in vivo situation upon engulfment by phagocytes.
2. Results:

2.1. *Candida glabrata* environmental stress response involves *Saccharomyces cerevisiae* Msn2/4 orthologues transcription factors

2.2. Autophagy supports *Candida glabrata* survival during phagocytosis

2.3. CgSkn7 and CgYap1 mediate key responses to oxidative stress in *Candida glabrata*

2.4. Major results obtained
Candida glabrata environmental stress response involves Saccharomyces cerevisiae Msn2/4 orthologous transcription factors

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Summary

We determined the genome-wide environmental stress response (ESR) expression profile of Candida glabrata, a human pathogen related to Saccharomyces cerevisiae. Despite different habitats, C. glabrata, S. cerevisiae, Schizosaccharomyces pombe and Candida albicans have a qualitatively similar ESR. We investigate the function of the C. glabrata syntenic orthologues to the ESR transcription factor Msn2. The C. glabrata orthologues CgMsn2 and CgMsn4 contain a motif previously referred to as HD1 (homology domain 1) also present in Msn2 orthologues from fungi closely related to S. cerevisiae. We show that regions including this motif confer stress-regulated intracellular localization when expressed in S. cerevisiae. Site-directed mutagenesis confirms that nuclear export of CgMsn2 in C. glabrata requires an intact HD1. Transcript profiles of CgMsn2/4 mutants and CgMsn2 overexpression strains show that they regulate a part of the CgESR. CgMsn2 complements a S. cerevisiae msn2 null mutant and in stressed C. glabrata cells, rapidly translocates from the cytosol to the nucleus. CgMsn2 is required for full resistance against severe osmotic stress and rapid and full induction of trehalose synthesis genes (TPS1, TPS2). Constitutive activation of CgMsn2 is detrimental for C. glabrata. These results establish an Msn2-regulated general stress response in C. glabrata.

Introduction

Adaptation of gene expression through regulation of transcription is a key mechanism in fungal response to fluctuating environmental conditions. Environmental stress causes activation of a variety of signalling mechanisms each responding to the particular situation, such as heat shock or osmotic stress, and in parallel evokes a stereotypic general response. In Saccharomyces cerevisiae, this response was first described and is referred to as general stress response or environmental stress response (ESR) (Gasch et al., 2000; Causton et al., 2001). Comparable ESR patterns have been characterized in Schizosaccharomyces pombe and to a certain extent in Candida albicans (Smith et al., 2004; Enjalbert et al., 2006; Gasch, 2007). Candida glabrata is more closely related to S. cerevisiae than C. albicans and S. pombe (Fitzpatrick et al., 2006), and is the second most common fungal pathogen isolated from humans (Kaur et al., 2005; Pfaller and Diekema, 2007). Infection rates are relatively low but have been constant during the last decade (Sandven et al., 2006). The ESR of C. glabrata is currently relatively unexplored.

For S. cerevisiae, C. albicans and S. pombe, one major mechanism for controlling general stress responses are p38-type SAP kinases (stress-activated mitogen-activated protein kinases). The SAPKs, Hog1, Sty1 and CaHog1 are all activated by hyperosmolality and oxidative stress, and to a varying degree by other stress agents, such as cadmium (Chen et al., 2003; Smith et al., 2004; Enjalbert et al., 2006). The HOG (high osmolarity glycerol) pathway of C. glabrata functions in a similar manner to S. cerevisiae (Gregori et al., 2007).
**Fig. 1.** Comparison of genome-wide expression levels in response to environmental changes in *C. glabrata* and *S. cerevisiae*. 
A. Hierarchical clustering. Transcript profiles were determined by hybridization to genome-wide *C. glabrata* microarrays. The sets represent average inductions of replicate profiles of *C. glabrata* wild-type strain (4166 ORFs) after treatment with 0.4 mM H$_2$O$_2$, upon glucose starvation, heat shock by incubation at 42°C and hyperosmolarity stress treatment with 0.5 M NaCl (left panel). All treatments were done at 30°C for 20 min. The developed profile was compared with corresponding *S. cerevisiae* expression data (Gasch *et al*., 2000) (right panel). Major clusters are labelled corresponding to induced and to repressed genes (labelled as 1 and 2), in both *C. glabrata* and *S. cerevisiae*. B. Genes involved in four different stress responses were clustered after specific selection (heat stress > 11-fold, osmotic stress > sixfold, oxidative stress > sixfold and glucose starvation > 10-fold). Identified clusters in both *C. glabrata* and *S. cerevisiae* are indicated. Gene names correspond to *C. glabrata* systematic ORF designations and their corresponding *S. cerevisiae* orthologues. C. The overlap between the CgESR and ScESR patterns is depicted as Venn diagram. CgESR was defined as described in Fig. 2A, with clusters are labelled corresponding to induced and to repressed genes (labelled as 1 and 2), in both *C. glabrata* and *S. cerevisiae*. 

In *S. cerevisiae*, a second general stress-mediating mechanism based on the transcription factor Msn2 and its parologue Msn4 exists (Martinez-Pastor *et al*., 1996; Estruch, 2000; Gasch *et al*., 2000; Causton *et al*., 2001; Hohmann, 2002). They are activated by a variety of stress conditions and changing nutrient supply situations, such as the exhaustion of the preferred carbon source glucose (Choo and Klug, 1994; Martinez-Pastor *et al*., 1996; DeRisi *et al*., 1997; Boy-Marcotte *et al*., 1998; Gasch *et al*., 2000; Rep *et al*., 2000; Causton *et al*., 2001; Hasan *et al*., 2002; Cameroni *et al*., 2004; Schüller *et al*., 2004; Teixeira *et al*., 2006). They also have a role in both chronological and replicative ageing (Fabrizio *et al*., 2001; 2004; Powers *et al*., 2006). During high-nutrient supply, Msn2 is inactivated by the PKA (protein kinase A) and TOR (target of rapamycin) pathways (Boy-Marcotte *et al*., 1998; Görner *et al*., 1998; Garreau *et al*., 2000; Görner *et al*., 2002). Activation of Msn2 and Msn4 causes their rapid accumulation in the nucleus and recruitment to chromatin. Msn2 has separate functional domains for nuclear import (nuclear localization signal, NLS), nuclear export (nuclear export signal, NES) and DNA binding. The C$_2$H$_2$ Zn finger DNA binding domain at the C-terminus recognizes the stress response element (STRE). The NLS is found adjacent to the DNA binding domain; it is phosphorylated and inactivated by PKA when glucose is available and rapidly dephosphorylated and activated by glucose starvation (Görner *et al*., 2002; De Wever *et al*., 2005). Stress signalling requires a region in the N-terminal part of Msn2 which includes its NES and a short stretch of high similarity to Msn4 designated homology domain 1 (HD1) (Görner *et al*., 1998; Görner *et al*., 2002; Durchschlag *et al*., 2004; Boy-Marcotte *et al*., 2006). A variety of stress conditions lead to inhibition of nuclear export of Msn2 by an unknown mechanism. The NES and its surrounding region might therefore represent a crucial determinant for the identification of stress-regulated Msn2 orthologues.

Msn2-like factors do not appear to play a role in regulating the stress response in *C. albicans* and *S. pombe*. The *C. albicans* Msn2 orthologue transcription factor designated CaMsn4 (orf 19.4752) is not involved in the ESR (Nicholls *et al*., 2004). In addition, the Hog1 Map kinase plays a more general role in stress response in *C. albicans* and *S. pombe*. These differences point to distinct strategies for regulating the stress response in different fungi.

Here we investigate ESR transcription patterns of *C. glabrata* and provide evidence that it uses an *S. cerevisiae*-like Msn2-directed stress response. We identify *C. glabrata* Msn2 and Msn4 orthologues based on a motif present in the Msn2/4 NES (HD1) and also in putative Msn2 orthologues of *Ashbya gossypii* and *Kluyveromyces lactis*. Furthermore, we find that *C. glabrata* and *S. cerevisiae* share many common Msn2 target genes. CgMsn2 is required for resistance against severe osmotic stress. In addition, comparison of ESR transcript patterns identifies core similarities and differences between the *S. cerevisiae*, *C. albicans*, *S. pombe* and *C. glabrata* stress responses.

**Results**

The ESR pattern of *C. glabrata* is orthologous to *S. cerevisiae*

The global immediate transcriptional response of *C. glabrata* to a set of environmental conditions was determined via microarray analysis. Conditions chosen were acute carbon starvation by removal of glucose from the medium, mild osmotic stress (0.5 M NaCl), heat stress (42°C) and mild oxidative stress (0.4 mM hydrogen peroxide). The treatment times were 20 min at 30°C to avoid indirect transcriptional responses. Transcript profiles were determined by hybridization to genome-wide *C. glabrata* microarrays. Expression data were filtered and averaged. From the 5063 genes spotted in duplicate, 4166 gave useful data under at least one tested condition. The entire data set was analysed for co-regulated genes by hierarchical clustering (Eisen *et al*., 1998).

Similar to the common stress response identified in *S. cerevisiae*, *C. glabrata* has a set of induced and repressed genes common to several stress conditions (Fig. 1A). To compare the *C. glabrata* expression pattern with *S. cerevisiae* data, we used a similarity-based annotation of orthologous genes (Feldmann, 2000) (http://cblab.life/cbi.lab.rl.fr/Genolevures/). Expression data for *S. cerevisiae* exposed to comparable conditions, such as glucose starvation, 0.32 mM H$_2$O$_2$, heat stress (37°C) and 1 M Sorbitol osmotic stress, were extracted from published ESR data.
Induced genes in ESRs:

CgESR

358

65

283

ScESR

Repressed genes in ESRs:

CgESR

424

203

585

ScESR

C. glabrata  S. cerevisiae

>4X induced

>4X repressed

A

oxidative stress

osmotic stress

heat stress

glucose starvation

B

Heat stress

response to stress

protein folding

C

Induced genes in ESRs:

CgESR

ScESR

605

603–620
(Gasch et al., 2000). *C. glabrata* has a optimal growth temperature of 37°C and was therefore heat-stressed at 42°C. Available evidence suggests that NaCl and Sorbitol are comparable in the concentrations used (Hirasawa et al., 2006). Analysis of both ESR data sets highlights clusters corresponding to induced and repressed genes for all environmental conditions (Fig. 1A). This indicates a conserved transcriptional response between *C. glabrata* and *S. cerevisiae*. More detailed comparison of individual stress conditions shows induction of expression of orthologous genes (Fig. 1B). For example, heat stress induces expression of conserved HSP genes in both organisms (HSP12, HSP42, HSP78, HSP31, HSP104), whereas oxidative stress affects a smaller set of genes in *C. glabrata*. The hydrogen peroxide concentration chosen (0.4 mM) is stressful for *C. glabrata* whereas oxidative stress affects a smaller set of genes in *S. cerevisiae* (Gasch et al., 2000). We extended this list by adding the corresponding *C. glabrata* genes with gene similarity data reported by the Genolevures consortium. Data used to generate these figures are available as supplementary files. Comparison of the specific expression profiles revealed a striking overlap between orthologous genes of the individual species for osmotic stress-induced genes (Fig. S2A) and oxidative stress-induced genes (Fig. S2B).

### Orthologues of general stress transcription factors Msn2 and Msn4 in *C. glabrata*

To explore the regulation of the *C. glabrata* ESR (referred hereafter as CgESR), we compared transcription patterns with the *S. cerevisiae* ESR (ScESR). We defined the CgESR by selecting genes from the *C. glabrata* data set which are induced or repressed more than fourfold in at least one condition. This selection resulted in a set of 760 CgESR genes. Of these, 268 genes overlap with the 868 ScESR genes (Gasch et al., 2000) (Fig. S3A and S3B). Many of the shared stress-induced genes are induced in *S. cerevisiae* upon overexpression of the general stress transcription factor Msn2 (Chua et al., 2006) (Fig. 2A column MSN2 OE). Furthermore, Msn2 binding sites (STRE; WAGGGG) are present in many CgESR genes. This suggested that the evolutionary conservation of the ESR is not limited to the set of regulated genes, but also extends to the transcriptional control of those genes.

To identify orthologues of *S. cerevisiae* Msn2 (ScMsn2) in *C. glabrata*, we searched for predicted open reading frames (ORF) comprising C,C,Zn-cluster DNA binding domains in available fungal genomes. One further recognizable feature of ScMsn2 is its central region, which confers regulated nuclear export. This is conserved in the paralogue Msn4, and was previously described as HD1 (Görner et al., 1998). We detected putative Msn2 (CAGL0F05995g) and Msn4 (CAGL0M13189g) orthologues with HD1 domains at synthetetic positions in the *C. glabrata* genome (Fig. 2B). *K. lactis* and *A. gossypii* contain a single orthologue of both genes that also contain an HD1 domain, which we have designated Msn2. The HD1 domain is not present in the single Msn2/4 orthologue in *C. albicans* and related species, nor
in similar proteins from Yarrowia lipolytica, nor S. pombe (Fig. 2B).

Stress-regulated nuclear export of both ScMsn2 and ScMsn4 requires an extended region, including the HD1. To pinpoint the HD1 region of the ScMsn2/4 orthologues as a functional component of the NES, we tested if these regions are sufficient to confer stress-regulated intracellular localization. We expressed parts containing the HD1 but not the NLS and the C-terminal Zn-finger DNA binding domains of the K. lactis, A. gossypii, and C. glabrata Msn2 orthologues as GFP fusions in S. cerevisiae (Fig. 3A). Sequences were amplified from genomic DNA and fused to an N-terminal nuclear localization signal (SV40NLS) to support constitutive nuclear import. Expression of the fusion proteins was driven by the ScADH1 promoter. Cells were grown to early exponential phase, exposed to osmotic stress (0.5 M NaCl) or weak acid stress (10 mM sorbic acid), and the intracellular distribution of the GFP fusion proteins was recorded by fluorescence microscopy. The GFP fusion proteins of the internal regions of Msn2 and Msn4 from S. cerevisiae and C. glabrata and Msn2 from K. lactis and A. gossypii, accumulated rapidly in the nucleus under stress conditions (Fig. 3A). We also analyzed the localization of orf19.4752 (CaMsn4), the C. albicans orthologue of both ScMsn2 and ScMsn4 (Nicholls et al., 2004). CaMsn4 has a similar Zn-finger DNA binding region to the S. cerevisiae and C. glabrata proteins, but lacks a significant similarity to HD1. The GFP fusion protein comprising the CaMsn4 N-terminal 517 amino acids was constitutively enriched in the nucleus in unstressed cells and stress-induced nuclear accumulation was not detectable. As presented below, point mutations in the conserved residues of the HD1 in CgMsn2 also abolish the NES function (Fig. 4C). These data suggest that regulated nuclear export requires the HD1 domain.

To verify if CgMsn2 functions as a stress-responsive transcription factor, we tested whether CgMsn2 can replace and complement the function of ScMsn2 in S. cerevisiae. We expressed full-length CgMSN2 in a S. cerevisiae strain lacking ScMSN2 and ScMSN4. The entire CgMSN2 reading frame was fused to CFP and its expression driven by the constitutive ADH1 promoter. In unstressed cells, CgMsn2–CFP was distributed mainly in the cytoplasm, similar to the analogous ScMsn2–GFP protein. Several environmental stress conditions induced

Fig. 2. CgESR is similar to ScESR and includes many Msn2-regulated genes.

A. The CgESR shown here includes 760 genes selected by being induced or repressed significantly (fourfold) in one of the tested conditions. Columns 5 (up in S.c.) and 6 (down in S.c.) show the corresponding ScESR genes from S. cerevisiae. Column 7 (MSN2 OE in S.c.) displays induction of the orthologous S. cerevisiae genes by MSN2 overexpression (Chua et al., 2006).

B. Alignment of Msn2 orthologous sequences including the HD1. The shared core motif corresponds to positions 284–314 of ScMsn2. The HD1 signature was detected only in close relatives of S. cerevisiae, circled in the phylogeny (taken from Fitzpatrick et al., 2006). The sequences used in the alignment are: S. cerevisiae (YMR037C, ScMsn2; YKL062W, ScMsn4), A. gossypii (ABR089C, AgMsn2), C. glabrata (CAGL0F05995g, CgMsn2; CAGL0M13189g, CgMsn4) and K. lactis (KLLA0F26961g, KIMsn2).
rapid nuclear concentration of CgMsn2 in S. cerevisiae (Fig. 3B). Next we investigated whether CgMsn2 can complement ScMsn2 and confer stress-regulated gene activation in S. cerevisiae. CgMsn2–CFP was expressed in a S. cerevisiae strain lacking both MSN2 and MSN4 genes (W303 msn2Δ msn4Δ) exposed to osmotic, weak acid and glucose starvation stress. mRNA levels of a target gene of ScMsn2, the CTT1 gene coding for catalase T, were analysed by Northern hybridization. As shown in Fig. 3C, CgMsn2 supported the stress induction of CTT1 transcripts in a very similar manner to ScMsn2.

CgMsn2 is regulated by stress in C. glabrata

These results suggested that CgMsn2 might be the functional orthologue of ScMsn2. To examine the regulation of CgMsn2 in C. glabrata, we first analysed its intracellular localization under stress conditions. CgMsn2 was expressed as CgMsn2–CFP fusion driven by the CgADH1 promoter (Fig. 4A). Live microscopy of CgMsn2–CFP localization revealed a rapid and reversible nuclear accumulation regulated by environmental stress conditions, such as acute glucose starvation, osmotic stress, heat shock, oxidative stress and ethanol stress (Fig. 4A). CgMsn2 activity as a transcriptional activator is not changed by the presence of the C-terminal CFP fusion. In contrast, CgMsn2–CFP failed to accumulate in the nucleus during weak organic acid stress (10 mM sorbic acid, 30 mM propionic acid). Interestingly, CgMsn2–CFP expressed in S. cerevisiae accumulates in the nucleus during weak acid stress. This difference in the response of CgMsn2 during weak acid stress could be either a consequence of the higher resistance of C. glabrata to weak acids compared with S. cerevisiae, which is not the case (Gregori et al., 2007), or the absence of a mechanism signalling weak acid stress to CgMsn2 in C. glabrata.

CgMsn2 accumulates in the nucleus within 4 min following acute carbon source starvation, and subsequent addition of glucose (2%) results in rapid nuclear export (Fig. 4B). The kinetics are very similar to those observed in S. cerevisiae (data not shown). To demonstrate that the integrity of the HD1 region is important for NES function, we constructed a mutant derivative by replacing two conserved serine residues S230 and S232 with alanine. The

Fig. 3. Regulated localization control is functionally conserved in Msn2-like factors.

A. Indicated regions of Msn2 and Msn4 orthologues were fused to GFP and a SV40-NLS. GFP fusion plasmids were expressed in S. cerevisiae strain W303-1A msn2Δ msn4Δ. Localization of GFP fusions was determined by fluorescence microscopy in unstressed cells and 10 min after exposure to weak acid stress (10 mM sorbic acid) and osmotic stress (0.5 M NaCl).

B. S. cerevisiae W303-1A msn2Δ msn4Δ strains containing plasmids expressing either CgMsn2–CFP or ScMsn2–GFP driven by the ScADH1 promoter (pAMG, pACgMC) were grown to exponential phase and exposed to conditions as indicated. Localization was recorded after 10 min by fluorescence microscopy of living cells.

C. mRNA levels of the Msn2-regulated gene CTT1 and the control IPP1 were visualized on Northern blots after 20 min stress treatment.
Fig. 4. *C. glabrata* CgMsn2 nuclear localization is stress-regulated.

A. The CgADH1-CgMsn2–CFP construct is illustrated schematically and the positions of used restriction sites are indicated. Localization of CgMsn2–CFP in the *C. glabrata* strain Cg msn2Δmsn4Δ was determined by fluorescence microscopy. CFP fluorescence was recorded in exponentially growing cells approximately 10 min after exposure to the indicated stress conditions. Nuclei were stained by addition of 2 μg ml⁻¹ 4,6 diamidino-2-phenylindol (DAPI) dye to the cultures 10 min prior to microscopy. In living cells, nucleic acids, such as the mitochondrial DNA, are also stained by DAPI, resulting in background staining. CgMsn2 localization during weak acid stress. CgMsn2–CFP accumulates in *S. cerevisiae* in the nucleus during weak acid stress (10 mM sorbic acid, 30 mM propionic acid). CgMsn2–CFP does not accumulate in the nucleus in *C. glabrata*. Arrows indicate stained nuclear DNA.

B. Nucleocytoplasmic shuttling of CgMsn2–CFP during glucose starvation and re-feeding. Localization of CgMsn2–CFP was visualized by fluorescence microscopy of *C. glabrata* cells fixed to a coverslip with Concanavalin A and localization of the CFP fusion was visualized by fluorescence microscopy.

C. Localization of CgMsn2 S230AS232A–CFP in unstressed cells. Cg msn2Δmsn4Δ cells expressing CgMsn2 S230AS232A–CFP were grown in rich media to early logarithmic phase and localization of the CFP fusion protein was determined by fluorescence microscopy.

D. Viability of Cg msn2Δmsn4Δ mutant cells expressing Msn2 variants. Cultures of Cg msn2Δmsn4Δ transformed with the empty vector as a control, or with a plasmid expressing CgMSN2 under the control of the native promoter, were incubated in selective media containing 2 M NaCl for 2 and 20 h. Cell suspensions were spotted in 10-fold dilutions on YPD plates and incubated at 37°C over night. Cultures of Cg msn2Δmsn4Δ transformed with the empty vector, or with plasmids expressing CgMSN2 under the control of the native or CgADH1 promoter, or expressing CgMSN2 S230AS232A–CFP, were grown in selective media at 30°C for 10 days. Cells were then spotted in 10-fold dilutions on YPD and SC-Trp plates incubated at 37°C over night and growth recorded.

E. High expression of CgMsn2 S230AS232A confers cold sensitivity. Replica-plated patches of Cg msn2Δmsn4Δ transformed with the above vectors were grown on selective plates overnight at 37°C, plates were kept at 4°C and room temperature as a control for 14 days. Plates were replica-plated to fresh and incubated at 37°C over night and growth recorded. Viability was also tested by colony-forming assay showing a threefold reduced colony-forming ability (0.38 of wild type; SD = 0.12; P = 0.05).
corresponding positions in the ScMsn2 ORF are indicated by arrows in Fig. 2B. As predicted, we find the CgMsn2 S230AS232A-CFP mutant constitutively enriched in the nucleus in unstressed cells, presumably as a result of impaired nuclear export and the basal activity of its nuclear localization signal (Fig. 4C).

We further tested the role of CgMSN2 for C. glabrata survival under extreme osmotic stress. We generated a strain (Cg msn2Δmsn4Δ) lacking both CgMsn2 and CgMsn4 by homologous gene replacement with the CgHIS3 and ScURA3 genes respectively. The correct integrations were confirmed by Southern blot (Fig. S1). The Cg msn2Δmsn4Δ strain has no obvious growth phenotype under laboratory conditions, similar to S. cerevisiae msn2Δmsn4Δ double mutants. Cultures of Cg msn2Δmsn4Δ cells transformed with the empty vector as a control, or with a plasmid expressing CgMSN2–CFP driven by the ADH1 promoter were grown for 10 days at 30°C in selective media. Viability was then assayed by spotting 10-fold dilutions. Cells expressing the constitutive nuclear mutant were significantly underrepresented compared with wild type and vector control (about two- to threefold, Fig. 4D, middle panel). The equal cell number on YPD suggests that the CgMSN2 S230AS232A–CFP plasmid, but not the other plasmids, was counter-selected in the culture. Cells carrying the mutant plasmid were also cold-sensitive (Fig. 4E).

Taken together, these data demonstrate that nuclear transport of CgMsn2 is highly regulated and requires the integrity of the HD1 region. CgMsn2 is beneficial under extreme stress conditions and its constitutive expression seems to be detrimental.

Expression of stress genes is tightly regulated and de-regulation has often adverse consequences. We therefore tested if high activity of CgMsn2 compromises long-term viability of C. glabrata. Cg msn2Δmsn4Δ cells transformed with plasmids expressing CgMSN2–CFP or CgMSN2 S230AS232A–CFP driven by the ADH1 promoter were grown for 10 days at 30°C in selective media. Viability was then assayed by spotting 10-fold dilutions. Cells expressing the constitutive nuclear mutant were significantly underrepresented compared with wild type and vector control (about two- to threefold, Fig. 4D, middle panel). The equal cell number on YPD suggests that the CgMSN2 S230AS232A–CFP plasmid, but not the other plasmids, was counter-selected in the culture. Cells carrying the mutant plasmid were also cold-sensitive (Fig. 4E).

Taken together, these data demonstrate that nuclear transport of CgMsn2 is highly regulated and requires the integrity of the HD1 region. CgMsn2 is beneficial under extreme stress conditions and its constitutive expression seems to be detrimental.

The CgMsn2 regulon is related to the ScMsn2 regulon
ScMsn2 has about 100–150 target genes depending on the conditions (Gasch et al., 2000; Schüller et al., 2004).
To define the targets of CgMsn2, we compared the expression profiles of C. glabrata wild type and Cg msn2Δmsn4Δ mutant strains during osmotic stress and acute glucose starvation. We also compared in parallel the mRNA profiles of cells carrying plasmids with the CgMsn2 gene under its own or the CgADH1 promoter (construct pCgADH1-CgMsn2–CFP). Many stress genes influenced by the absence of Msn2 are also induced by the overexpression of CgMSN2 (Fig. 5A, right panels, supplementary data). Furthermore, we find that C. glabrata and S. cerevisiae share a set of 21 Msn2 targets that are also part of the 65 induced genes shared between the ScESR and CgESR (Fig. 5B). All the genes found in this core set possess at least one STRE site in their promoters. STRE-like sequences were detected among the CgMsn2-regulated genes by an unbiased heuristic search using AlignAce in the upstream regions of the selected CgMsn2-regulated genes (Hughes et al., 2000) and are depicted in the form sequence logo (Fig. 5A, lower panel). The most salient gene functions are connected to stress response (HSP42, HSP12, SML1, DDR48), glycogen and trehalose metabolism (TPS1, NTH1, TPS2, YGP1) or DNA repair (SML1, ribonucleotide reductase) based on GO-terms analysis (P-values < 0.005). Detailed data are available as supplementary files. However, genes regulated only in C. glabrata by CgMsn2 include the glyoxylate cycle enzyme MDH3 (cytoplasmic malate dehydrogenase), the glycolytic enzyme FBP26 (fructose-2,6-bisphosphatase) and PHM8 involved in phosphate metabolism, suggesting that there is different wiring of some metabolic pathways between the two species. In addition, there are changes in expression of signalling components, such as the casein kinase YCK1 involved in cell morphogenesis, which are specific to C. glabrata. Together, these data show that CgMsn2 has a broad set of target genes and that many of them are conserved between S. cerevisiae and C. glabrata. A smaller set of CgMsn2 and ScMsn2 target genes are conserved stress genes also in S. pombe and C. albicans (Fig. S2C). C. glabrata is a nicotinamide adenine dinucleotide (NAD+) auxotroph and its growth is dependent on exogenous supply of NAD+ precursors. A main part of the NAD+ metabolism is the Preiss-Handler pathway, where the nicotinamide Pnc1 ultimately converts precursors to NAD+ (Ma et al., 2007). PNC1 is upregulated in S. cerevisiae and C. glabrata during oxidative and osmotic stress (Fig. S2C).

CgMsn2 is required for rapid induction of osmotic stress-induced transcription of trehalose synthesis genes in C. glabrata

To confirm the function of CgMsn2 as a stress-regulated transcription factor, we measured the expression of

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CgMsn2 is required for rapid induction of transcription after osmotic stress. A. Northern blot analysis of CgTPS1, CgTPS2 and CgUBP15 transcripts during 0.5 M NaCl induced osmotic stress. C. glabrata wild type, Cg msn2Δmsn4Δ and Cg msn2Δmsn4Δ transformed with pCgMCgMsn2–CFP or pCgADH1CgMsn2–CFP were grown to exponential phase before 0.5 M NaCl was added. Samples for RNA extraction were taken at indicated time points. mRNA levels were visualized by hybridization of radio-labelled probes and autoradiography. CgACT1 mRNA was used as loading control. B. Quantification of mRNA levels of CgTPS1, CgTPS2 and CgUBP15 normalized to CgACT1 and expressed relative to the highest wild-type level.

CgTPS1, CgTPS2 and CgUBP15 over several time points following exposure to osmotic stress. TPS1 and TPS2, encoding trehalose-6-phosphate synthase and phosphatase respectively, are both required for the synthesis and the storage of the carbohydrate trehalose. UBP15, coding for an ubiquitin-specific protease, is induced by heat and osmotic stress in S. cerevisiae. Northern blot analysis of CgTPS1, CgTPS2 and CgUBP15 showed rapid induction upon treatment with 0.5 M NaCl (30°C and 37°C gave similar results) in the C. glabrata wild type, and also in C. glabrata msn2Δmsn4Δ mutant cells supplemented with CgMsn2 on a plasmid under its own promoter (pCgMCgMSN2) (Fig. 6A). CgADH1 promoter-driven expression of CgMsn2 in this strain (C. glabrata msn2Δmsn4Δ, pCgADH1CgMsn2–CFP) enhanced the signals of all genes in untreated and treated conditions. The CFP tag of CgMsn2 did not disrupt its activity (Fig. S4). In the absence of CgMsn2 and CgMsn4, mRNA levels were severely reduced. Stress-induced expression is not completely abolished in the double mutant cells, suggesting the parallel action of other factors reminiscent of S. cerevisiae (Reiser et al., 1999). These data confirm a direct role for CgMsn2 during osmostress induction. The impact of CgMsn4 remains to be elucidated. We conclude that general stress response mechanisms are at least partially conserved between C. glabrata and S. cerevisiae.

CgMsn2 is not required for virulence in a Drosophila melanogaster infection model

Candida glabrata is a human opportunistic pathogen. To test for a possible role of CgMsn2 in virulence, we tested the C. glabrata msn2Δmsn4Δ mutant in a D. melanogaster model of infection. Wild type flies are resistant to injection of 7500 C. glabrata cells (ΔHTU) (Fig. 7). In contrast, MyD88 mutant flies, in which Toll signalling, and thus the humoral arm of the antifungal response, are blocked, succumb in a few days to the same dose of cells. C. glabrata mutants lacking a functional Hog1 pathway have reduced virulence in this infection model, demonstrating its applicability for stress response mutants (not shown). However, we find that there is no difference in the survival of wild-type or immunosuppressed flies when injected either with wild type (ΔHTU) or Cg msn2Δmsn4Δ mutants. Similarly, we find no difference between the virulence of strains expressing CgMsn2–CFP under the control of the endogenous promoter or the strong constitutive CgADH1 promoter, and the empty control plasmid. These findings are in line with a role of Msn2 during...
extreme stress situation probably not encountered in the fly hemocoel.

Discussion

Despite their very different environmental niches and several hundred million years of phylogenetic distance, the transcriptional responses of *S. cerevisiae*, *C. albicans* and *S. pombe* to diverse environmental conditions (ESR) share significant similarities (Enjalbert et al., 2006; Gasch, 2007). *C. glabrata* is phylogenetically related to *S. cerevisiae*, but it is adapted to a mammalian host environment. This is reflected by adhesin-mediated adherence to surfaces, the absence of certain biosynthetic pathways (Domergue et al., 2005; Kaur et al., 2005) and different physiology, for example, optimal growth at 37°C. We report an analysis of part of the CgESR. The CgESR is similar to the ScESR and we identify a conserved role for the general stress transcription factor Msn2.

In many organisms, p38 MAP kinases are central to the control of environmental responses. The *S. pombe* Sty1, *C. albicans* CaHog1 and *S. cerevisiae* Hog1 SAPK mediate the response to a variety of environmental conditions (Toone and Jones, 1998; Wysong et al., 1998; Alonso-Monge et al., 1999; Hwang et al., 2002; Enjalbert et al., 2006). *S. pombe* Sty1 is required for most SpESR regulation (Chen et al., 2003). *S. cerevisiae* Hog1 is most strongly induced by osmotic stress and activated to lower levels by other stresses, including oxidative stress and weak acid stress (Bilsland et al., 2004; Lawrence et al., 2004; Sotelo and Rodriguez-Gabriel, 2006). However, many ScESR genes are not exclusively dependent on Hog1, but rely also on the general stress factors Msn2 and Msn4. The *C. albicans* ESR is influenced by CaHog1 and in parallel also by other pathways, but Msn2 does not play any role (Enjalbert et al., 2006). This raises the question as to whether *S. cerevisiae* has developed a unique stress response mechanism involving Msn2 and Msn4.

Do functional Msn2 orthologues exist in other ascomycete fungi apart from *S. cerevisiae*? One characteristic feature of Msn2 is its DNA binding domain recognizing the corresponding recognition element STRE. Many fungal genome sequences contain ORFs with high similarity to ScMsn2. However, these genes usually have very little other sequence conservation to ScMsn2. Zn-finger proteins binding to STRE-like

**Fig. 7.** Survival of *D. melanogaster* to wild type or msn2Δmsn4Δ mutant *C. glabrata* infection.

A. Flies were injected with 7500 *C. glabrata* cells. MyD88 mutant flies succumbed rapidly to a challenge with either wild-type *C. glabrata* or Cg msn2Δmsn4Δ mutant strain. The genotypes of the infected flies are indicated (A5001: wild-type).

B. Immunosuppressed flies succumb rapidly when challenged with Cg msn2Δmsn4Δ transformed with either the empty pACT plasmid or plasmids expressing CgMsn2 (pCgMCgMSN2 or pCgADH1CgMSN2). Survival was monitored at 29°C. The survival rate is expressed as a percentage. These survival experiments are representative of at least three independent experiments for each panel. The slight difference observed between wild-type flies injected with wild-type or mutant yeasts is not reproducible. Similar results were observed with a lower dose of injected *C. glabrata* (5000).
sequences were reported from *S. pombe* and *Trichoderma atroviride* (Kunitomo et al., 2000; Seidl et al., 2004), although these factors are not mediating stress responses. The most significant similarity between ScMsn2 orthologues apart from the DNA binding domain (Fig. 2B) is a region designated earlier as Msn2 HD1 (Görner et al., 1999). This motif is not present in the Msn2 orthologue from *C. albicans* or its close relatives, including *Candida parapsilosis*, *Candida tropicalis*, *Candida lusitaniae*, *Candida guilliermondii*, *Lodderomyces elongisporus*, *Debaryomyces hansenii* and *Pichia stipitis*.

The intracellular localization of ScMsn2 rapidly changes from the cytoplasm to the nucleus in response to nutrient and stress conditions. (Görner et al., 1998; Beck and Hall, 1999; Görner et al., 2002). This switch is the result of two independent activities within ScMsn2. The NLS near the C-terminus drives nuclear import. Nuclear export of Msn2 requires the exportin Msn5 which also transports other transcription factors, such as Mig1, Crz1 and Pho4 (Kaffman et al., 1998; DeVit, 1999; Chi et al., 2001; Boustany and Cyert, 2002). Activation of Msn2 by stress and nutrient starvation requires HD1 which overlaps with the NES function (Görner et al., 2002; Boy-Marcotte et al., 2006). PKA and TOR inhibit Msn2 activity through this region. Interestingly, all close Msn2 orthologues have an embedded conserved PKA phosphorylation site (Fig. 2B). However, the function of PKA has not been explored in *C. glabrata*.

We have shown that the region including the HD1 motif of Msn2/4 from *K. lactis*, *A. gossypii*, *S. cerevisiae* and *C. glabrata* is sufficient for nuclear export. We provide direct evidence by mutation of two conserved serine residues to alanine in the CgMsn2 HD1, which leads to constitutive nuclear enrichment. Similar changes in the ScMsn2 abolish its NES function (W. Reiter, G. Ammerer and C. Schüller, unpubl. obs.). In contrast, a large part of CaMsn4 (1–517) which is devoid only of its NLS and Zn-finger region at the C-terminus does not support regulated nuclear export.

The NES of ScMsn2 is much larger than the generic exportin 1-driven signal (Wen et al., 1995; Kutay and Gutttinger, 2005). Importantly, nuclear export of CgMsn2 expressed in *S. cerevisiae* also requires Msn5 and PKA (A. Roetzer and C. Schüller, unpubl. results). These data support our hypothesis that the HD1 is the target site for the exportin Msn5, and that the conserved PKA phosphorylation site within the HD1 site has a regulatory role. Our results suggest that the Saccharomyces can be divided into two groups according to their stress response mechanisms. The HD1 motif and the stress-mediating role of Msn2 orthologues appear after the separation of *C. albicans* from the lineage leading to *S. cerevisiae* (Fig. 2B). The functional conservation of ScMsn2 and CgMsn2 highlights the important regulatory motifs of both proteins and will be used as a guide for further structure—function analysis.

*Candida glabrata* as a human commensal and occasional pathogen exists in very different environmental niches compared with *S. cerevisiae*. Our global transcript analysis of *C. glabrata* cells exposed to the generic stress types carbon starvation, heat, osmotic and oxidative stress reveals a transcription pattern related to *C. albicans*, *S. pombe* and *S. cerevisiae*. The most conserved component of the ESRs is dependent on Msn2-like factors in *C. glabrata* and *S. cerevisiae*. Several lines of evidence presented here suggest that CgMsn2 is an orthologue of ScMsn2. It can functionally substitute for ScMsn2 in *S. cerevisiae* for stress-dependent induction of an Msn2 target gene. Intracellular localization of a CgMsn2–CFP fusion protein is stress-regulated in both *S. cerevisiae* and *C. glabrata*. CgMsn2 is required for rapid and full induction of several target genes tested CgTPS1, CgTPS2 and CgUBP15 which all contain putative STRE sequences in their promoter region. Conserved Msn2-dependent genes, such as TPS1, NTH1, TPS2, HSP42, HSP12, SML1, DDR48 and YGP1, are involved in stress response, glycocon and trehalose metabolism or DNA repair (SML1, ribonucleotide reduction) based on GO-terms analysis (P-values < 0.005). *C. glabrata* stress genes were also found in a global analysis of the CgPdr1-regulated drug response (Vermitsky et al., 2006).

CgMSN2 and CgMSN4 are not required for virulence in a *Drosophila* infection model. This may reflect an absence of extreme stressful conditions for *C. glabrata* in the fly hemocoel. Alternatively, other pathways may allow *C. glabrata* to adapt to the host environment. Finally, the fly model might not reflect all host niches *C. glabrata* is adapted to. Adhesion is essential for *C. glabrata* virulence (Cormack et al., 1999). We found no difference of adhesion to a plastic surface (Iraqui et al., 2005) in *C. glabrata* Msn2/4 deletion and Msn2 overexpression strains (data not shown). The adhesins encoded by EPA genes are regulated differently during environmental stress conditions. Two genes *EPA3/CAGL0E06688g* and *CAGL0I00220g* are highly induced by osmotic stress and glucose starvation; however, with a minor role for CgMsn2.

Our results highlight the conserved regulation of Msn2 between *S. cerevisiae* and *C. glabrata*, but we also find differences that require further investigation. Stress regulation of many Msn2 target genes is conserved up to *S. pombe*, indicating a selective advantage for such regulation regardless of the particular transcription factors. It will be of particular interest to analyse the role of Msn2 orthologues which we suspect to be stress-regulated factors in the pre-whole-genome duplication clade, such as *Kluyveromyces* species and *A. gossypii*. We suggest
that *C. glabrata* Msn2 functions to improve survival under severe stress conditions.

**Experimental procedures**

**Yeast strains and plasmids**

Yeast strains used in this study are listed in Table 1. Rich medium (YPD) and synthetic medium (SC) supplemented with appropriate auxotropic components were prepared as described elsewhere (Current Protocols in Molecular Biology; Wiley). Unless otherwise indicated, all strains were grown at 30°C.

Plasmids and oligonucleotides used in this study are listed in Table 2 and Table S1 respectively. *Cg msn2Δ* strain was obtained by genomic integration. PCR products of *ScURA3* for *CgMSN4* and *CgHIS3* for *CgMSN4* were amplified from the plasmids pRS316 (Sikorski and Hieter, 1989) and pTW23 (Kamran et al., 2004) using fusion PCR (CgMsn4, primer series MSN4; CgMsn2, primer series MSN2) (Noble and Johnson, 2005). Correct genomic integration of all fragments was verified by genomic PCR (primer series Ctrl) followed by Southern analysis using labelled probes generated by amplification with primers MSN2-5′/MSN2-5′ and MSN4-1/MSN4-3 from genomic DNA.

Cloned PCR fragments used in this study were confirmed by sequencing. Plasmid pASMG1, which was described in Görner et al. (1998), is based on vector YCP lac111 (Gietz and Sugino, 1988) containing the *S. cerevisiae* ADH1 promoter followed by the SV40 NLS (PKKKRKV), and a part of MSN2 coding for amino acid position 267–568 and GFP. To create a unique Sall site after the SV40 NLS, the plasmid was modified by site-directed mutagenesis using a Quick Change Site-directed Mutagenesis Kit (Stratagene) and primers Re-Sall/Re-Sall-3 resulting in plasmid pASNScM2G. Sequence fragments from Msn2 orthologues were exchanged with the ScMsn2 sequences by excision with Sall/NcoI. Plasmid pASNKIM2G was created by a Sall/NcoI digest of pASNScM2G and integration of a Sall/NcoI cut fragment obtained by PCR using primers KIM2Sall and KIM2NcoI from genomic *K. lactis* DNA as a template. Plasmid pASNAgM2G was created by using an Xhol/BsmBI fragment of a PCR product generated with primers AgM2XhoI and AgM2NcoI and plasmid pAG1334 as a template. The *C. glabrata* Msn2 and Msn4 orthologues (CAGL0F05999g and CAGL0M13189g) sequences were amplified by PCR from genomic DNA from strain ΔHTU. The CgMsn2 PCR fragment obtained with primers CgM2XhoI and CgM2NcoI was cut with Xhol/NcoI digest of pASNScM2G and integration of a SalI/NcoI cut fragment obtained by PCR using primers KIM2Sall and KIM2NcoI from genomic *K. lactis* DNA as a template. Plasmid pASNAgM2G was created by using an Xhol/BsmBI fragment of a PCR product generated with primers AgM2XhoI and AgM2NcoI and plasmid pAG1334 as a template. The *C. glabrata* Msn2 and Msn4 orthologues (CAGL0F05999g and CAGL0M13189g) sequences were amplified by PCR from genomic DNA from strain ΔHTU. The CgMsn2 PCR fragment obtained with primers CgM2XhoI and CgM2NcoI was cut with Xhol/NcoI, the CgMsn2 sequences by excision with Sall/NcoI.

**Table 1.** Yeast strains used in this study.

<table>
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<td>W303-1A</td>
<td>a ura3 leu2 his3 trp1 ade2 can1</td>
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<td></td>
<td>W303 msn2Δ</td>
<td>msn2Δ::TRP1 msn4Δ::HIS3</td>
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<tr>
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<td>K. lactis</td>
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<td>C. albicans SC5314</td>
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**Table 2.** Plasmids used in this study.

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<td>ScADH1-ScMsn2-GFP (pAMG)</td>
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<td>ScCTT1 PCR fragment</td>
<td>This study</td>
</tr>
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<td>This study</td>
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<td>CgADH1-Promoter CgMsn2–CFP (SphI/SacII and SacII/NsiI); CgTRP1 marker.</td>
<td>This study</td>
</tr>
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CgM4Ncol) was cut with Sall/Ncol and both were inserted into the Sall/Ncol-cut pASN5ScM2G. pASN5ScM4G was created by insertion of a Sall/Ncol-digested PCR fragment obtained using primers ScM4Sall and ScM4Ncol and genomic DNA from W303-1A. The CaMs4n fragment was amplified via PCR (primers CaMs4n-5 and CaMs4n-3) from genomic C. albicans DNA and cut with Sall and Ncol. pACgMC was cloned by integration of a Sall/Ncol-cut PCR fragment containing the CgMs4n ORF into pAMC. pCgACgMs4n–CFP is a derivative of pGEM-ACT (Gregori et al., 2007). Nine hundred base pairs of the CgADH1 promoter were inserted as a Sphl/SacII PCR product obtained with primers CgAdhPro-Sphl and CgAdhPro-SacII. The coding sequence for CgMs4n–CFP was amplified from pACgMC using primers CgMs4n2Cfp-SacII and CgMs4n2Cfp-Nsil, and inserted as a SacII/Nsil-cut fragment. The native promoter (860 base pairs) was inserted as a Sphl/SacII PCR product obtained with primers Sphl-msn2nat and SacII-msn2nat. Exchange of single amino acids in CgMs4n was done via site-directed mutagenesis using a Quick Change Site-directed Mutagenesis Kit (Stratagene) using the primers 5-SASA and 3-SASA. Probes for Northern and Southern blot analysis were amplified by PCR from genomic DNA: IPP1 from S. cerevisiae, CgACT1, CgTPS1, CgTPS2 and CgUBP15 from C. glabrata. For ScCTT1, a KpnI/SacII fragment from the plasmid pKSCTT1 was used.

Fly strains and survival experiment

Stocks were raised on standard cornmeal-agar medium at 25°C. wA5001 flies were used as wild type throughout the experiments because the MyD88 mutant was generated in this background. Batches of 25–30 wild type and mutant strains were challenged with 7500 cells of wild type or mutant C. glabrata using a Nanoject II apparatus (Drummond Scientific). Overnight cultures of C. glabrata were collected and washed once; cell pellets were re-suspended in RNA isolation buffer (50 mM Tris pH 7.5/5 mM EDTA/5% SDS/130 mM NaCl), 200 μl PCI solution (Roth) and glass beads (2/3 of total volume) were added and total RNA was extracted using FastPrep (2 x 12”, speed 6, Thermo Savant). RNA samples (20 μg) were separated on a 1.1% agarose gel in FGRB (5x: 0.1 M MOPS/40 mM NaAc pH 7/5 mM EDTA) containing 2.2 M formaldehyde. After transfer to nylon membranes and UV cross-linking, the quality and amount of RNA were determined by staining with Methylen Blue. Hybridization of [32P-α]-ATP-labelled probes occurred over night in hybridization buffer (0.5 M sodium phosphate buffer pH 7.2/7% SDS/1 mM EDTA) at 65°C. After washing, the membrane was exposed to an X-ray film. For DNA extraction, yeast cells were grown to an OD600 of 6 (10 ml), collected and washed once; cell pellets were re-suspended in Lysis buffer (2% Triton X-100/1% SDS/100 mM NaCl/10 mM Tris pH 8/1 mM EDTA). Genomic DNA was isolated by PCI extraction. Digestion of 10 μg of genomic DNA was done over night with Scal and BglII (5 U μg⁻¹ DNA). The digests were separated and blotted. After cross-linking, the radioactive labelled probes were hybridized over night at 65°C. Bands were detected by X-ray film and Phospholmager.

Microscopy

Fluorescence microscopy was performed as described previously (Görner et al., 1998). GFP and CFP were visualized in live cells without fixation. Nuclei were stained by addition of 2 μg ml⁻¹ 4,6-diamidino-2-phenylindol dye to the cultures 10 min prior to microscopy. All cells were viewed using a Zeiss Axioplan 2 fluorescence microscope. Images were captured with a Spot Pursuit (Sony) CCD camera using MetaVue (Molecular Devices) and Spotbasic software. Time-lapse imaging of life cell was done by adhering the cells to a coverslip with Concanavalin A (Sigma) fixed above a chamber allowing continuous flow of medium driven by a pump. Incoming medium could be switched between two sources containing rich medium without glucose and with 2% glucose. Medium flow rate was about 500 μl min⁻¹.

Candida glabrata long-term viability

Cg msn2Δmsn4Δ transformed with the above plasmids were grown on selective plates, replica-plated to YDP grown over night and then stored for 14 days at 4°C and on room temperature as a control. Plates were then replica-plated and incubated at 37°C over night and growth recorded. Cell patches expressing CgMSN2 S230A232A–CFP failed to grow, suggesting loss of viability. Viability was also verified by colony-forming assay by plating showing a threefold reduced colony-forming ability (0.38 of wild type; SD = 0.12; P = 0.05).

Microarray analysis

Microarrays were produced (G. Butler lab) by spotting 5908 69- or 70-mer oligonucleotides synthesized at the Pasteur Institute to Corning UltraGAPS slides. Slides were pre-incubated in 0.5% NaBH₄ (in 75% PBS and 25% EtOH) solution, washed three times with water and dipped in isopropanol. CDNA was synthesized using 15–20 μg of RNA and Superscript III kit (Invitrogen), including either Cy3-3-dCTP or Cy5-3-dCTP (Amersham Biosciences). RNA was hydrolysed in 50 mM NaOH at 65°C for 15 min, the solution was neutralized with acetic acid and CDNA was purified using a GFX purification kit (GE Healthcare). Labelled cDNAs were con-
centrated, pooled and hybridized in 60 µl in DiGEEasyHyb solution (Roche Diagnostics) with 0.1 mg ml⁻¹ salmon sperm DNA (Sigma) at 37°C for 14–16 h. Microarrays were disassembled in 1× SSC, washed two times in 1× SSC, 0.1% SDS at 50°C for 20 min, followed by a 1 min wash in 1× SSC at room temperature. Slides were spun dry for 5 min at 700 r.p.m. Slides were scanned immediately on an Axon4000B scanner (Axon Instruments) and analysed using GenePix Pro4.1 software (Axon Instruments). All standard protocols were provided by the Ontario Cancer Institute (Toronto; http://www.microarrays.ca/). Each experiment was repeated twice.

**Analysis of microarray data**

The raw data set of this study is available as supplemental material and has been deposited at array express (http://www.ebi.ac.uk/arrayexpress/; accession number: E-MEXP-1427). Microarrays were analysed with GenePixPro4.1. Values of not found features were excluded from further analysis. Mean ratios were calculated for features with at least four data points and their quality was approximated by their coefficient of variation (CV) values and subsequent exclusion of values with CV smaller than 1. Only genes with at least four data points and a CV > 1 were included in subsequent analysis. Genes assigned as dubious ORFs by the Genolevures consortium analysis were removed from analysis. The filtered median of ratio values were normalized. The normalized values used for further analysis are available as supplementary file Cg_array_data.xls. Cluster analysis (Eisen et al., 1998) was performed with cluster3 and TreeView (see http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/index.html). Association to GO terms was analysed with the T-Profiler (Boorsma et al., 2005) by using the orthologous S. cerevisiae genes. Values of genes associated with the most significant terms were visualized by Cluster analysis using complete linkage and correlation as similarity metric. The cluster results were confirmed by K means and SOM clustering. P-values of overlapping gene sets were calculated by hypergeometric distribution. Of the 5063 gene features spotted in duplicate on our arrays, 4166 gave useful data under at least one tested condition. Systematic C. glabrata IDs were used from the Genolevures resource (5215 ORFs) and linked to systematic names of S. cerevisiae. The ESR was defined as the set of genes with values above a fourfold induction and below a fourfold repression after applying different stresses. To estimate the contribution of the transcription factors Cgsn2p/Cgsn4p, the ratio of the wild type versus double mutant and induced genes was calculated. In addition, data from an all-against-all BLASTp search of protein sequences from S. cerevisiae, C. albicans and S. pombe (Enjalbert et al., 2006) were compared with C. glabrata data for osmotic and oxidative stress. Normalized values with canonical gene names are available in plain text format as supplementary data as well as Cluster3 output files corresponding to the figures. Sequence patterns were found by AlignAce (Hughes et al., 2000) using the C. glabrata genomic DNA. The most recent annotation as of 09.Nov.2007 was used to define ORF and promoter regions. Sequence logo was generated at http://weblogo.berkeley.edu/Crooks et al., 2004).

**Acknowledgements**

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**References**


Rim15 orchestrates g(0)-associated antioxidant defense mechanisms. *Cell Cycle* 3: 462–468.


### Supplementary material

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-2958.2008.06301.x

(This link will take you to the article abstract).

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Figure S1

probe MSN2

wt msn2Δ msn2Δ msn2Δ msn2Δ

6.2kb 3.6kb

BglII BglII BglII BglII

Cagl0F CgMSN2 probe CgHIS3

BglII

3.6kb 6.2kb

probe MSN4

wt msn2Δ msn2Δ msn2Δ msn2Δ

3.7kb 1.3kb

Scal Scal

Cagl0M CgMSN4 Scal probe ScURA3

3.7kb 1.3kb
Figure S2

A

Osmotic stress

C.g. 330 > 2
S.c. 120 > 1.5
C.a. 74 > 1.5
S.p. 124 > 1.5

B

Oxidative stress

C.g. 317 > 1.5
S.c. 268 > 1.5
C.a. 159 > 1.5
S.p. 174 > 1.5

C

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Mat2-OE in C. glabrata
Mat2-OE in S. cerevisiae

YDR533c  HSP31
YDR074w  TPS2
YBR126c  TPS1
YFL014w  HSP12
YCR021c  HSP30
YDL124w  YDL124w
YGL037c  PNC1
YPR061c  JID1
YMR297w  PRC1
YBR026c  ETR1
YIL155c  GUT2
YJR096w  YJR096w
YOL084w  PHM7
Figure S4

![Graphs of CgTPS1 and CgTPS2](Image)

- **CgTPS2**
- **CgTPS1**

Legend:
- **Blue** - no stress
- **Red** - 20 min 0.5M NaCl
- **Green** - 20 min Glucose

*Figure shows expression levels of CgTPS1 and CgTPS2 under different stress conditions.*

- **rRNA**

*Additional image showing RNA expression levels.*

Legend for RNA image:
- **0** - no stress
- **os** - 0.5M NaCl
- **glc** - Glucose starvation
**Figure S1. Southern analysis of CgMSN2 and CgMSN4 deletion strains.** CgMSN2 was replaced by CgHIS3 and CgMSN4 was replaced by ScURA3. Genomic DNA from wild type and the msn2Δ and msn2Δmsn4Δ deletion strains was digested with either BglII or Scal. Chromosomal restriction sites are indicated. DNA fragments were detected with radio-labeled probes as indicated. Replacement of CgMSN2 with CgHIS3 strain abolishes two BglII cleavage sites leading to a 6.2kb fragment instead of the 3.6kb wildtype fragment (left panel). Replacement of CgMSN4 with ScURA3 strain introduces a Scal cleavage site changing the 3.7kb wildtype fragment to a 1.3kb fragment (right panel).

**Figure S2. ESR gene expression levels of four different yeasts is similar.** (A, B) Pairwise overlap of stress induced orthologous genes between C. glabrata, C. albicans, S. cerevisiae, and S. pombe. Cut-off values are indicated. Expression levels of all four organisms were compared to each other during osmotic (A) and oxidative stress (B). Specific induction levels were chosen (C. glabrata >2-fold after osmotic stress and >1.5-fold upon oxidative stress; S. cerevisiae >1.5-fold upon osmotic stress and oxidative stress; C. albicans >1,5-fold upon osmotic stress and oxidative stress; S. pombe >1,5-fold upon osmotic stress and oxidative stress) and used for a selection among all expressed genes (>1) of the four yeasts during oxidative and osmotic stress, respectively (C. glabrata: 945, 1126; C. albicans: 934, 1058; S. cerevisiae: 956, 360; S. pombe: 916, 1065; genes oxidative, osmotic stress). (C) Common Msn2 response of orthologous genes. 13 genes are induced significantly (4-fold) by at least one of the tested conditions and by overexpression of ScMsn2 and CgMsn2.

**Figure S3. ESR genes are conserved between C. glabrata and S. cerevisiae.** (A) Comparison of genes comprising the CgESR of C. glabrata versus the ScESR of S. cerevisiae. A set of 268 genes shared between C. glabrata and S. cerevisiae has a similar stress induction profile for the conditions tested. Color code indicates common Msn2 regulated genes. CgMsn2
regulated genes are highlighted with a green letter (M), whereas genes from core response (see Figure 5B) are in red. (B) CgESR from C. glabrata (760 genes) is compared to the expression set of S. cerevisiae (~ 6200 genes) (left panel), whereas in the right panel the ScESR from S. cerevisiae (868 genes) was aligned against the expression set of C. glabrata (~ 4200 genes). Data from corresponding stress treatments were chosen: 0.4mM H₂O₂ (C.g.) versus 0.32mM H₂O₂ (S.c.), 20 minutes of glucose depletion (C.g.) versus a corresponding time point of a starvation time course (S.c.), temperature shift from 30°C to 42°C (heat shock, C.g.) versus shift from 25°C to 37°C (S.c.) and 0.5M NaCl (osmotic shock, C.g.) versus 1M Sorbitol (S.c.). S. cerevisiae data were extracted from (Gasch et al., 2000)

**Figure S4.** CgMsns2 and CgMsns2-CFP display similar rapid induction of transcription after applying stress. (A) mRNA levels of CgTPS1 and CgTPS2 were quantified and normalized to large rRNA. (B) Northern blot analysis of CgTPS1 and CgTPS2 transcripts during 0.5M NaCl induced osmotic stress and glucose starvation. Cg msn2Δmsn4Δ transformed with empty vector and Cg msn2Δmsn4Δ transformed with pCgMCgMSN2-CFP, pCgMCgMSN2, pCgADH1CgMSN2-CFP and pCgADH1CgMSN2 were grown to exponential phase. Cells were treated with 0.5M NaCl or washed twice and incubated in YP without glucose. Samples for RNA extraction were taken at indicated time points. mRNA levels were visualized by hybridization of radio-labeled probes and autoradiography. Staining of rRNA was used as a loading control.
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Autophagy supports *Candida glabrata* survival during phagocytosis.

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**Running title:** *Candida glabrata* phagocytosis response

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Abstract

The opportunistic human fungal pathogen *Candida glabrata* is confronted with phagocytic cells of the host defence system. Survival of internalized cells is thought to contribute to successful dissemination. We investigated the reaction of engulfed *C. glabrata* cells using fluorescent protein fusions of the transcription factors CgYap1 and CgMig1 and catalase CgCta1. The expression level and peroxisomal localization of catalase was used to monitor the metabolic and stress status of internalized *C. glabrata* cells. These reporters revealed that the phagocytosed *C. glabrata* cells were exposed to transient oxidative stress and starved for carbon source. Cells trapped within macrophages increased their peroxisome numbers indicating a metabolic switch. Prolonged phagocytosis caused a pexophagy-mediated decline in peroxisome numbers. Autophagy, and in particular pexophagy, contributed to survival of *C. glabrata* during engulfment. Mutants lacking CgATG11 or CgATG17, genes required for pexophagy and non-selective autophagy, respectively, displayed reduced survival rates. Furthermore, both CgAtg11 and CgAtg17 contribute to survival, since the double mutant was highly sensitive to engulfment. Inhibition of peroxisome formation by deletion of CgPEX3 partially restored viability of CgATG11 deletion mutants during engulfment. This suggests that peroxisome formation and maintenance might sequester resources required for optimal survival. Mobilization of intracellular resources via autophagy is an important virulence factor that supports the viability of *C. glabrata* in the phagosomal compartment of infected innate immune cells.

Keywords: Candida glabrata, catalase, peroxisomes, autophagy, pexophagy, phagocytosis
Introduction

_Candida glabrata_ belongs to the diverse group of human fungal pathogens and is phylogenetically closely related to _Saccharomyces cerevisiae_ (Kaur et al., 2005, Marcet-Houben et al., 2009). The high similarity of _C. glabrata_ to _S. cerevisiae_ suggests that also for fungi, relatively small genetic changes may be sufficient for adaptation to a pathogenic life style (Dujon et al., 2004). _C. glabrata_ is a common commensal, but can turn into an opportunistic pathogen with a rising frequency of isolates among immunocompromised patients and elder people (Pfaller et al., 2007, Presterl et al., 2007, Li et al., 2007). In the host environment, _C. glabrata_ has to evade or survive attacks of the cell-mediated immune system (Nicola et al., 2008). Counterstrategies of fungal pathogens differ between species. _Candida albicans_ destroys macrophages by hyphal outgrowth. Alternatively, _Cryptococcus neoformans_ either lyses macrophages or escapes via phagosomal extrusion (Alvarez et al., 2006, Ma et al., 2006). _C. glabrata_ engulfed by macrophages do not undergo morphological transitions such as _C. albicans_ (Leberer et al., 2001). An open question concerns how _C. glabrata_ is coping with cells of the immune system, such as macrophages.

The phagosome is a hostile environment for fungi (reviewed in (Nicola et al., 2008)). After internalization of microbial cells, the organelle maturates into the phagolysosome containing mature hydrolytic enzymes and a more acidic pH 4.5-5.5 (Geisow et al., 1981, Levitz et al., 1999). Additionally, the NADPH oxidase complex generates reactive oxidative species to attack internalized microorganisms (for review see Segal, 2005, Romani, 2004)). Thus, commensal and pathogenic fungi are exposed to reactive oxygen species (ROS) produced by polymorphonuclear leucocytes, macrophages and dendritic cells (Miller et al., 1997, Missall et al., 2004, Gildea et al., 2005). On the fungal side, antioxidant defence enzymes such as catalase, superoxide dismutase, thioredoxin- and glutathione-dependent peroxidases and reductases guard
against oxidative stress and are thus considered virulence factors (Johnson et al., 2002, Cox et al., 2003, Chaves et al., 2007, Missall et al., 2005). Oxidative stress as defence strategy is not restricted to combat fungal infections. Invading bacteria, such as Staphylococcus aureus, or the malaria parasites Plasmodium sp., face ROS stress upon engulfment (Voyich et al., 2005, Becker et al., 2004). ROS sensed by microbes act also as signalling molecules. The C. albicans catalase, an enzyme which decomposes hydrogen peroxide, has been investigated more closely. C. albicans induces catalase when engulfed in neutrophils or in macrophages (Rubin-Bejerano et al., 2003, Lorenz et al., 2004, Enjalbert et al., 2007). Moreover, hydrogen peroxide promotes the morphological transition of C. albicans cells to hyphal growth, a form invading the host tissue (Nakagawa, 2008, Nasution et al., 2008). Finally, C. albicans devoid of catalase was eliminated more efficiently in a mouse infection model (Nakagawa et al., 2003). The filamentous fungus Aspergillus fumigatus lacking the catalases expressed in the mycelium exhibited delayed infection in a rat model of invasive aspergillosis (Paris et al., 2003). In contrast, C. glabrata catalase was not a virulence determinant in an immunocompromised mouse model (Cuellar-Cruz et al., 2008). In mice infected with a C. neoformans mutant devoid of all four catalases, mortality was unchanged (Giles et al., 2006). Thus the relative importance of individual ROS scavenging enzymes varies between fungal pathogens.

Besides being exposed to oxidative stress, cells engulfed by macrophages adjust their metabolic program (Fan et al., 2005, Barelle et al., 2006). Engulfed C. albicans cells induce many genes involved in non-fermentative carbon metabolism (Prigneau et al., 2003, Lorenz et al., 2004). Phagocytosed C. glabrata induces genes encoding enzymes involved in β-oxidation, the glyoxylate cycle and gluconeogenesis (Kaur et al., 2007). Moreover, the glyoxylate cycle, which is required to channel fatty acid-derived two carbon units into metabolism, was early recognized as a virulence determinant for C. albicans (Lorenz et al., 2001). Other human fungal pathogens also induce glyoxylate cycle
components during infection conditions (Derengowski et al., 2008, Rude et al., 2002, Canovas et al., 2006). However, A. fumigatus and C. neoformans do not require the glyoxylate cycle for virulence (Schöbel et al., 2007, Idnurm et al., 2007). Some of the enzymes of the glyoxylate cycle are localized in the peroxisomal matrix (for review see e.g. (Kunze et al., 2006)). Peroxisomes are inducible, single membrane organelles which harbour enzymes for the oxidative catabolism of fatty acids, the glyoxylate cycle and others. Generally, peroxisome number and size vary according to metabolic needs (for review see (Yan et al., 2005, Platta et al., 2007)).

Autophagy continuously recycles almost all constituents of the cell (for review see (Kraft et al., 2009, Mizushima et al., 2007)). Different types of autophagy help organisms to overcome periods of nutrient starvation by recycling intracellular components to sustain vital cellular functions and seems to be linked to the unique niches and morphology of these pathogens (for review see (Palmer et al., 2008)). For certain pathogenic fungi, autophagy has been identified as a virulence factor. C. neoformans requires an intact autophagy pathway during infection (Hu et al., 2008). Peroxisomes and their contents are delivered to the vacuole by the pexophagy pathway, a specialized form of autophagy (Kim et al., 2001, Guan et al., 2001, Farre et al., 2004). In S. cerevisiae selective pexophagy is dependent on Atg11 and partly on Atg17 which is also important for non-selective autophagy (Cheong et al., 2005, Yorimitsu et al., 2005).

Here we investigated responses of C. glabrata during its encounter with the macrophage phagosome compartment from which it cannot escape. We developed in vivo reporters to track fungal responses to this environment. To detect oxidative and glucose starvation stress of cells, we used fluorescent protein fusions of the C. glabrata orthologues of the S. cerevisiae transcription factors Yap1 and Mig1 (Kuge et al., 1997, Carlson, 1999). We found the C. glabrata catalase gene CgCTA1 and catalase activity regulated by oxidative stress and glucose starvation. Additionally, we demonstrated GFP-
Results

CgCta1 localization to peroxisomes. *C. glabrata* peroxisomes have not been described so far and were here defined by several independent criteria. We found that *C. glabrata* cells engulfed by mouse macrophages experience a mild oxidative stress and sustained carbon starvation. Additionally, peroxisomes became transiently induced in engulfed cells. We explored the role of peroxisomes with various mutants lacking peroxisome biogenesis or autophagy pathways mediating destruction of peroxisomes. We report here that autophagy and, surprisingly, pexophagy is a likely virulence factor for *C. glabrata*. Mutants lacking CgAtg11 and/or CgAtg17 were killed more efficiently by macrophages during engulfment. Thus, for engulfed *C. glabrata* cells, nutrient deprivation represents a serious challenge and mobilization of intracellular resources via autophagy is a major contributor to sustain viability.
Results

C. glabrata catalase CgCta1 is induced by hydrogen peroxide and carbon starvation.

C. glabrata harbours one catalase gene (CgCTA1, CAGL0K10868g), related to the yeast peroxisomal catalase CTA1 gene. The two catalase genes of S. cerevisiae are regulated differently. CTT1, coding for the cytoplasmic catalase, is induced by stress conditions (Marchler et al., 1993). The CTA1 gene, is expressed only during growth on non-fermentable carbon sources (Hartig et al., 1986, Cohen et al., 1985). To find out the regulatory pattern of the C. glabrata catalase, we assayed its activity in crude protein extracts from cells grown either on glucose or a non-fermentable carbon source. Cells adapting to ethanol as carbon source showed a substantial induction of catalase activity (Figure 1A). Moreover, mild oxidative stress of 0.4mM H$_2$O$_2$ induced C. glabrata catalase activity about ten-fold suggesting regulation by both glucose starvation and oxidative stress (Figure 1B). To verify if the CgCTA1 gene encodes the only catalase activity in C. glabrata, we replaced the open reading frame with the S. cerevisiae URA3 gene (Figure S1). Catalase activity was undetectable in extracts derived from the mutant strain (Figure 1A, B). A centromeric plasmid (pCgCTA1) harbouring the CgCTA1 ORF including a 1.8kb upstream region fully restored wild type level catalase activity to the cta1Δ mutant (Figure 1A, B). To demonstrate that the regulation of catalase activity occurs at the level of transcription, CgCTA1 mRNA levels were analyzed from cells shifted to medium lacking glucose or exposed to 0.4mM H$_2$O$_2$. Glucose starved cells displayed a continuous increase of CgCTA1 mRNA immediately after shift to glucose free medium (Figure 1C). Hydrogen peroxide stress caused a rapid increase within 10 minutes. Taken together, regulation of the C. glabrata catalase gene CgCTA1 by carbon source availability and oxidative stress combines elements of both S. cerevisiae catalases.
**Results**

1. **CgCta1 confers hydrogen peroxide stress resistance.**

   The similarity of the *CgCTA1* gene to the *S. cerevisiae CTA1* gene suggested its peroxisomal localization. To clarify the intracellular localization, we fused a green fluorescent protein (GFP) to the CgCta1 N-terminus (*GFP-CgCTA1*) to preserve the putative peroxisomal targeting sequence 1 (PTS1) (Figure 1C, lower panel). The preceding 1.8kb of the *CgCTA1* 5′ untranslated region conferred a wild type-like expression pattern in hydrogen peroxide stressed cells (Figure 1C). Basal catalase activity of GFP-CgCta1 was detectable in unstressed cells, whereas hydrogen peroxide stress induced activity was reduced to about 20% of the wild type level (Figure 1B).

   We assessed if the reduced activity of GFP-Cta1 interferes with hydrogen peroxide stress resistance. *C. glabrata cta1Δ* mutant cells transformed with either p*GFP-CgCTA1*, p*CgCTA1* or the empty plasmid (pACT) were grown in synthetic medium, the cultures were split and one part treated with 0.4mM H$_2$O$_2$. Both were subsequently exposed to higher doses of hydrogen peroxide. Growth was scored after 24 hours (Figure 1D). The cta1Δ mutant cells containing the empty plasmid failed to grow in medium containing 5mM H$_2$O$_2$. In contrast, the strain carrying the p*CgCTA1* plasmid was resistant to medium supplemented with up to 20mM H$_2$O$_2$, whereas pre-incubation with 0.4mM H$_2$O$_2$ pushed the growth limit to 40mM H$_2$O$_2$, similar to the wild type parent strain (ΔHTU). Cells expressing the GFP-CgCta1 derivative displayed lower basal resistance. However, naive cells without pre-treatment tolerated 5mM H$_2$O$_2$ and failed to grow only at about 20mM H$_2$O$_2$. Pre-treatment with 0.4mM H$_2$O$_2$ shifted tolerance to about 30mM H$_2$O$_2$. Thus, the GFP tagged CgCta1 derivative, when compared to the untagged version, conferred resistance to oxidative stress to reduced but overall high level. These results suggested that H$_2$O$_2$ stress resistance of strains carrying the plasmid-encoded catalase derivatives
encompasses the oxidative stress load of 0.4mM H$_2$O$_2$ determined for the in vivo situation (Enjalbert et al., 2007). Our data also showed that the C. glabrata strains tolerated a substantial higher oxidative stress load compared to S. cerevisiae laboratory strains, which failed to grow at concentrations higher than 3mM H$_2$O$_2$ (Davies et al., 1995, Cuellar-Cruz et al., 2008).

Localisation of GFP-CgCta1 is dependent on the carbon source.

Cells expressing GFP-CgCTA1 were exposed to different stress conditions. In rich medium, GFP-CgCta1 fluorescence was hardly detectable, reflecting its low basal expression of CgCTA1. Oxidative stress caused induction of the GFP-CgCta1 fluorescence signal. To compare different expression levels directly, unstressed cells were marked by staining their nucleic acids with DAPI. For microscopy these marked unstressed cells were mixed to cells from the same culture treated for 1 hour with 0.4mM H$_2$O$_2$. The micrograph demonstrates induction of the fusion protein by oxidative stress and its initial localization in the cytoplasm (Figure 2A). GFP-CgCTA1 became also induced after the glucose concentration in the growth medium dropped below 0.03% (Figure S2A).

We then investigated GFP-CgCta1 distribution in cells growing on non-fermentable carbon sources. Cells expressing GFP-CgCTA1 were grown in medium supplemented with 0.5% glucose and 1.5% ethanol. After 5 hours, glucose was exhausted, and cells were switching to the non-fermentable carbon source. (Figure 2B, left panel). Although the vast majority of GFP-CgCta1 was still located in the cytoplasm, small vesicles accumulating catalase became visible (see insert). After 20 hours, almost all GFP-CgCta1 was accumulated in vesicular structures (Figure 2B, middle panel).

CgCta1 can localize to peroxisomes.
We suspected that the vesicles accumulating GFP-CgCta1 were peroxisomes. The PTS1 of CgCta1 was a boundary case compared to \textit{S. cerevisiae} Cta1 (Figure 2B, lower panel). To interfere with \textit{C. glabrata} peroxisome assembly, we chose to eliminate the \textit{CgPEX3} gene (CAGL0M01342g). The \textit{S. cerevisiae} orthologue Pex3 has an essential function for peroxisome biogenesis (Hohfeld \textit{et al.}, 1991). The \textit{CgPEX3} open reading frame was replaced with the \textit{ScURA3} gene and the correct integration was tested by Southern blot (Figure S1). In these \textit{pex3Δ} mutant cells, GFP-CgCta1 remained distributed in the cytoplasm, even in 1.5\% ethanol grown cells (Figure 2B, right panel). With oleic acid as sole carbon source, \textit{S. cerevisiae} cells increase number and size of peroxisomes (Thieringer \textit{et al.}, 1991). GFP-CgCta1 accumulated in vesicles in cells growing in medium containing 0.2\% oleic acid, whereas in \textit{pex3Δ} mutant cells fluorescence was dispersed in the cytoplasm (Figure 2C). The number of stained vesicles also increased substantially in cells growing on a non-fermentative carbon source (1.5\% ethanol) (Figure 2D). These data suggest that \textit{C. glabrata} accumulates GFP-CgCta1 in CgPex3-dependent structures resembling peroxisomes.

To visualize peroxisomal structures in \textit{C. glabrata}, we fused a generic peroxisomal targeting signal peptide (KNIESKL) derived from the \textit{S. cerevisiae} citrate synthase to the C-terminus of YFP (Lewin \textit{et al.}, 1990, Kragler \textit{et al.}, 1993). The \textit{YFP-KNIESKL} fusion gene expression was driven by the strong \textit{CgADH1} promoter. YFP fluorescence marked peroxisomes, which increased their number during growth on ethanol (Figure 3A, upper panel) and were absent in \textit{pex3Δ} mutant cells (Figure 3A, lower panel). This result confirmed the requirement of CgPex3 for \textit{C. glabrata} peroxisome biogenesis.

The above results indicated a partial organellar localisation of catalase, depending on the type of carbon source. To show this, we prepared cell extracts of \textit{cta1Δ} mutant cells expressing \textit{CgCTA1}. We separated these in an organellar pellet and a cytosolic
supernatant fraction by centrifugation and tested the fractions for catalase activity. After oxidative stress, the entire induced catalase activity was found in the cytoplasmic supernatant (Figure 3B). In extracts from cells growing with ethanol as main carbon source, catalase activity was found in the cytosolic supernatant, but about one fourth of total activity was present in the pellet fraction (Figure 3C, left panel). To confirm that the pellet fraction contained organelles, we used cytochrome C oxidase activity as marker enzyme for mitochondria. Most of the cytochrome C oxidase activity was found in the pellet fraction (Figure 3C, right panel). Separation of extracts derived from cells grown in medium containing 0.2% oleic acid showed a further shift of catalase activity towards the pellet fraction (Figure 3D). Activity of CgCta1 in the various fractions was distributed corresponding to the previously observed intracellular localisation of GFP-CgCta1. Together, these results showed a dual localization of C. glabrata catalase depending on the presence of peroxisomes.

Phagocytosis induces GFP-CgCta1.

Fungal pathogens are exposed to a stressful environment, when they come into contact with phagocytic cells (Nicola et al., 2008). The regulation and localisation of GFP-CgCta1 made it useful to report the environmental conditions during phagocytosis. C. glabrata cta1Δ mutant cells expressing GFP-CgCTA1 grown to exponential phase were used for infection of primary mouse macrophages. We used time lapse live microscopy to follow the fate of individual engulfed cells (Figure 4A). Freshly phagocytosed C. glabrata cells reacted to this environment with a detectable GFP-CgCta1 fluorescence signal within 40 minutes. (Figure 4A and Figure S2C). Furthermore, during prolonged phagocytosis, GFP-CgCta1 accumulated in peroxisomes. To support the idea of peroxisome proliferation during phagocytosis, we followed localisation of the YFP-KNIESKL fusion protein during
infection of macrophages. Cells were fixed and stained for microscopy immediately after infection and after 2.5, 5, 10 and 24 hours (Figure 4B). We counted cells with visible peroxisomes per macrophage at various time points (Figure 4C, left panel; Figure S3). The number of cells with peroxisomes and the number of peroxisomes within these cells transiently increased, reaching a peak after 5 hours (Figure 4C). After 24 hours, the vast majority of cells displayed a cytoplasmic/vacuolar YFP-KNIESKL fluorescence signal. Thus, engulfed cells show transient proliferation of peroxisomes.

Localization of GFP-CgYap1 and CgMig1-CFP in phagocytosed cells

The localization of CgCta1 suggested that engulfed C. glabrata cells might experience oxidative stress and/or carbon source starvation. To confirm this independently, we created additional fluorescent reporter constructs. In S. cerevisiae, the glucose-regulated transcriptional repressor Mig1 is rapidly exported from the nucleus in cells starved for glucose (De Vit et al., 1997). S. cerevisiae Yap1 accumulates rapidly in the nucleus in cells exposed to mild oxidative stress (Kuge et al., 1997). To preserve the localization signals of the orthologous transcription factors, CgYap1 was N-terminally fused to GFP whereas CgMig1 was C-terminally fused to CFP. To be detectable, both fusion genes were expressed from centromeric plasmids and driven by the CgADH1 promoter. Nuclear localization was confirmed by simultaneous staining of nucleic acids with DAPI (Figure 5A and 5B).

GFP-CgYap1 was located in the cytoplasm in unstressed C. glabrata ΔHTU cells. Upon exposure to mild oxidative stress (0.4mM H₂O₂), GFP-CgYap1 rapidly accumulated in the nucleus (Figure 5A, upper panel). The fusion gene could complement the transcription defects of the corresponding deletion mutant (our unpublished observation). Within the first hour upon engulfment, cells with nuclear GFP-CgYap1 were visible (Figure
Results

We determined the percentage of yeast cells with nuclear GFP-Yap1 per macrophage after 30 minutes, 1 hour and 5 hours (Figure 5A, lower panel) and found a peak at about 1 hour. The CgMig1-CFP fluorescence signal accumulated in the nucleus after addition of glucose (2%) to the medium of glucose starved cells, and was also nuclear in the glucose-rich environment of the macrophage culture medium (DMEM) (Figure 5B, upper panel). Immediately after phagocytosis, CgMig1-CFP accumulated in the cytoplasm and remained there constantly, indicating glucose starvation (Figure 5B, lower panel). These data showed that within the phagosome oxidative stress is transient, whereas macrophages are highly effective in depriving the carbon source.

Peroxisomes are transiently induced during phagocytosis.

Peroxisome numbers declined at later stages of engulfment (Figure 4B). In S. cerevisiae, key factors for pexophagy are Atg11 (Yorimitsu et al., 2005) and Atg17, which is also essential for non-selective autophagy (Cheong et al., 2005). We deleted the C. glabrata CgATG11 and CgATG17 homologues (CAGL0H08558g, CAGL0J04686g) in wild type (ΔHTU) and pex3Δ cells (Figure S1). We investigated engulfed C. glabrata cta1Δ, pex3Δ, atg11Δ, atg17Δ, pex3Δatg17Δ, pex3Δatg11Δ, and atg11Δatg17Δ mutant cells expressing GFP-CgCta1 after 5 and 24 hours (Figure 6A, E). After 5 hours, GFP-Cta1 was located in peroxisomes in the cta1Δ, atg11Δ, and atg17Δ mutant cells, and, in contrast, accumulated in the cytoplasm of pex3Δ, pex3Δatg11Δ, and pex3Δatg17Δ mutant cells. However, after 5 hours, wild type, atg11Δ, and atg17Δ, had similar numbers of cells with peroxisomes, whereas after 24 hours, peroxisomes were more abundant in atg11Δ and atg17Δ mutants (Figure 6B). In atg11Δ mutants, peroxisome numbers remained constant between 5 and 24 hours engulfment. C. glabrata atg17Δ cells displayed a slight reduction of peroxisomes after 24 hours of engulfment, similar to S.
Results

cerevisiae atg17Δ cells during prolonged starvation conditions (Cheong et al., 2005),
Upon internalization, the cytoplasmic localization of CgMig1-CFP demonstrated the same
2 glucose starvation status in the atg11Δ, pex3Δatg11Δ mutants and wild type (Figure S2B).

We investigated if the turnover of peroxisomes and mobilization of internal
resources is relevant for survival during engulfment. Indeed, the atg11Δ and atg17Δ
mutants had a significantly reduced viability after 24 hours compared to wild type, cta1Δ
and pex3Δ strains. Furthermore, in pex3Δatg11Δ and pex3Δatg17Δ double mutants the
absence of pexophagy might be compensated by absence of peroxisome biogenesis.
Consistently, we found that the loss of Pex3 partially reversed the effect of atg11Δ with
respect to survival during engulfment (Figure 6C). In contrast, the double mutant
pex3Δatg17Δ did not show this phenotype, indicating a broader function for CgAtg17-
dependent non-selective autophagy during engulfment. Strikingly, the atg11Δatg17Δ
double mutant, lacking both selective and non-selective autophagy, was highly sensitive
to phagocytosis.

To simulate the phagosome environment in vitro we combined nutrient starvation
and acidic pH. We incubated C. glabrata wild type, atg11Δ, pex3Δatg11Δ, atg17Δ,
pex3Δatg17Δ, and atg11Δatg17Δ mutant cells in medium lacking nitrogen- and carbon-
sources at pH 3.5 at 37°C for 24 hours. The survival was determined by counting colony
forming units after 24h hours relative to 2 hours treatment (Figure 6D). In comparison to
the wild type, all mutants showed diminished survival. Intriguingly, the pex3Δatg11Δ strain
survived better than the atg11Δ strain. Furthermore, the double mutant atg11Δatg17Δ
displayed the lowest survival rate, similar to the macrophage model. In the macrophage,
after 24 hours, most of the engulfed atg11Δatg17Δ cells had lost GFP-CgCta1
fluorescence presumably due to cell death (not shown). However, after 5 hours the GFP-
CgCta1 fluorescence signal indicated numerous peroxisomes (Figure 6E). These results
indicated that autophagy is beneficial for survival of *C. glabrata* during engulfment in macrophages, possibly counteracting acute nutrient starvation.
Results

Discussion

Phagocytic cells internalize microbial cells and attack them with a range of microbicidal strategies (Chauhan et al., 2006, Nicola et al., 2008). Microbial pathogens have developed a number of strategies to improve their survival in the host environment (Urban et al., 2006). Here we used three reporters (CgCta1, CgYap1 and CgMig1) to visualize aspects of the response of the human fungal pathogen \textit{C. glabrata} to macrophage engulfment. We found that \textit{C. glabrata} cells engulfed by primary mouse macrophages suffer from transient oxidative stress, show signs of carbon source starvation, and transiently induce peroxisomes. Our results revealed that the recycling of internal resources, especially peroxisomes, plays an important protective role for \textit{C. glabrata} during engulfment in the phagosome.

The presence and/or proliferation of peroxisomes in fungal cells points to adjustment of carbon metabolism. We demonstrated accumulation of peroxisomes in \textit{C. glabrata} during growth on non fermentable carbon sources and during engulfment in macrophages. Peroxisomes were visualized using two fluorescent reporter constructs GFP-CgCta1 and YFP-KNIESKL and further confirmed by other criteria. They were induced on medium containing ethanol and oleic acid as carbon source. Furthermore, peroxisomes were dependent on the \textit{CgPEX3} gene, a peroxisomal integral membrane protein, whose orthologue in \textit{S. cerevisiae} is essential for peroxisomal biogenesis (Hohfeld et al., 1991). Peroxisomal catalases such as \textit{S. cerevisiae} Cta1 (Simon et al., 1991) are scavengers of hydrogen peroxide generated during peroxisomal $\beta$-oxidation. We find that \textit{C. glabrata} catalase expression is regulated by oxidative stress and carbon source, and its intracellular localization correlates with the presence of peroxisomes. This combines the regulation of both yeast catalases. The \textit{CgCTA1} gene lacks synteny with the yeast \textit{CTA1} gene and other fungal catalases (Gordon et al., 2009). It is tempting to
speculate that the shuffling of the *C. glabrata* genome fostered the accumulation of regulatory elements for oxidative stress and carbon source response.

In a phagocytosis model using bone marrow-derived mouse macrophages, GFP-CgCta1 expressed under the control of the *CgCTA1* promoter was induced in the earliest stages after internalization. This could be due to oxidative stress or acute carbon starvation. Intracellular localization of two other fluorescent reporters (CgYap1 and CgMig1) supported rather low oxidative stress load and starvation for glucose of engulfed *C. glabrata* cells. High level expression was necessary for detection of GFP-transcription factor fusions and could potentially interfere with signalling. However, both factors are tightly regulated by post translational modifications and thus buffered for expression level.

We found that in a population of engulfed cells a minor fraction displayed signs of acute oxidative stress. This is consistent with other reports. Only a small portion of *C. albicans* cells derived from mouse kidneys displayed an acute oxidative stress response when examined for *CaCTA1* expression (Enjalbert et al., 2007).

The *C. glabrata* transcriptional response might have been selected to the specific conditions of phagocytosis. Microarray data indicated induction of a group of about 30 genes by both oxidative stress and glucose starvation (Roetzer et al., 2008). Moreover, phagocytosed *C. glabrata* cells induce genes involved in gluconeogenesis, β-oxidation, glyoxylate cycle, and transporters for amino acids and acetate (Kaur et al., 2007). Induction of peroxisomes after internalization by macrophages indicated adjustment of metabolism within the phagosome. Cells utilizing non fermentable carbon sources e.g. fatty acids or ethanol require peroxisomal β-oxidation and the partly peroxisomal glyoxylate cycle. The induction of non-fermentative carbon metabolism genes is beneficial for the survival of *C. albicans* (Barelle et al., 2006, Lorenz et al., 2004). In a mouse infection model, Fox2, the second enzyme of the β-oxidation pathway, and isocitrate lyase
(Icl1) an enzyme of the glyoxylate cycle, were required for *C. albicans* virulence (Lorenz et al., 2001, Piekarska et al., 2006). However, *C. albicans* mutants defective in the import receptor of PTS1 targeted peroxisomal proteins, CaPex5, displayed no attenuation of virulence (Piekarska et al., 2006). The survival of *C. glabrata* devoid of peroxisomes in a *pex3Δ* mutant was not compromised in our infection model. Also, *C. neoformans* *pex1Δ* deletion mutants were not attenuated for virulence (Idnurm et al., 2007). These data support the view that peroxisomes are not a major virulence determinant. Instead, the peroxisomal metabolic pathways, which can function to sufficient extent in the cytosol, appear to contribute to virulence.

In engulfed *C. glabrata* cells peroxisome numbers declined at later time points. Also at later time points GFP-CgCta1 accumulated partly in the cytosol. Peroxisomes are not known to export proteins, thus the cytosolic fluorescence was most probably due to de novo synthesis or peroxisome turnover. Peroxisomes are degraded by pexophagy, a selective autophagic pathway (Hutchins et al., 1999, Farre et al., 2004). In *S. cerevisiae*, mutants lacking Atg11 and Atg17 had a severe delay of pexophagy (Cheong et al., 2005, Kim et al., 2001, Cheong et al., 2008). In *C. glabrata*, we found that mutants lacking *atg11Δ* or *atg17Δ* had reduced survival in macrophages and *in vitro* during starvation. Moreover, the *C. glabrata* double mutant *atg11Δatg17Δ* displayed a striking additive decrease of survival. In *S. cerevisiae*, the *atg11Δatg17Δ* double mutant strain did not contain any detectable autophagic bodies and had a severe autophagy defect (Cheong et al., 2008). We suggest that *C. glabrata* *atg11Δatg17Δ* is unable to induce autophagic processes in order to sustain prolonged phagocytosis. Notably, homologues of proteins of the autophagy core machinery have been found from yeast to mammals, but both Atg11 and Atg17 are not conserved and might be a target for antifungal drugs (reviewed by (Xie et al., 2007)).
Autophagy is required for \textit{C. neoformans} virulence (Hu \textit{et al.}, 2008). Furthermore, \textit{C. neoformans} genes involved in autophagy, peroxisome function, and lipid metabolism became also induced during infection (Fan \textit{et al.}, 2005). \textit{C. neoformans} could escape from macrophages through extrusions of the phagosome, without killing the phagocytic cell (Alvarez \textit{et al.}, 2006). It has been suggested that this is a pathway for dissemination within the host. Therefore, survival in the macrophage indirectly contributes to virulence. A \textit{C. albicans} mutant lacking CaATG9 was defective for autophagy, but nevertheless was able to kill macrophages (Palmer \textit{et al.}, 2007). In contrast to \textit{C. albicans}, \textit{C. glabrata} is trapped inside the phagosome. In \textit{C. glabrata} pex3\textsuperscript{Δ}atg11\textsuperscript{Δ} cells, we found the sensitivity of atg11\textsuperscript{Δ} partially reversed. We suggest from this genetic observation, that autophagy of peroxisomes is beneficial for engulfed \textit{C. glabrata} cells. \textit{C. glabrata} pex3\textsuperscript{Δ}atg17\textsuperscript{Δ} mutants did not display this effect. Selective pexophagy, which is affected in both atg11\textsuperscript{Δ} and atg17\textsuperscript{Δ} mutants, might help to mobilize intracellular resources during prolonged engulfment. \textit{S. cerevisiae} uses autophagy to recycle proteins to overcome nitrogen starvation (Onodera \textit{et al.}, 2005). Autophagic processes, such as pexophagy, are contributing to virulence of important fungal plant pathogens (Asakura \textit{et al.}, 2009, Veneault-Fourrey \textit{et al.}, 2006). However, an \textit{A. fumigatus} mutant strain lacking Atg1 also remained virulent (Richie \textit{et al.}, 2007). Thus the role of autophagy for fungal pathogens is also dependent on their morphology (Palmer \textit{et al.}, 2008).

Beside the carbon and nitrogen starvation conditions inside the phagosome, other restrictions, such as pH, hydrolytic enzymes, and antimicrobial peptides might act in a synergistic manner. We infer from the phenotype of our autophagy mutants that macrophage engulfment is essentially a starvation situation in combination with acidic pH. Acidification of the phagosome aids the destruction of some microbes, but it might also contribute to the escape of others. For example, lysosomal acidification induced germ
tube formation of *C. albicans* and therefore contributed to its escape from the macrophage (Kaposzta *et al.*, 1999). The observed oxidative stress response of *C. glabrata* might result from a switch of metabolism rather than a macrophage-derived oxidative burst. It has been reported that in *S. cerevisiae*, a shift to oleic acid as carbon source induced a specific Yap1-dependent subset of oxidative stress response genes (Koerkamp *et al.*, 2002). However, we believe that the importance of autophagy for survival suggests a starvation situation. Furthermore, in our model system, the transient induction and degradation of peroxisomes is not supporting substantial metabolism in the phagosome.

Our results demonstrate that monitoring of the intracellular localization of proteins tagged with fluorescent reporters is a highly informative tool to reveal intracellular signalling and metabolic conditions. Here we show that the macrophage is efficiently depriving engulfed *C. glabrata* cells from nutrient sources. Autophagic processes, prolonging the survival of engulfed cells, are potentially aiding the dissemination of *C. glabrata* and the establishment of infection.
Experimental Procedures

Yeast strains and Plasmids: Yeast strains used in this study are listed in Table 1. Rich medium (YPD), synthetic medium (SC) and yeast nitrogen base medium (YNB) without amino acids and ammonium sulfate were prepared as described elsewhere (Current Protocols in Molecular Biology; Wiley). All strains were grown at 30°C or 37°C as indicated. Oleate medium contained 0.2% oleic acid, 0.3% yeast extract, 0.5% peptone and 0.5% KH₂PO₄ (pH6). Oleate plates were incubated at 37°C for 7 days. Glucose concentration between 0.5% and 0.03% (w/v) was determined using the Freestyle mini (Abbott). To assess viability of cells during starvation (Figure 6D, colony forming units were determined by spreading on rich medium, usually after 2 hours of incubation at 37°C and after the indicated time (24h). Oligonucleotides used in this study are listed in Table S1. C. glabrata strains AR_Cg cta1Δ, AR_Cg pex3Δ, AR_Cg atg11Δ, AR_Cg pex3Δ atg11Δ, AR_Cg atg17Δ, AR_Cg pex3Δ atg17Δ, and AR_Cg atg11Δ atg17Δ were obtained by replacing the open reading frames with the S. cerevisiae URA3 gene or HIS3 gene generated by genomic integration. Knock out cassettes were synthesized using fusion PCR according to Noble (Noble et al., 2005) from the plasmids pRS316 and pRS313 (Sikorski et al., 1989) with the oligonucleotides CTA1-1 to 6, PEX3-1 to 6 ATG11-1 to 6 and ATG17-1 to 6. Correct genomic integration was verified by genomic PCR (primer series Ctrl) followed by Southern analysis using probes generated with primers CTA1-4/CTA1-6, PEX3-1/PEX3-3, ATG11-4/ATG11-6 and ATG17-1/ATG17-3 or ATG17-4/ATG17-6. Probes for Southern and also for Northern analysis (CTA1-5/CTA1-3 and ACT1-5/ACT1-3) were amplified by PCR from genomic DNA.

Plasmids used in this study are listed in Table 2. To generate pGEM-ACT-CgCTA1, 1800 basepairs of the CgCTA1 promoter were inserted as a SphI/NotI PCR product obtained with primers CTAPro-up and CTAPro-down into the plasmid pGEM-ACT
Results

(Gregori et al., 2007). The coding sequence for CgCTA1 was amplified from genomic DNA using primers CTA-up-Not and CTA-down-Nsi, cut and inserted as a NotI/NsiI fragment. GFP was inserted as a NotI/NotI fragment at the N-terminus of CgCTA1. To generate pYFP-KNIESKL YFP was inserted as a NotI/NotI fragment obtained by PCR with primers YFP-Not-Start and YFP-SKL-Stop into the plasmid pGEM-ACT-CgADH1 (Roetzer et al., 2008). CgYAP1 was amplified using primers CgYap5/CgYap3 containing a NotI or a NsiI site, GFP was inserted as NotI/NotI fragment into the plasmid pGEM-ACT-CgADH1 at the N-terminus of CgYAP1. CgMIG1 was amplified using primers Mig1-5sac/Mig1-3nco and inserted into NcoI and SacII cut pGEM-ACT-CgADH1-MSN2-CFP (Roetzer et al., 2008). All cloned PCR fragments used in this study were controlled by sequencing.

Catalase and Cytochrome C oxidase assay: Crude extracts were prepared by breakage of yeast cells with glass beads. Catalase activity was assayed spectrophotometrically at 240nm as described in (Durchschlag et al., 2004); protein concentrations were assayed at 280nm. For the Cytochrome C oxidase assay, 0.5g/l Sodium dithionite was added to reduce Cytochrome C (0.1mg/ml) solution. Cytochrome C has a sharp absorption band at 550nm in the reduced state. Absorption spectra of Cytochrome C were recorded between 410nm and 570nm. 5 minutes after addition of crude extracts, spectra were measured to determine the oxidized state of Cytochrome C (Lemberg, 1969).

Separation of organelles: Cells were resuspended in washing buffer (20mM HEPES pH7.4, 50mM NaCl, 0.6M sorbitol), incubated with protease inhibitor PMSF and broken using glass beads. The supernatant was centrifuged for 12 minutes at 6900 rcf to separate (post-mitochondrial) supernatant and the organellar pellet.

Northern and Southern blot analysis: RNA extraction and separation followed essentially the described protocol (Current Protocols In Molecular Biology; Wiley).
Hybridization of $^{32}$P-αATP labelled probes occurred overnight in hybridization buffer (0.5M Sodium phosphate buffer pH 7.2 / 7% SDS / 1mM EDTA) at 65°C. For DNA extraction, 10ml yeast cells (grown to an OD$_{600}$=6) were collected, washed once and resuspended in Lysis buffer (2% Triton X-100 / 1% SDS / 100mM NaCl / 10mM Tris pH8 / 1mM EDTA). Genomic DNA was isolated by PCI (phenol/chloroform/isoamyl alcohol) extraction. Digestion of 10µg genomic DNA was done overnight with XcmI for CgPEX3, EcoRV for CgCTA1 and Clal/Ncol for CgATG11 (5U/µg DNA). The labelled probes were hybridized overnight in hybridization buffer at 65°C. Signals were visualized by autoradiography.

**Microscopy:** GFP-fluorescence microscopy was performed as described previously (Görner *et al.*, 1998). GFP was visualized in live cells without fixation. All cells were monitored using a Zeiss Axioplan 2 fluorescence microscope. Images were captured with a Spot Pursuit (Sony) CCD camera using Spotbasic software. Time lapse microscopy was performed on an Olympus cell-imager system (IX81 inverted microscope) equipped for cell culture observation. Cells were incubated in a glass chamber at 37°C connected to an active gas mixer (Ibidi, Martinsried, Germany). Pictures were taken with a Hamamatsu ORCA-ER camera and analysed using cellM&cellR software (Olympus). Nomarski contrasted, bright field microscopy pictures are indicated as BF. Quantification and statistical analysis of peroxisomes in *C. glabrata* cells (Figures 2D, 4C and 6B) have been added in figure S3.

**Macrophage cell culture:** Primary bone marrow derived macrophages (BMDMs) were obtained from the femur bone marrow of 6 – 10 weeks old C57Bl/6 mice. Cells were cultivated in DMEM supplemented with 10% FCS in the presence of L cell-derived CSF-1 as described (Baccarini *et al.*, 1985). Mice were housed under specific pathogen-free conditions. For infection assays, BMDMs were seeded at 5 x 10$^5$ cells per dish in 3.5-cm dishes containing medium without antibiotics. Log-phase *C. glabrata* cells were washed
with PBS supplemented with 0.1% glucose) and added to macrophages in a 4:1 ratio and
incubated at 37°C. For microscopy, cells were fixed with 2% formaldehyde for 5 minutes.
After washing with PBS, cells were incubated in 1% triton X-100 for 1 minute. After
washing with PBS, cells were dyed with Phalloidin Texas-Red for 30 minutes. Cover slips
were fixed to slides with Mowiol. For CFU assays, BMDM were seeded at 2 x 10^5 cells per
dish. Exponentially growing \textit{C. glabrata} cells were washed with PBS supplemented with
0.1% glucose and added to macrophages in a 1:2 ratio and incubated at 37°C. After 45
minutes, cells were washed three times with PBS to remove not phagocytosed yeast cells
and fresh medium was added. At the indicated times deionised water was added to lyse
macrophage cells. \textit{C. glabrata} cells were spread on YPD plates, colonies were counted
after incubation at 37°C for 2 days.
Acknowledgements:

We thank Andreas Hartig, Fabian Rudolf, Claudine Kraft and especially Wolfgang Reiter for discussions, Christophe d’Énfert for critical reading and advice and Josef Gotzmann and Doris Mayer for technical support. C.S. was supported by the Herzfelder Foundation, and the Vienna Hochschuljubiläumsstiftung. This work was supported by the Austrian Research Foundation (FWF) through grants P16726-B14, I27-B03 and SFB F28 to P.K. and P19966-B12 to CS.
### Table 1

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### Supplemental Table S1

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Results

Figure legends

Figure 1. Oxidative stress and carbon source stress regulate Cg catalase CgCTA1. (A) To measure catalase activity upon glucose depletion, cells were grown to log phase in YPD and shifted to medium with 2% or 0.1% glucose and grown for 4 hours. Catalase activity was determined as described in materials and methods. (B) Cells were incubated in YPD with 0.4mM H$_2$O$_2$ for 45 minutes. Crude cell extracts were prepared and then assayed for catalase activity. (C) Northern blot analysis of CgCTA1 mRNA levels from wild type, ARCg cta1Δ mutant, and complemented mutant strain was performed under stress conditions (glucose starvation, 0.4mM H$_2$O$_2$). Samples were taken at the indicated time points. CgACT1 mRNA levels were used as loading control. mRNA levels were visualized by hybridization of radioactive probes and autoradiography. The pCgC-GFP-CgCTA1 construct with GFP inserted at the N-terminus is illustrated. (D) C. glabrata ARCg cta1Δ mutant complemented with pGFP-CgCTA1, pCgCTA1 or an empty plasmid were grown in synthetic medium to log phase, adjusted to 10$^5$ cells/ml and exposed to indicated doses of hydrogen peroxide. Optical density after 24 hours of incubation at 37°C is indicated.

Figure 2. Intracellular localization of C. glabrata catalase. (A) Localization of GFP-CgCta1 was determined by fluorescence microscopy in ARCg cta1Δ cells transformed with pCgCTA1-GFP-CgCTA1. Cells were incubated for 1 hour after induction of oxidative stress with 0.4mM H$_2$O$_2$. Unstressed cells were stained with DAPI (2µg/ml) for 10 minutes. Aliquots of both cultures were pooled prior to microscopy. White arrows indicate unstressed cells. (B) ARCg cta1Δ and ARCg pex3Δ mutant strains transformed with pCgC-GFP-CgCTA1 were grown in synthetic medium with 0.5% glucose and 1.5%...
ethanol for 20 hours. White arrows indicate vesicular structures. Inserts show enlarged pictures of single cells. Possible peroxisomal targeting signals 1 (PTS1) detected at the C-terminus of CgCta1, ScCta1 and CaCct1 (Q6FM56, P15202, Q5AAT2; (Neuberger et al., 2003)). (C) Fluorescence signals of strains as in (B) after growth in medium with 0.2% oleic acid for 20 hours. (D) Number of peroxisomes in *C. glabrata* cells during growth with ethanol (1.5%) and oleic acid (0.2%) as main carbon source.

Figure 3. CgCta1 localizes to peroxisomes upon glucose depletion. (A) *C. glabrata* ΔHTU and ARCg pex3Δ mutant cells expressing YFP-KNIESKL driven by the *CgADH1* promoter were grown in synthetic medium with 2% glucose or 1.5% ethanol for 20 hours. Localization of YFP was recorded by fluorescence microscopy and bright field (BF) microscopy. An overlay of YFP and BF microscopy is shown in the left panel. (B) The ARCg cta1Δ strain carrying pCgC-CgCTA1 was exposed for 1 hour to oxidative stress (0.4mM H₂O₂). Pellets containing mitochondria and small organelles and post-mitochondrial supernatants were assayed for catalase activity. (C) The same strain was grown in synthetic medium with 1.5% ethanol for 20 hours. Catalase activity was measured in pellets and supernatants. Activity of Cytochrome C oxidase was measured in pellet and supernatant fractions as described in materials and methods (right panel). (D) Catalase activity in pellets and supernatant fraction collected from ARCg cta1Δ containing pCgC-CgCTA1 grown in synthetic medium with 0.2% oleic acid for 20 hours.

Figure 4. GFP-CgCTA1 is induced upon phagocytosis and is located in both cytoplasm and peroxisomes. (A) *C. glabrata* cells before and after being phagocytosed. Exponentially growing ARCg cta1Δ cells transformed with pCgC-GFP-CgCTA1 were washed in PBS containing 0.1% glucose and added to macrophages in a 4:1 ratio at
Results

37°C. Still pictures at the indicated times are shown as overlay of bright field and fluorescence signals. (B) Exponentially growing wild type cells transformed with pCgADH1-YFP-KNIESKL1 were washed in PBS containing 0.1% glucose and added to macrophages in a 4:1 ratio at 37°C. Cells were fixed and stained with Phalloidin Texas-Red after 0, 2.5, 5, 10 and 24 hours for fluorescence microscopy. (C) Percentage of phagocytosed C. glabrata cells with visible peroxisomes per macrophage from the total cell number of C. glabrata cells per macrophage after 0, 2.5, 5, 10 and 24 hours (left panel). Number of visible peroxisomes within phagocytosed C. glabrata cells after 0, 2.5, 5, 10 and 24 hours (right panel).

**Figure 5. Localization of GFP-CgYap1 and CgMig1-CFP during early stage of phagocytosis.** (A) C. glabrata wild type cells transformed with pCgADH1-GFP-CgYAP1 were grown in synthetic medium. Cells were stressed by addition of 0.4mM H$_2$O$_2$ for 10 minutes. Nuclei were stained with DAPI. An overlay of GFP and DAPI staining is shown in the right panel. GFP-CgYap1 visualized by fluorescence microscopy under phagocytosis conditions (lower panel). Cells were washed in PBS 0.1% glucose and added to macrophages in a 4:1 ratio and incubated at 37°C for 10 minutes to follow the route of tagged transcription factors. Samples were fixed and stained with Phalloidin Texas-Red. Percentage of cells with nuclear GFP-Yap1 was calculated after 30 minutes, 1 hour and 5 hours. White arrows point to nuclear GFP-CgYap1 in yeast inside the phagosome. (B) C. glabrata wild type cells transformed with pCgADH1-CgMIG1-CFP were grown in synthetic medium until glucose depletion. Cells were incubated in fresh medium containing 2% glucose for 10 minutes or 1X DMEM. Lower panel depicts localisation of CgMig1-CFP under phagocytosis conditions. Cells were treated as described in (A).
Figure 6. Induction and pexophagy of peroxisomes upon phagocytosis. (A) Log-phase *C. glabrata* ARCg *cta1Δ*, ARCg *pex3Δ*, ARCg *atg11Δ*, ARCg *atg17Δ*, ARCg *pex3Δatg17Δ*, and ARCg *atg11Δatg17Δ* mutant cells transformed with pGFP-CgCTA1 were used to infect mouse macrophages in a 4:1 ratio at 37°C. Cells were fixed for microscopy after 5 hours and 24 hours. (B) Percentage of cells with visible peroxisomes after phagocytosis in macrophages after 5 hours and 24 hours. (C) Log-phase *C. glabrata* ARCg *cta1Δ*, ARCg *pex3Δ*, ARCg *atg11Δ*, ARCg *atg17Δ*, ARCg *pex3Δatg17Δ*, and ARCg *atg11Δatg17Δ* mutant cells were used to infect mouse macrophages in a 1:2 ratio at 37°C. The viability of the engulfed cells was assessed by hypotonic lysis of the macrophages and quantification of colony formation (cfu) on rich medium. Assays were done in triplicate. A one-way ANOVA was performed and P values were calculated comparing the numbers of recovered colonies of the indicated strains (**, P < 0.005). (D) *C. glabrata* wild type, ARCg *atg11Δ*, ARCg *pex3Δatg11Δ*, ARCg *atg17Δ*, ARCg *pex3Δatg17Δ* and ARCg *atg11Δatg17Δ* mutant cells were grown to exponential phase in rich medium; after washing with PBS supplemented with 0.1% glucose, 2×10⁵ cells were incubated in selective medium without nitrogen-sources and glucose and pH 3.5 at 37°C. After 24 hours colony formation (cfu) of mutant cells was determined. Percentage of viable cells was calculated relative to 2 hours treatment. (E) Log-phase ARCg *atg11Δatg17Δ* mutant cells transformed with pGFP-CgCTA1 were used to infect mouse macrophages in a 4:1 ratio at 37°C. Cells were fixed for microscopy after 5 hours. Overlay of GFP/Texas-Red and BF is shown.
Supplementary Figures

Figure S1. Southern blot analysis of cta1Δ, pex3Δ, atg11Δ and pex3Δatg11Δ deletion strains. Both CgCTA1 and CgPEX3 were replaced by ScURA3. CgATG11 was replaced by ScURA3 in the single mutant and by ScHIS3 in the double mutant ARCg pex3Δatg11Δ. CgATG17 was replaced by ScURA3 in the single mutant and by ScHIS3 in the double mutants ARCg pex3Δatg17Δ and ARCg atg11Δatg17Δ. Amplified probes and chromosomal restriction enzyme locations are indicated. Chromosomal DNA derived from ARCg cta1Δ digested with EcoRV and ARCg pex3Δ with XcmI resulted in shortened fragments relative to wild type. Digestion of chromosomal DNA from the ARCg atg11Δ strain with Ncol and from the ARCg pex3Δatg11Δ double mutant strain with ClaI led to shorter fragments in both cases, since CgATG11 contains neither a Ncol site nor a ClaI site. Picture is a composite of two exposures of the same blot, due to different amounts of DNA (lower panel). Chromosomal DNA derived from ARCg atg17Δ digested with AflIII and ARCg pex3Δatg17Δ or ARCg atg11Δatg17Δ with Ndel resulted in shortened fragments.

Figure S2. Glucose depletion leads to induction of GFP-CgCTA1 and cytoplasmic localization of CgMig1-CFP. (A) GFP-CgCTA1 is induced upon glucose depletion and is located in the cytoplasm. C. glabrata ARCg cta1Δ cells transformed with pCgC-GFP-CgCTA1 were grown in rich medium with glucose to exponential phase and washed twice and incubated in rich medium including 0.5% glucose. Every 10 minutes concentration of glucose was determined (see Material and Methods) and samples were fixed for microscopy. GFP fluorescence was visible at about 40 minutes after glucose exhaustion. (B) Localization of CgMig1-CFP in ARCg atg11Δ and ARCg pex3Δatg11Δ mutants during internalization by macrophages. CgMig1-CFP was visualized by fluorescence microscopy.
under phagocytosis conditions. *C. glabrata* wild type cells transformed with pCgADH1-CgMIG1-CFP were grown to exponential phase, washed in PBS 0.1% glucose and added to macrophages in a 4:1 ratio and incubated at 37°C for 1 hour. Samples were fixed and stained with Phalloidin Texas-Red. (C) GFP-CgCTA1 is induced upon phagocytosis. Still pictures from time lapse analysis are shown as overlay of bright field and fluorescence signals. Exponentially growing ARGctalΔ cells transformed with pCgC-GFP-CgCTA1 were washed in PBS containing 0.1% glucose and added to macrophages in a 4:1 ratio at 37°C.

**Figure S3. Quantification details.**


Results


Figure 2

A. *C. glabrata cta1Δ + GFP-CgCTA1*

- 1h 0.4mM H₂O₂
- BF, GFP, DAPI (of unstressed cells)

B. *C. glabrata cta1Δ + GFP-CgCTA1* and *C. glabrata pex3Δ + GFP-CgCTA1*

- 5h 1.5% Ethanol
- 20h 1.5% Ethanol
- 20h 1.5% Ethanol

C. *C. glabrata cta1Δ + GFP-CgCTA1* and *C. glabrata pex3Δ + GFP-CgCTA1*

- 20h 0.2% Oleic acid

D. *C. glabrata cta1Δ + GFP-CgCTA1*

- Bar graph showing number of GFP-stained vesicles in cells over time with 1.5% Ethanol and 0.2% Oleic acid.
Figure 3

A

**pCgADH1-YFP-KNIESKL**

<table>
<thead>
<tr>
<th></th>
<th>2% Glucose</th>
<th>20h 1.5% Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>wt</strong></td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
<tr>
<td><strong>pex3Δ</strong></td>
<td>[Image]</td>
<td>[Image]</td>
</tr>
</tbody>
</table>

BF / YFP  | YFP | BF

B

60min 0.4mM H₂O₂

<table>
<thead>
<tr>
<th></th>
<th>Pellet</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CgCTA1</strong></td>
<td>[Graph]</td>
<td>[Graph]</td>
</tr>
<tr>
<td>Vector</td>
<td>[Graph]</td>
<td>[Graph]</td>
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</table>

C

4h 1.5% Ethanol

<table>
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<tr>
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<th>Pellet</th>
<th>Supernatant</th>
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<td><strong>Cytochrome C oxidase</strong></td>
<td>[Graph]</td>
<td>[Graph]</td>
</tr>
<tr>
<td>Catalase U/mg/min</td>
<td>[Graph]</td>
<td>[Graph]</td>
</tr>
</tbody>
</table>

D

0.2% Oleic acid

<table>
<thead>
<tr>
<th></th>
<th>Pellet</th>
<th>Supernatant</th>
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</thead>
<tbody>
<tr>
<td><strong>Catalase U/mg/min</strong></td>
<td>[Graph]</td>
<td>[Graph]</td>
</tr>
</tbody>
</table>
Figure 4

A

C. glabrata cta1Δ + GFP-CgCTA1

B

C. glabrata wild type + CgADH1-YFP-KNIESKL

C

% C.g. cells in macrophages with peroxisomes

Visible peroxisomes in C.g. cells
Figure 5

A

*C. glabrata* wild type + GFP-CgYAP1

<table>
<thead>
<tr>
<th>Condition</th>
<th>Image 1</th>
<th>Image 2</th>
<th>Image 3</th>
</tr>
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<tbody>
<tr>
<td>No stress</td>
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<td><img src="image2" alt="Image" /></td>
<td><img src="image3" alt="Image" /></td>
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<tr>
<td>0.4mM H₂O₂ (10min)</td>
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<td><img src="image6" alt="Image" /></td>
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GFP

<table>
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</thead>
<tbody>
<tr>
<td>1h</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
<tr>
<td>5h</td>
<td><img src="image9" alt="Image" /></td>
<td><img src="image10" alt="Image" /></td>
</tr>
</tbody>
</table>

C. glabrata wild type + GFP-CgYAP1

<table>
<thead>
<tr>
<th>Condition</th>
<th>Image 1</th>
<th>Image 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5h, 1h, 5h</td>
<td><img src="image11" alt="Image" /></td>
<td><img src="image12" alt="Image" /></td>
</tr>
</tbody>
</table>

% engulfed cells with nuclear CgYap1

B

*C. glabrata* wild type + CgMIG1-CFP

<table>
<thead>
<tr>
<th>Condition</th>
<th>Image 1</th>
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<th>Image 3</th>
</tr>
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<td>Glucose starvation</td>
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<td><img src="image14" alt="Image" /></td>
<td><img src="image15" alt="Image" /></td>
</tr>
<tr>
<td>2% Glucose (10min)</td>
<td><img src="image16" alt="Image" /></td>
<td><img src="image17" alt="Image" /></td>
<td><img src="image18" alt="Image" /></td>
</tr>
<tr>
<td>1X DMEM</td>
<td><img src="image19" alt="Image" /></td>
<td><img src="image20" alt="Image" /></td>
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CFP

<table>
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<th>Image 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1h</td>
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<td><img src="image23" alt="Image" /></td>
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Figure 6

A

<table>
<thead>
<tr>
<th>5h</th>
<th>24h</th>
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</thead>
<tbody>
<tr>
<td>cta(^{1}) &amp; pex(^{3}) &amp; atg(^{11}) &amp; pex(^{3})&amp;atg(^{11}) &amp; atg(^{17}) &amp; pex(^{3})&amp;atg(^{17})</td>
<td></td>
</tr>
</tbody>
</table>

GFP T-R

BF

B

% C.g. cells with peroxisomes in macrophages

5h

24h

C

cfu recovery after 24h relative to wt

D

% cfu after 24h in YNB (pH 3.5)

E

atg\(^{11}\)&atg\(^{17}\)

GFP T-R / BF
Figure S1
Figure S2

A

Glucose consumption

mg/dl Glucose

Time in minutes

B

atg11Δ + CgMIG1-CFP
pex3Δ atg11Δ + CgMIG1-CFP

1h

CFP
T-R

BF

C

C. glabrata cta1Δ + GFP-CgCTA1

0min

20min

40min

60min

80min
### Figure S3

#### Figure 2D

**C. glabrata cta1Δ + GFP-CgCTA1**

<table>
<thead>
<tr>
<th>Time Points</th>
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<th>20h</th>
</tr>
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<tr>
<td>1.5% Etanol</td>
<td>x</td>
<td>y</td>
<td>z</td>
</tr>
<tr>
<td>0.2% Oleic acid</td>
<td>x</td>
<td>y</td>
<td>z</td>
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</tbody>
</table>

**Counts of peroxisomes per single cell**

<table>
<thead>
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<th>Time Points</th>
<th>5h (ethanol)</th>
<th>24h (ethanol)</th>
<th>24h (oleate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>3.64705882</td>
<td>10</td>
<td>9.375</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>1.22173936</td>
<td>3.44480285</td>
<td>1.86063337</td>
</tr>
<tr>
<td>n(cytoplasm)</td>
<td>n=17</td>
<td>n=16</td>
<td>n=24</td>
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</tbody>
</table>

#### Figure 4C

**% C.g. cells with peroxisomes in macrophages**

<table>
<thead>
<tr>
<th>Time Points</th>
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<th>2.5h</th>
<th>5h</th>
<th>10h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (percentages)</td>
<td>11.6597688</td>
<td>45.474793</td>
<td>60.4209427</td>
<td>28.7447479</td>
<td>15.2678571</td>
</tr>
<tr>
<td>n(cytoplasm)</td>
<td>14</td>
<td>18</td>
<td>11</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>n(cytoplasm)</td>
<td>130</td>
<td>174</td>
<td>97</td>
<td>68</td>
<td>111</td>
</tr>
<tr>
<td>n(cytoplasm w peroxi)</td>
<td>16</td>
<td>82</td>
<td>56</td>
<td>21</td>
<td>18</td>
</tr>
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</table>

**Visible peroxisomes in C.g. cells**

<table>
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<th>Time Points</th>
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<th>5h</th>
<th>10h</th>
<th>24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (percentages)</td>
<td>11.6597688</td>
<td>45.474793</td>
<td>60.4209427</td>
<td>28.7447479</td>
<td>15.2678571</td>
</tr>
<tr>
<td>n(cytoplasm)</td>
<td>14</td>
<td>18</td>
<td>11</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>n(cytoplasm)</td>
<td>130</td>
<td>174</td>
<td>97</td>
<td>68</td>
<td>111</td>
</tr>
<tr>
<td>n(cytoplasm w peroxi)</td>
<td>16</td>
<td>82</td>
<td>56</td>
<td>21</td>
<td>18</td>
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</table>

#### Figure 6B

**% C.g. cells with peroxisomes in macrophages**

<table>
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<tr>
<th>Strains</th>
<th>wt</th>
<th>atg11Δ</th>
<th>atg17Δ</th>
<th>wt</th>
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<th>atg17Δ</th>
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<tbody>
<tr>
<td>5h (o/n)</td>
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<td>15,5166747</td>
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<td>60,4093823</td>
<td>57,8152403</td>
<td>41,4793693</td>
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<tr>
<td>Standard Deviation</td>
<td>12,9468752</td>
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<td>19,2853736</td>
<td>17,9638125</td>
<td>22,232647</td>
<td>13,9497083</td>
</tr>
<tr>
<td>n(cytoplasm)</td>
<td>11</td>
<td>9</td>
<td>9</td>
<td>8</td>
<td>13</td>
<td>16</td>
</tr>
<tr>
<td>n(cytoplasm)</td>
<td>136</td>
<td>102</td>
<td>73</td>
<td>71</td>
<td>102</td>
<td>145</td>
</tr>
<tr>
<td>n(cytoplasm w peroxi)</td>
<td>82</td>
<td>16</td>
<td>41</td>
<td>42</td>
<td>61</td>
<td>60</td>
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<tr>
<td>P-values (5h - o/n)</td>
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<td>0.864138</td>
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</tbody>
</table>
Results

CgSkn7 and CgYap1 mediate key responses to oxidative stress in *Candida glabrata*.

Andreas Roetzer\textsuperscript{a}, Eva Klopf\textsuperscript{a}, Nina Gratz\textsuperscript{b}, Marina Marcet-Houben\textsuperscript{c}, Toni Gabaldon\textsuperscript{c}, Pavel Kovarik\textsuperscript{b} and Christoph Schüller\textsuperscript{a}\textsuperscript{*}

From

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\textsuperscript{c} Comparative Genomics Group, Bioinformatics and Genomics Programme, Centre for Genomic Regulation, 88 08003 Barcelona, Spain

**Running title:** *Candida glabrata* oxidative stress response

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Dr. Bohr-Gasse 9/5  
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E-mail: christoph.schueller@univie.ac.at
Abstract:

In this work we addressed the wiring of the transcriptional oxidative stress response regulon and its main regulators of *C. glabrata*. We showed that CgSkn7 and CgYap1 are completely interdependent for regulation of a number of genes encoding important antioxidant functions. Chromatin immunoprecipitation data revealed that this interdependence occurs at the level of promoter recognition. CgYap1 was required for protection against peroxides and peroxynitrite however not for protection against superoxide anions. Accordingly, we discovered that superoxide dismutase gene *CgSOD1* was constitutively expressed in *C. glabrata* in a CgSkn7/CgYap1 independent manner. In addition, many oxidative stress-associated genes were also upregulated during carbon source depletion, a condition met by *C. glabrata* upon engulfment by phagocytes. Furthermore, we also found CgSod1 and CgSod2 to be strongly induced by carbon source starvation. Our results support the view that *C. glabrata* has adapted the regulation of oxidative stress response genes to a host-pathogen situation. However, the *C. glabrata skn7Δyap1Δ* double mutant had a similar survival rate compared to the wild type in a primary mouse macrophage model. Taken together, we conclude that the *S. cerevisiae*-like oxidative stress response is either dispensable for prolonged survival upon engulfment, or other starvation-associated factors can induce this regulon to a significant extent.
Introduction:

The human fungal pathogen *Candida glabrata* is found as a common commensal in gastrointestinal and genitourinary tracts, but can turn into an opportunistic pathogen in immunocompromised patients and elder people (52, 66, 67, 73). It is phylogenetically much closer related to baker’s yeast *Saccharomyces cerevisiae* than *Candida albicans* (38). Consistently, *C. glabrata* engulfed by phagocytic cells does not undergo characteristic morphological transitions such as *C. albicans* (47). Therefore, cells had to develop other strategies to withstand phagocytosis, which are currently under investigation.

In the mammalian host, cell-mediated immunity, which is based on efficient engulfment of pathogens by phagocytic cells, is crucial to counteract fungal infections (55). In the phagolysosome of phagocytic cells, such as macrophages and neutrophils, the NADPH oxidase complex consists of enzymes, which mediate oxidative destruction of engulfed microorganisms (7, 75). It catalyzes the production of superoxide anions (O$_2^-$), serving as initial source for the production of ROS, which damage the cell constituents including proteins, lipids and DNA. Other reactive oxidants produced are hydrogen peroxide (H$_2$O$_2$), which is generated by the breakdown of superoxides (O$_2^-$), hypochlorous acid (HOCl), which is formed by the myeloperoxidase-catalyzed oxidation of Cl$^-$ by H$_2$O$_2$, hydroxyl radicals (OH$^-$), produced by the reduction of H$_2$O$_2$ by Fe$^{3+}$ or Cu$^{+}$, peroxynitrite (ONOO$^-$), formed by the reaction between O$_2^-$ and NO$^-$, and many others (3, 81). Thus, for successful dissemination, pathogenic fungi have to counteract a broad spectrum of reactive oxidants during the oxidative burst.

Simple fungal organisms face complex oxidative stress environments inside the host, and therefore established various, often redundant, antioxidant systems (57), including a broad variety of antioxidant enzymes and cofactors (21, 48). These are catalases, superoxide dismutases, thioredoxins and glutathione-dependent peroxidases and reductases. These enzymes are known to be relevant for the survival
of several fungal pathogens (10, 14, 25, 58, 61). The loss of superoxide dismutases decreased virulence of *C. neoformans* in mice and diminished survival of *C. albicans* in macrophages (14, 25). Further it has been shown that catalase Cct1 and glutaredoxin Grx2 are necessary for full virulence of *C. albicans* in mouse infection models (10, 61). In contrast, the loss of CgCta1 did not affect survival of *C. glabrata* in mice (15). Furthermore, it has been shown recently, that *C. albicans* and *C. glabrata* can actively suppress production of ROS upon engulfment by phagocytes (88). Thus, it is essential for internalized fungal pathogens to counteract emerging reactive oxidants during phagocytosis.

Oxidative stress causes rapid changes in transcription of many gene encoding antioxidant functions. This is well described for the oxidative stress regulon of *S. cerevisiae* where this regulon is almost completely under control of the transcription factors Skn7 and Yap1 (31, 48). Yap1 contains a basic leucine zipper, and relocalizes from the cytoplasm to the nucleus upon stress induction (42). Activation of Yap1 during oxidative stress involves a C-terminal cysteine-rich domain, which contains a NES nuclear export signal (17, 18). Certain Cysteine residues in Yap1 become oxidized by the glutathione peroxidase Gpx3 which leads in turn to the formation of inner disulfide bonds, causing a conformational change that causes Yap1 to accumulate in the nucleus and activate its target genes (18, 42). Yap1 mediates not only resistance to oxidative stress but is also involved in the response to heat, metal ions and drugs (79, 89, 90). Its role in diverse stress responses has been elucidated in several important fungi, such as *Kluyveromyces lactis*, *Ustilago maydis*, *C. albicans* and *Aspergillus fumigatus* (1, 5, 59, 68, 86). The *S. cerevisiae* transcription factor Skn7 is part of a branched two-component signaling system, involved in oxidative stress response, osmoregulation and integration of cell wall synthesis (8, 34, 53, 60, 72). The Skn7 protein localizes mainly to the nucleus, and it is thought, that Skn7 undergoes a post-translational modification by phosphorylation following exposure to reactive oxygen species (ROS), which is dependent on Yap1 (32). Furthermore, also a direct interaction
Results

between Skn7 and Yap1 seems to be necessary for efficient induction of many oxidative stress response genes in *S. cerevisiae* (31, 32). Mutations introduced in the two component receiver domain of Skn7 were found to compromise the association of Skn7 but also Yap1 to upstream regions of oxidative stress-associated genes (31, 60). However, the precise mechanism by which these two factors interact and co-operate during oxidative stress induction of transcription is not yet clear.

Yap and Skn7 comprise a conserved regulatory unit modulating oxidative stress response in the phylum of Ascomycota. In *C. glabrata*, genes encoding putative orthologues of Yap1 and Skn7 were discovered (15). Similar to other fungi, resistance to hydrogen peroxide was found to be linked to the corresponding genes CgSkn7 and CgYap1 (12, 15). In *C. albicans*, the lack of the Skn7 orthologue led to a slight attenuation of virulence (77), whereas the impact of CaYap1 on virulence remains unclear. In contrast, in *Cryptococcus neoformans*, CnSkn7 is not required for pathogenicity (91); in addition, no obvious Yap1 orthologue was found. In *A. fumigatus*, both strains lacking AfYap1 or AfSkn7 displayed no attenuated virulence in a mouse infection model (45, 51). In the fungal plant pathogen *U. maydis*, loss of the Yap1 orthologue led to reduced virulence (59). The role of Yap1 and Skn7 is currently not fully explored in pathogenicity and may be dependent on specific host niches.

In this work we addressed the wiring of the transcriptional oxidative stress response regulon and its main regulators of *C. glabrata*. We found that CgSkn7 and CgYap1 are completely interdependent for regulation of a number of genes encoding important antioxidant functions. Chromatin immunoprecipitation data show that this interdependence occurs at the level of promoter recognition. CgYap1 was required for protection against hydrogen peroxide and peroxynitrite however not against superoxide due to menadione treatment. In addition, many oxidative stress-associated genes are also upregulated during carbon source depletion, a condition met by *C. glabrata* upon engulfment by phagocytes. Surprisingly, we observed that the superoxide dismutase
genes (CgSod1, CgSod2) are strongly induced by carbon source starvation and are not under CgYap1 and CgSkn7 control. Our results support the view that *C. glabrata* has adapted the regulation of oxidative stress response genes to a host-pathogen situation.
Materials and Methods:

Yeast strains and Plasmids: Yeast strains used in this study are listed in Table 1. Rich medium (YPD) and synthetic medium (SC) were prepared as described elsewhere (Current Protocols in Molecular Biology; Wiley). All strains were grown at 30°C or 37°C as indicated. Gradient plates were prepared as described first in (80). Square plastic petri dishes (Singer) were used to establish a concentration gradient of. Plates containing menadione (Sigma), hydrogen peroxide (Merck) and hypochlorite (Neuber) were poured at room temperature; plates containing peroxynitrite (CaymanChem) - due to its instability - were poured at 4°C.

Susceptibility of Candida glabrata strains to CTBT was determined by zone inhibition assay, using YPD (rich medium with glucose), YPGE (rich medium with glycerol and ethanol) and YNB (minimal medium with glucose) media. The filter discs (diameter of 6 mm) soaked with 10 µg CTBT were placed on the plates which were incubated at 30°C for 2 (rich medium) or 5 days (minimal medium) before determination of the diameter of the zone of growth inhibition.

Oligonucleotides used in this study are listed in Table S1. C. glabrata strains ARGcg skn7Δ, ARGcg yapΔ and ARGcg skn7Δyap1Δ were obtained by replacing the respective open reading frames with the S. cerevisiae genes URA3 or HIS3 generated by genomic integration of knock out cassettes synthesized using fusion PCR according to Noble (64) from the plasmids pRS316 and pRS313 (76) with the oligonucleotides SKN7-1 to 6 and YAP1-1 to 6. Correct genomic integration was verified by genomic PCR (primer series Ctrl) followed by Southern analysis using probes generated with primers listed below.

Plasmids used in this study are listed in Table 2. To generate pGEM-ACT-CgYAP1, 1200 basepairs of the promoter were inserted as a SphI/NotI PCR product obtained with primers ProNatYap1-5 and ProNatYap1-3 into the plasmid pGEM-ACT (29). The coding sequence for CgYAP1 was amplified from genomic DNA using
primers CgYap5/CgYap3, cut and inserted as a NotI/NsiI fragment into pGEM-ACT-ProYAP1 and pGEM-ACT-ProADH1. GFP was inserted as a NotI/NotI fragment at the N-terminus of CgYAP1. To generate pGEM-ACT-CgSKN7, CgSKN7 was amplified using primers SKN7-5sac/Skn7-3nco and inserted into Ncol and SacII cut pGEM-ACT-CgADH1-MSN2-CFP (70). 1200 base pairs of the promoter were inserted as Sphi/SaclI PCR (using primers ProNatSkn7-5/ProNatSkn7-3) fragment into pGEM-ACT-CgADH1-SKN7-CFP. CFP was removed through cutting with Ncol and NsiI, refilled and ligated. To generate pGEM-ACT-CgYAP1CgSKN7, CgSKN7 including its promoter, was amplified using the primers Skn7-5Nsi/Skn7-3Nsi, and inserted into the plasmid pGEM-ACT-CgYAP1. Truncated versions of the CgCTA1 promoter were amplified using according primers (see Table 2) and cut with Sphi/NotI for insertion into the plasmid pCgC-CgCTA1.

HA-tags were amplified through PCR using the primers Skn7_HA6-5/Skn7_HA6-3 and Yap1_HA6-5/Yap1_HA6-3. For YAP1-HA, the product was cut with NotI, for SKN7-HA the product was cut with Ncol. The proper insertion was tested via western blotting using an anti-HA antibody. All cloned PCR fragments used in this study were controlled by sequencing.

Probes for Northern (see supplemental Table S1) and Southern analysis (SKN7-1/ SKN7-3 and YAP1-1/YAP1-3) were amplified by PCR from genomic DNA.

Northern and Southern blot analysis: RNA extraction and separation followed essentially the described protocol (Current Protocols In Molecular Biology; Wiley). Hybridization of $^{32}$P-αATP labelled probes occurred over night in hybridization buffer (0.5M Sodium phosphate buffer pH 7.2 / 7% SDS / 1mM EDTA) at 65°C. For DNA extraction, 10ml yeast cells (grown to an OD$_{600}$=6) were collected, washed once and resuspended in Lysis buffer (2% Triton X-100 / 1% SDS / 100mM NaCl / 10mM Tris pH8 / 1mM EDTA). Genomic DNA was isolated by PCI extraction. Digestion of 10µg genomic DNA was done over-night with MfeI and MscI for CgSKN7 and BstXI for
Results

CgYAP1 (5U/µg DNA). The digests were separated on a 1% TAE agarose gel. After cross linking (50mJ), the radioactive labelled probes were hybridized over night in hybridization buffer at 65°C. Signals were visualized by autoradiography.

Catalase assay: Crude extracts were prepared by breakage of yeast cells with glass beads. Catalase activity was assayed spectrophotometrically at 240nm, protein concentrations were assayed at 280nm as described in (19).

Microscopy: GFP-fluorescence microscopy was performed as described previously (28). GFP/CFP was visualized in live cells without fixation. All cells were monitored using a Zeiss Axioplan 2 fluorescence microscope. Images were captured with a Spot Pursuit (Sony) CCD camera using Spotbasic software.

Macrophage cell culture: Primary bone marrow derived macrophages (BMDMs) were obtained from the femur bone marrow of 6 – 10 weeks old C57Bl/6 mice. Cells were cultivated in DMEM supplemented with 10% FCS in the presence of L cell-derived CSF-1 as described (4). Mice were housed under specific pathogen-free conditions. For infection assays, BMDMs were seeded at 5 x 10^5 cells per dish in 3,5-cm dishes containing medium without antibiotics. Log-phase C. glabrata cells were washed with PBS supplemented with 0.1% glucose) and added to macrophages in a 4:1 ratio and incubated at 37°C. After washing with PBS, cells were fixed with 2% formaldehyde for 5 minutes, and incubated in 1% triton for 1 minute the cover-slips were incubated with Phalloidin Texas-Red and fixed to slides with Mowiol. For CFU assays, BMDM were seeded at 2 x 10^5 cells per dish. Exponentially growing C. glabrata cells were washed with PBS supplemented with 0.1% glucose and added to macrophages in a 2:1 ratio and incubated at 37°C. At the indicated times deionised water was added to lyse macrophages. Cells were spread on YPD plates, incubated at 37°C and counted.

Chromatin immunoprecipitation assay: ChIP was performed essentially as described previously (2, 43). Briefly, 50 ml of C. glabrata culture (OD ~ 0.4) were
treated with 0.4mM hydrogen peroxide for times indicated, followed by crosslinking with 1% Formaldehyde for 10 minutes. Cell extracts were sonicated to reach a fragment resolution of approximately 500bp. For immunoprecipitation, a commercial Anti-HA monoclonal antibody (12CA5) was used. Precipitated DNA was analyzed by quantitative Real Time PCR (Eppendorf Mastercycler) with the following primer pairs: TRR2 (-639/-511) for CgTRR2 (CAGL0I01166g) and GPX2 (-787/-617) for CgGPX2 (CAGL0C01705g). As a negative control a centromeric region of Chromosome B was used (fwdCGAACTCATAACCAATAGCTCG/rev GAATCGTTGGGAAGTATATTCC).
Results:

*C. glabrata* reacts differently to various oxidative stress causing agents.

For the close *C. glabrata* relative *S. cerevisiae*, different responses and survival rates to specific oxidative stress causing agents such as menadione, hypochlorous acid or hydrogen peroxide have been found (23, 35, 40). We determined the role of the *C. glabrata* oxidative stress-associated transcription factors CgSkn7 and CgYap1 in the response against agents causing different oxidative stress types. Therefore, we generated strains lacking either CgSkn7 (CAGL0F09097g), CgYap1 (CAGL0H04631g) or both by homologous gene replacement with *ScURA3* and *ScHIS3* (Figure S1). We used gradient plates with concentration ranges of the ROS-generating agent menadione or with gradients of Reactive Oxygen/Nitrogen/Chloride Species (RCS = hypochlorite, RNS = peroxynitrite, ROS = hydrogen peroxide). The growth assays revealed that *C. glabrata* strains exhibit different sensitivities (Figure 1A). We observed that the loss of CgSkn7 had only minor effects on survival, whereas the lack of CgYap1 diminished growth significantly, dependent on the applied chemical agent. On plates containing menadione, we found only minimal growth differences between *C. glabrata* wild type, *skn7Δ* mutant cells and *yap1Δ* mutants. In contrast, we could not detect any varying sensitivities between these strains on plates containing hypochlorite. In addition, in the presence of peroxynitrite, which is generated in the phagosome by the reaction of nitric oxide with superoxide (16, 82), survival of *yap1Δ* and *skn7Δyap1Δ* mutants was severely diminished. We observed that, with an initial inoculum of 500 colony forming units (cfu), both *yap1Δ* and *skn7Δyap1Δ* mutants displayed decreased survival on gradient plates with only 0.4mM hydrogen peroxide (Figure 1A, lower left panel). The *skn7Δ* mutant showed sensitivity only at a high concentration of 10mM hydrogen peroxide. Similar to *S. cerevisiae* (35), we found that *C. glabrata* cells from over-night grown cultures which lacked glucose in the culture medium became increasingly resistant to hydrogen peroxide (Figure 1A, lower right panel). Stationary
cells displayed this phenotype on plates supplemented with 2% glucose or supplemented with 2% ethanol/1% glycerol, indicating that pre-starved cells retain their high resistance state sufficiently long even in the presence of glucose. Furthermore, the carbon source starvation induced oxidative stress resistance was also observed in the absence of CgYap1/CgSkn7 demonstrating a parallel signal contributing to oxidative stress resistance.

Further, *S. cerevisiae yap1Δ* mutants were hypersensitive to superoxide stress induced by treatment with 7-chlorotetrazolo(9)benzo(1)triazine (CTBT) (9). Therefore, we tested the susceptibility of *C. glabrata* strains lacking CgSkn7 and/or CgYap1 to CTBT using a zone inhibition assay (Figure 1B). In contrast to *S. cerevisiae*, the *C. glabrata yap1Δ* mutant strains did not display different susceptibilities. This result was fitting to observations reported recently (49) suggesting that the roles of Yap1 and CgYap1 have partly diverged. Taken together, we conclude that the functionality of CgYap1 is crucial for *C. glabrata* cells to overcome high loads of oxidative stress causing agents.

**The core oxidative stress response of *C. glabrata* is similar to *S. cerevisiae***.

To investigate the oxidative stress regulon of *C. glabrata*, the immediate transcriptional response to 0.4mM hydrogen peroxide was determined via microarray analysis (Figure 2). To avoid indirect transcriptional responses, the chosen treatment time was 20 minutes at 30°C. Transcript profiles were determined by hybridization to genome-wide *C. glabrata* microarrays. Expression data were filtered and averaged. The entire data set was analyzed for co-regulated genes by hierarchical clustering (20). Expression levels of stressed *C. glabrata skn7Δ* and *yap1Δ* mutants were compared directly against expression levels of the stressed wild type. These patterns were aligned to the *C. glabrata* wild type oxidative stress transcriptome.
To compare the *C. glabrata* expression pattern with *S. cerevisiae* data we used a similarity based annotation of orthologous genes (22) (http://cbi.labri.fr/Genolevures/). Orthologous genes, induced by oxidative stress in *S. cerevisiae* (26), are highlighted in violet. Further, expression data from *S. cerevisiae skn7Δ* and *yap1Δ* mutants exposed to 0.2mM H$_2$O$_2$, from two-dimensional gel electrophoresis data, was extracted for comparison (48) (Figure 1 highlighted in red). Subsets of genes dependent on CgYap1/CgSkn7 or both were classified into three groups. Group 1 comprised genes dependent on both CgSkn7 and CgYap1 and included many generic oxidative stress response genes (*CgTRR1/2, CgTRX2, CgTSA1/2, CgGPX2, CgCCP1* and *CgCTA1*) (33, 48). We designate this group of genes as "core oxidative stress response" (COR). Further, we found a group of genes, only dependent on CgYap1 (Figure 1 Group 3), including *CgGRE2, CgADH6* and *CgOYE2*, which have aldo-keto reductase and oxidoreductase activity in *S. cerevisiae* (11, 46, 63).

Furthermore, we discovered a group of 18 genes upregulated in the wild type during oxidative stress however, not dependent on Skn7 and Yap1 (Figure 1 Group 2). Group 2 contained genes associated to mitochondrial processes (*CgACP1, CgOPI3, CgHSP10* and *CgMRP10*) (36, 56, 71, 74) similar to *S. cerevisiae* (26). Two scenarios could explain their regulation. Either, transcription of these genes is independently activated by CgSkn7 or CgYap1, or their expression is dependent on other factors. Therefore, we determined the transcription pattern upon oxidative stress of the double mutant *skn7Δyap1Δ*. Indeed most genes of Group 2 were highly induced in the *skn7Δyap1Δ* double mutant (Figure 2). Therefore we suggest the involvement of other factors during the oxidative stress response.

Stationary *C. glabrata* cells displayed an increased resistance against oxidative stress. Thus, we compared the transcript pattern of oxidative stress and glucose starvation (70). Indeed, 26 genes of the oxidative stress regulon were found to be upregulated (Figure 2, right panel). This group also includes *CgCTA1, CgGPX2* and
CgTRX2, indicating an at least partially overlapping response. We suggest that *S. cerevisiae* and *C. glabrata* share a highly similar core oxidative stress response pattern, which is regulated by CgSkn7 and CgYap1.

**Conserved consensus sequences for CgSkn7 and CgYap1 are present in genes involved in oxidative stress response in *C. glabrata*.

The above analysis suggested that *C. glabrata* and *S. cerevisiae* share a common set of genes involved in the oxidative stress response. Further, we found a number of genes dependent on CgSkn7 and CgYap1. We performed an *in silico* analysis of the upstream regions in *C. glabrata* to identify putative *S. cerevisiae* Yap1 and Skn7 consensus sites in their promoters (Figure 3A). Consensus-like sequences shared by the CgSkn7 and CgYap1 regulated genes were found in the upstream regions (2kB) by a systematic search allowing one deviation from the known consensus sequences in *S. cerevisiae* (31, 62, 84). Exact Yap1 and Skn7 consensus sequences were counted in the upstream regions (< -1200bp) of Groups 1, 2 and 3 (Figure 3A, for a schematic view see Figure S2). We found a highly conserved Yap1 target sequence (TTASTMA) in the majority of identified CgYap1 dependent genes in *C. glabrata*. Skn7 is known to have a broader range of consensus sequences in *S. cerevisiae* (OSREs, Skn7 oxidative stress responsive elements) (30, 31); nevertheless we also identified conserved Skn7 target sequences (GNCNGSCS or GGCTGGC). Discovered consensus sequences from Group 1 and Group 3 are depicted in the form of sequence logos (Figure 3A, lower panel). We also found both, Yap1 and Skn7 consensus sites, but to a lesser extent in Group 2. Either CgSkn7 or CgYap1 can contribute partially to expression of these genes during oxidative stress beside other transcription factors, or those sites are not corresponding with the sites where CgSkn7 and CgYap1 bind in *C. glabrata*. Consistently, we discovered slightly altered Yap1-like and Skn7-like sites allowing one deviation at any position (see Figure S2, indicated as light blue and pink.
bars) in Groups 1, 2 and 3. Another explanation for the significant number of Yap1 consensus sites in Group 2 might be that other factors of the YAP family, which can potentially recognize the Yap1 consensus site in *S. cerevisiae* (13, 85), regulate transcription of those genes.

To correlate the putative CgYap1 and CgSkn7 binding sites with regulatory elements, we investigated the oxidative stress regulation of the Cg*CTA1* promoter. Therefore, we created a series of truncations of the catalase promoter and fused them to the catalase open reading frame. We used the catalase activity which is tightly coupled to its mRNA levels (our unpublished observations) as a convenient read out for the activity of the promoter fragments. We transformed the promoter deletion series into a *C. glabrata* cta1Δ mutant strain and tested activity of catalase in extracts upon induction by oxidative stress (Figure 3B). We found that promoter activity was significantly diminished in the construct containing up to -1000bp of the promoter and was abolished when truncated version with about 900bp. Interestingly, the perfect *S. cerevisiae* Yap1 consensus sites are located at positions -732bp and -1676bp (Figure 3B, lower panel), whereas we found a Yap1-like and a Skn7-like sequence indicated as light blue and pink bars within a -1100bp to -900bp. Therefore, we suggest that binding of both, CgYap1 and CgSkn7, is not restricted to known *S. cerevisiae* consensus sequences, but favors variants of these sites. However, the composition of the putative binding sites in the upstream regions of oxidative stress genes pointed to a predominantly CgYap1 controlled oxidative stress regulon.

**Expression of important key enzymes to overcome oxidative stress is dependent on CgSkn7 and CgYap1.**

To confirm the microarray results and to demonstrate the function of CgSkn7 and CgYap1 for oxidative stress regulated transcription, we measured the expression levels of several exemplary genes of Groups 1, 2 and 3 over several time points
following exposure to hydrogen peroxide (Figure 4). We chose \textit{CgCTA1}, \textit{CgTRR2}, \textit{CgGPX2} and \textit{CgTSA1} of Group 1 comprising CgSkn7 and CgYap1 dependent genes (see Figure 4A). Northern blot analysis showed rapid induction upon treatment with 0.4mM H$_2$O$_2$ in \textit{C. glabrata} wild type and in \textit{C. glabrata skn7}$\Delta$ and \textit{yap1}$\Delta$ mutant cells supplemented with \textit{CgSKN7} or \textit{CgYAP1} on a plasmid under their own promoters (pCgSkn7, pCgYap1). In \textit{C. glabrata} wild type cells, expression of \textit{CgTRR2} and \textit{CgTSA1} decreased after 20 minutes again, whereas expression of \textit{CgCTA1} and \textit{CgGPX2} was still induced after 40 minutes. In the absence of CgSkn7 and CgYap1, mRNA levels were severely reduced. However, stress-induced expression of \textit{CgTRR2} and \textit{CgGPX2} is not completely abolished in \textit{skn7}$\Delta$ mutant cells, suggesting either the parallel involvement of other factors or that CgYap1 can induce those genes to a certain extent alone. Further, we found that \textit{C. glabrata} strains lacking CgYap1 did not display a significantly increased susceptibility to superoxide anions (see Figure 1). Therefore we wanted to know whether expression of the superoxide dismutase \textit{CgSOD1} is affected in \textit{yap1}$\Delta$ or \textit{skn7}$\Delta$ mutant cells during hydrogen peroxide induced oxidative stress. We found that \textit{CgSOD1} was constitutively expressed, and expression was not impaired in mutant strains. We conclude that the superoxide dismutase \textit{CgSOD1} has a housekeeping function in \textit{C. glabrata}, which is not part of CgSkn7/CgYap1 mediated oxidative stress regulon.

Group 2 comprises genes regulated independently of CgSkn7 and CgYap1. Therefore we used the double mutant \textit{skn7}$\Delta$\textit{yap1}$\Delta$ supplemented with pCgSKN7, pCgYAP1 or pCgYAP1-CgSKN7 to perform northern blot analysis (Figure 4B). We chose \textit{CgHSP78} and \textit{CgZWF1} to measure expression levels. Expression of \textit{CgHSP78}, a mitochondrial matrix chaperone (50), occurred independently of CgSkn7/CgYap1, revealing the involvement of other transcription factors in the regulation of Group 2. In addition, we investigated expression pattern of \textit{CgZWF1}, encoding the glucose-6-phosphate dehydrogenase, which is also involved in adapting to oxidative stress in \textit{S.
cerevisiae (37). It was constitutively expressed due to its role as housekeeping gene (65). Interestingly, CgZWF1 maximum expression was dependent on the presence of either CgSkn7 or CgYap1 in C. glabrata. Both factors contributed independently of each other to full expression upon oxidative stress induction, which is consistent with a 550bp gap between putative consensus sites of CgSkn7 and CgYap1 in the corresponding upstream region. Microarray data predicted a group of genes to be solely dependent on CgYap1 (Group 3). We chose CgHSP31 to follow its expression pattern upon oxidative stress induction (78). Indeed, we observed a strict dependency on CgYap1. These data confirm a direct role for CgSkn7 and CgYap1 during induction of transcription by oxidative stress. Nevertheless, our analysis showed that other factors are involved in oxidative stress induced gene expression in C. glabrata.

Since nutrient starvation-associated genes are known to be also upregulated during phagocytosis (39), we investigated expression levels of oxidative stress genes during glucose depletion (Figure 4C). CgCTA1 and CgGPX2 displayed the same transcriptional pattern: after one hour, both occurred to be upregulated in a CgSkn7 and CgYap1 independent manner. Further, we wanted to know whether expression of the superoxide dismutases CgSOD1 and CgSOD2 is affected in yap1Δ mutant cells during glucose starvation (Figure 4C, lower panel). Indeed, elevated expression of CgSOD2 and CgSOD1 did not change in C. glabrata skn7Δ, yap1Δ and skn7Δyap1Δ mutants, pointing to a different regulation pattern of the C. glabrata orthologues of SOD1 and SOD2 than in S. cerevisiae (6). Members of the SOD family were also reported to be upregulated in C. albicans cells upon entering stationary phase (44).

Therefore, even genes belonging to core oxidative stress regulon might be expressed in vivo upon engulfment of C. glabrata cells due to the involvement of other transcription factors.
Catalase activity increases during glucose starvation and is not dependent on the presence of CgSkn7/CgYap1.

Since carbon starvation induced a large part of the oxidative stress regulon in C. glabrata, we measured catalase activity upon glucose depletion (Figure 5). Overnight cultures of C. glabrata wild type, skn7Δ, yap1Δ and skn7Δyap1Δ cells displayed high catalase activities; as a control we used the C. glabrata catalase cta1Δ knock out strain (Figure 5A). CgCta1 activity was not affected by the loss of CgSkn7 or CgYap1 CgSkn7. Further cells were diluted, grown to an OD$_{600}$ of 1 and shifted to medium containing 1.5% ethanol/0.5% glucose. After several hours we collected the cells measured the catalase activity. Again we observed an increased catalase activity, unaffected by the loss of CgSkn7/CgYap1. These results were in line with the expression levels observed in Figure 4, indicating the presence of different starvation-associated factors, which can induce genes belonging to the oxidative stress regulon.

Further, we wanted to uncover the part of the upstream region of CgCTA1 which is crucial for full induction during glucose starvation (Figure 5B). Truncated promoter versions expressing CgCTA1 (similar to Figure 3B), were used to measure remaining catalase activity. We discovered an area between -950bp and -900bp to be essential for full induction upon glucose depletion. Taken together, we assume that during glucose starvation, C. glabrata cells switch their transcriptional pattern including at least part of the oxidative stress regulon, which is then under control of other factors than CgSkn7/CgYap1.

CgSkn7 and CgYap1 are dependent on each other to induce gene expression of oxidative stress genes.

Genes comprising Group 1 are dependent on both CgSkn7 and CgYap1 for full expression. This cooperative activation of transcription might be dependent on, e.g. the
interaction with basal transcription machinery, or these factors might be interdependent for promoter recognition. Therefore we tested whether CgSkn7 and CgYap1 are able to bind independently to oxidative stress gene promoters. CgSkn7 was tagged with HA epitopes at the C-terminus, whereas CgYap1 was tagged at the N-terminus, to ensure their proper nuclear localization and promoter binding. The expression of the respective fusion proteins was checked on Western Blots (not shown). Both constructs were expressed from centromeric plasmids under the control of the native promoter. To elucidate the binding of these factors to the promoters, we performed chromatin immunoprecipitations. Precipitated DNA was analyzed by quantitative Real Time PCR measurements (Figure 6).

As target gene promoters we chose CgTRR2 and CgGPX2, since mRNA analysis showed that the stress-induced expression of both was not completely abolished in the C. glabrata skn7Δ mutant (Figure 4A). Therefore, we suggested that CgYap1 alone might be able to bind to these promoters to a certain extent. HA-CgYap1, expressed in the yap1Δ mutant, was detected at the CgTRR2 promoter within three minutes upon stress induction (Figure 6 upper left panel). After initial recruitment, HA-CgYap1 dissociated gradually from the CgTRR2 promoter (< 6 minutes). In the case of CgGPX2, binding was detected within 3 minutes upon stress induction, but the HA-CgYap1 signal declined more slowly (> 17 minutes, Figure 6 upper right panel). However, when HA-CgYap1 was expressed in absence of CgSkn7 in the skn7Δyap1Δ double mutant, we failed to detect recruitment to the CgTRR2 promoter (Figure 6 upper left panel), whereas we observed a slightly increased recruitment to the CgGPX2 promoter (Figure 6 upper right panel).

In the skn7Δ single mutant, CgSkn7-HA bound with similar kinetics as HA-CgYap1 within 3 minutes to the CgTRR2 promoter. Notably, CgSkn7-HA stayed slightly longer at the CgTRR2 promoter than HA-CgYap1 (> 9 minutes, Figure 6 lower left panel). The binding kinetics of CgSkn7-HA to the CgGRX2 promoter was the same as
for HA-CgYap1 (> 17 minutes, Figure 6 lower right panel). Finally, in the double mutant
\textit{skn7}\textsuperscript{Δ}yap1\textsuperscript{Δ}, CgSkn7-HA was not detectable in the promoter regions (Figure 6 lower
colors). Taken together, upon induction through oxidative stress, binding of CgSkn7 and CgYap1, to certain promoters, is strongly interdependent.

\textbf{CgSkn7 and CgYap1 are not required for survival in a primary mouse macrophage infection model.}

Since the oxidative burst is known to be part of the strategy of phagocytic cells
to erase engulfed cells, we analyzed the importance of the oxidative stress
transcription factors CgSkn7 and CgYap1 during phagocytosis. First, we investigated
their localization \textit{in vitro} and in our \textit{ex vivo} macrophage model. Both factors were fused
to fluorescent proteins. To preserve their nuclear localization signals CgSkn7 was C-
terminally fused to CFP, whereas CgYap1 was N-terminally fused to GFP. Both fusion
genes were expressed from pACT derived centromeric plasmids and driven by the
\textit{CgADH1} promoter.

The CgSkn7-CFP signal was detected in the nucleus in \textit{skn7}\textsuperscript{Δ} mutant cells in
rich medium (Figure 7A, upper panel). This observation is consistent with the
localisation data in \textit{S. cerevisiae} (69). The nuclear localisation pattern did not change
during phagocytosis by bone marrow derived primary mouse macrophages. In contrast,
GFP-CgYap1 was located in the cytoplasm in unstressed cells. Upon short exposure to
oxidative stress (0.4mM H\textsubscript{2}O\textsubscript{2} and 2mM As\textsubscript{2}Cl\textsubscript{3}), GFP-CgYap1 rapidly accumulated in
the nucleus (Figure 7A, lower panel). Hydrogen peroxide is produced during the
oxidative burst in a comparable concentration (21). Arsenic is known to induce a fast
oxidative stress response (92). In addition, within the first hour upon macrophage
engulfment, cells with nuclear GFP-CgYap1 were visible. However, we found that the
nuclear accumulation occurred only transiently in the early phase of phagocytosis (Roetzer et al., submitted revised). Nevertheless, *C. glabrata* cells seemed to experience oxidative stress inside the phagosome of macrophages.

To verify if CgSkn7 and CgYap1 are required for prolonged survival upon phagocytosis, we infected macrophages with *C. glabrata skn7Δyap1Δ* double mutant cells carrying either the plasmid pCgSKN7-CgYAP1 or the empty vector (Figure 7B). *C. glabrata* cells were added to macrophages in a 2:1 ratio. After 24 hours and 48 hours, engulfed *C. glabrata* cells were recovered on YPD plates. However, the double mutant *skn7Δyap1Δ* complemented with pCgSKN7-CgYAP1 displayed the same phenotype as *skn7Δyap1Δ* carrying the empty vector. Therefore, we suggest that oxidative stress is efficiently alleviated during phagocytosis of *C. glabrata* and that CgYap1 and CgSkn7 function for induction of the oxidative stress defence response is complemented by independent other environmental cues.
Discussion:

Upon phagocytosis, microbes need to counteract reactive oxygen and nitrogen species produced through the oxidative burst inside the phagosome of phagocytic cells (3, 7, 75). How does the fungal pathogen *C. glabrata* change its oxidative stress-associated transcriptional pattern to sustain a massive increase of ROS and RNS upon internalization? We explored here the oxidative stress regulon of *C. glabrata*, and subsequently analysed the contribution of the two prominent transcription factors CgSkn7 and CgYap1. In this study, we defined a *C. glabrata* core set of the oxidative stress response (COR), and we showed that CgSkn7 and CgYap1 are interdependent for regulation of at least some of these genes, indicating that the interaction is conserved between *C. glabrata* and *S. cerevisiae* (31, 48). However, we found that, in a primary mouse macrophage model, the loss of CgSkn7 and CgYap1 had no impact on the survival rate compared to the wild type. This suggests that the conserved regulatory system consisting of Yap1 and Skn7 plays a dispensable role during acute phagocytosis conditions. Strikingly, a large set of oxidative stress-associated genes was also upregulated during carbon starvation, suggesting that carbon source starvation provides a parallel pathway for induction of oxidative stress response genes.

*The *C. glabrata* oxidative stress response is similar to *S. cerevisiae*. In *S. cerevisiae*, at least 71 genes displayed increased expression within minutes upon exposure to hydrogen peroxide (27). Furthermore, Yap1 controlled at least 32 genes of the identified oxidative stress stimulon in *S. cerevisiae* (33, 48). 15 of these proteins required both Skn7 and Yap1 for induction. Two distinct Yap1 regulons were defined in *S. cerevisiae*, one covering the oxidative stress response, the second involved in the metabolic pathways regenerating the main cellular reducing power, GSH and NADPH (48). In *C. glabrata*, 38 genes were upregulated more than two-fold during oxidative stress. Among the oxidative stress-induced regulon we found genes to be dependent on both CgSkn7 and CgYap1 or on CgYap1 alone, respectively. In addition, a set of genes appeared to be regulated independently of CgSkn7/CgYap1. Induction of the*
Results

core response to oxidative stress (COR), which included thioredoxin peroxidases (CgTsa1, CgTsa2), thioredoxin reductases (CgTrr1, CgTrr2), the thioredoxin cofactor CgTrx2, the glutathione peroxidase CgGpx2, the mitochondrial cytochrome-c peroxidase CgCcp1 and the catalase CgCta1, was dependent on the presence of both, CgSkn7 and CgYap1. A second CgYap1-associated set of genes was encoding proteins displaying aldo-keto reductase and oxidoreductase activity (CgADH6, CgGRE2, CgSCS7 and CgOYE2). Therefore, we conclude that Skn7 and Yap1 have conserved functions in the class of Saccharomycetes.

*CgSkn7 and CgYap1 cooperate for promoter binding to induce the oxidative stress regulon.* In S. cerevisiae, a direct interaction between Skn7 and Yap1 seemed to be crucial for induction of oxidative stress response genes (31, 32). The mutated receiver domain of Skn7 affected the association of Yap1 and Skn7 to upstream regions of oxidative stress genes (31, 60). However, the precise mechanism by which these two factors co-activate oxidative stress response was not clear yet. To assess the interaction of CgSkn7 and CgYap1 in C. glabrata, we tested whether binding of CgSkn7 and CgYap1 to oxidative stress gene promoters in C. glabrata is dependent on the presence of each other. Chromatin immunoprecipitation data showed that a strict interdependence occurs at the level of promoter recognition. In the presence of CgYap1, CgSkn7 was able to bind the upstream sequences of CgTRR2 and CgGPX2, whereas in the absence of CgYap1, CgSkn7 could not bind to these promoters. Similarly, CgYap1 bound only in the presence of CgSkn7 to the tested upstream regions in a significant manner. This kind of interdependence was also observed in S. cerevisiae: an electrophoretic mobility shift assay demonstrated the presence of a Skn7-Yap1 complex at the promoter of TSA1 (48). Here, we showed the first time, that, in C. glabrata, CgSkn7 needs CgYap1 and vice versa to bind to the upstream region of core oxidative stress genes. Recruitment to promoter occurred immediately upon stress induction (< 3 minutes). Both factors were detectable at the promoter for the same period of time (CgTRR2 < 10 minutes, CgGPX2 > 10 minutes). Although CgYap1
displayed minimal binding properties in \textit{skn7}Δ mutant cells, we suggest, that remaining \textit{CgGPX2} expression in \textit{skn7}Δ mutants is carried out by other components. This is consistent to the regulation found in \textit{S. cerevisiae}, where \textit{GPX2} is also induced in a calcineurin/Crz1-dependent manner during oxidative stress (83).

Interestingly, we found a significant number of Yap1 consensus sites throughout the oxidative stress regulon, including the set of genes, which appeared to be upregulated even in the absence of CgYap1. To test binding properties of upstream regions we measured catalase activity of \textit{CgCTA1} expressed by truncated promoter versions. The identified upstream region, which was crucial for full catalase activity, did not comprise \textit{S. cerevisiae} Skn7 and Yap1 consensus sites. Present \textit{S. cerevisiae} Skn7- and Yap1-like sequences pointed to an altered recognition pattern of CgYap1.

\textit{C. glabrata yap1Δ} cells displayed increased susceptibility against hydrogen peroxide and peroxynitrite. However, in contrast to \textit{S. cerevisiae} (6), the loss of CgYap1 appeared to have only minimal effects on susceptibility to superoxide anions: both menadione and CTBT induced a similar stress response pattern (our unpublished results). Consistently, we found that the superoxide dismutase gene \textit{CgSOD1} was expressed independently of CgSkn7/CgYap1. This is in line with results found by Lelandais \textit{et al}, that CgYap1 has a distinct recognition pattern in \textit{C. glabrata} during benomyl response in comparison to Yap1 in \textit{S. cerevisiae} (49). Further, we found that the superoxide dismutase CgSod1 is constitutively expressed in \textit{C. glabrata} in a CgSkn7/CgYap1 independent manner. Therefore we conclude that the specific responses dependent on CgYap1 have further evolved in \textit{C. glabrata} after separation from \textit{S. cerevisiae}.

\textit{The oxidative stress response genes are under dual control}. 26 genes of the oxidative stress regulon were found to be upregulated during glucose starvation (70). This group also includes \textit{CgCTA1}, \textit{CgGPX2} and \textit{CgTRX2}, which were expressed independently of CgSkn7/CgYap1 upon glucose depletion. In addition, \textit{CgSOD1/2}
Results

appeared to be upregulated during starvation. It has been reported, that *S. cerevisiae*
cells grown to stationary phase exhibit increased resistance against menadione and
hydrogen peroxide (35). Contrary, we could not find a *C. glabrata*-like expression
pattern in *S. cerevisiae* during the diauxic shift (26). Notably, in a *C. albicans* batch
culture, stationary phase cells also exhibited induction of proteins involved in oxidative
stress response (44). Adaptation to these environmental changes requires a
simultaneous upregulation of a set of genes, beneficial for both, oxidative stress and
glucose starvation. Interestingly, nutrient limitation can also increase resistance to acid
shock and oxidative stress in bacteria, such as *Staphylococcus aureus* and *Salmonella
typhimurium* (24, 87). Therefore we suggest the existence of dual control mechanisms
in *C. glabrata*, which could be important inside the phagosome due to acute oxidative
stress and prolonged starvation.

*The impact of the oxidative stress regulon on virulence remains contradictory.*
Since CgSkn7 and CgYap1 regulated the core oxidative stress response, the loss of
these factors might attenuate virulence. However, the *C. glabrata skn7Δyap1Δ* double
mutant did not display a diminished survival rate compared to the wild type situation in
our primary mouse macrophage model. Accordingly, we observed that low numbers of
starved *skn7Δyap1Δ* cells can overcome 0.4mM H$_2$O$_2$, the concentration that *C.
glabrata* cells experience inside the mammalian host (15, 21). Microarray analysis of *C.
glabrata* cells engulfed by macrophages, revealed a rather inconsistent oxidative stress
response: *CgCTA1, CgGDB1, CgHEM15* and *CgGLO1* displayed an increased
expression, whereas *CgTTR1/2* and *CgTSA1/2* were downregulated during
phagocytosis (39). Notably, *CgTRR1/2* and *CgTSA1/2* were also strictly downregulated
during glucose depletion in our microarray experiments.

This might be explained by at least two models. One assumes a mild oxidative
stress load for *C. glabrata* inside the phagosome of phagocytic cells and thus the
induced expression of the respective target genes is not immediately necessary.
Wellington et al have shown recently, that *C. glabrata* cells can suppress ROS production upon internalization by macrophages (88). Alternatively, the crucial genes might be induced by parallel pathways (Figure 8). This latter idea is supported by the observed upregulation of oxidative stress-associated genes by environmental conditions which *C. glabrata* might encounter as well inside a phagocytic vesicle. This might be primarily starvation for nutrients and especially carbon source (39, 54). This model implies an adaptation of stress gene regulation to the prevailing situation in the host. Both scenarios suggest that *C. glabrata* can efficiently counteract the oxidative burst.
Table 1

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### Supplemental Table S1

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Results

Figure legends:

Figure 1. *C. glabrata* responds to oxidative stress in a CgYap1 and partially CgSkn7 dependent manner. (A) *C. glabrata* resistance against oxidative stress causing agents. *C. glabrata* wild type, skn7Δ, yap1Δ and skn7Δyap1Δ mutant cells were grown to an OD_{600} of 1 in YPD. 5x10^2 cells were dropped on gradient plates containing changing concentrations of menadione, hypochlorite, peroxynitrite and hydrogen peroxide (see Material and Methods). Plates were incubated at 37°C overnight. Further, saturated over-night cultures were diluted and 5x10^2 cells were dropped on gradient plates containing 0.4mM hydrogen peroxide (lower right panel). Plates were incubated at 37°C for 48 hours. (B) Susceptibility of *Candida glabrata* strains to CTBT assessed using a zone inhibition assay (10 µg CTBT/disc) Approximately 7.5x10^6 cells were plated onto YPD (rich medium with glucose, A), YPGE (rich medium with glycerol and ethanol, B), YNB (minimal medium with glucose, C) a YNGE (minimal medium with glycerol and ethanol, D) media.

Figure 2. Comparison of genome-wide expression levels in response to oxidative stress in *C. glabrata*. Transcript profiles were determined by hybridization to genome-wide *C. glabrata* microarrays. The sets represent average inductions of replicate profiles of *C. glabrata* wild type strain (4166 ORFs), and comparison of wild type strain versus *C. glabrata* skn7Δ and yap1Δ mutant strains, and the skn7Δyap1Δ double mutant strain alone after treatment with 0.4mM H_2O_2. All treatments were done at 30°C for 20 minutes. Genes involved in oxidative stress response were clustered after specific selection (>2-fold). Gene names correspond to *C. glabrata* systematic ORF designations and their corresponding *S. cerevisiae* orthologues. Major cluster are labeled corresponding to dependence on Skn7 and Yap1: Group 1 represents genes dependent on CgSkn7 and CgYap1, Group 2 represents genes expressed
independently of CgSkn7 or CgYap1, Group 3 represents genes dependent on CgYap1. Genes found to be upregulated in *S. cerevisiae* upon oxidative stress are highlighted in violet. Genes discovered to be dependent on Skn7 and Yap1 or Yap1 alone in *S. cerevisiae* are highlighted in red. Induction profile of corresponding genes in the *C. glabrata* wild type strain during glucose depletion is included (right lane).

**Figure 3.** CgSkn7 and CgYap1 dependent genes possess highly conserved consensus sequences. (A) Percentage of identified *S. cerevisiae* Skn7 and Yap1 consensus sites present within -1200bp upstream regions of *C. glabrata* genes of Groups 1, 2 and 3. For a schematic view of promoters of Group 1 and 3 see Figure S2. Verified logo representation consensus sequence patterns among the CgSkn7 and/or CgYap1 regulated genes are shown. (B) To measure catalase activity, *C. glabrata* wild type and *cta1*Δ cells bearing plasmids with *CgCTA1* including various truncated promoter versions were incubated in YPD with 0.4mM H₂O₂ for 45 minutes. Crude cell extracts were prepared and then assayed for catalase activity. Schematic representation of -2000bp upstream region of *CgCTA1* displaying all identified *S. cerevisiae* Skn7-like and Yap1-like sequences and Yap1 consensus sites (lower panel).

**Figure 4.** CgSkn7 and CgYap1 are required for rapid induction of transcription after oxidative stress. (A) Northern blot analysis of *CgCTA1, CgTRR2, CgGPX2, CgTSA1, CgSOD1* and *CgSOD2* transcripts during 0.4mM H₂O₂ induced oxidative stress. *C. glabrata* wild type, *C. glabrata skn7Δ* transformed with pCgSKN7 and *C. glabrata yap1Δ* transformed with pCgYAP1 were grown to exponential phase before 0.4mM H₂O₂ was added. Samples for RNA extraction were taken at indicated time points. mRNA levels were visualized by hybridization of radio-labeled probes and
Results

autoradiography. *CgACT1* mRNA was used as loading control. (B) Northern blot analysis of *CgHSP78, CgZWF1* and *CgHSP31* transcripts during 0.4mM H$_2$O$_2$ induced oxidative stress. *C. glabrata* wild type and *C. glabrata skn7Δyap1Δ* transformed with empty vector, p*CgSKN7*, p*CgYAP1* or p*CgYAP1-CgSKN7* were grown to exponential phase and treated as described in (A). (C) Northern blot analysis of *CgCTA1, CgGPX2, CgSOD1* and *CgSOD2* transcripts during glucose starvation. *C. glabrata* wild type and *C. glabrata skn7Δyap1Δ* transformed with empty vector, p*CgSKN7*, p*CgYAP1* or p*CgYAP1-CgSKN7* were grown to exponential phase, washed twice and incubated in rich medium lacking glucose. Samples for RNA extraction were taken at indicated time points. mRNA levels were visualized by hybridization of radio-labeled probes and autoradiography.

**Figure 5.** During glucose starvation catalase *CgCta1* is induced in a *CgSkn7/CgYap1* independent manner. (A) To measure catalase activity, *C. glabrata* wild type, *cta1Δ, skn7Δ, yap1Δ* and *skn7Δyap1Δ* cells the plasmid p*CgC-CgCTA1* were grown over-night, dilute to an OD = 0.1, grown to exponential phase (OD = 1) and shifted to medium containing 1.5% ethanol/0.5% glucose for 4 hours. Crude cell extracts were prepared and then assayed for catalase activity. (B) *C. glabrata* wild type and *cta1Δ* cells bearing plasmids with *CgCTA1* including various truncated promoter versions were incubated in YP plus 1.5% ethanol/0.5% glucose for five hours. Crude cell extracts were used for catalase activity measurements.

**Figure 6.** *CgSkn7* and *CgYap1* are interdependent for an binding to oxidative stress promoters. *C. glabrata skn7Δ* and *skn7Δyap1Δ* mutant strains transformed with p*CgSKN7-HA* or p*HA-CgYAP1* were grown to early log phase (OD ~ 0.4) and treated with 0.4mM H$_2$O$_2$ for times indicated, followed by crosslinking with 1% Formaldehyde for 10 minutes. Cell extracts were sonicated to reach a fragment resolution of ~800bp.
Results

For immunoprecipitation, an Anti-HA monoclonal antibody (12CA5) was used. Precipitated DNA was analyzed by Quantitative Real Time PCR.

**Figure 7. C. glabrata skn7Δyap1Δ double mutant display a similar survival rate compared to the wild type.** (A) C. glabrata skn7Δ and yap1Δ mutant cells transformed with pCgADH1-CgSKN7-CFP or pCgADH1-GFP-CgYAP1 were grown in synthetic medium (left panels). Cells were stressed by addition of 0.4mM H$_2$O$_2$ or 2mM As$_2$Cl$_3$ for 10 minutes. CgSkn7-CFP and GFP-CgYap1 visualized by fluorescence microscopy under phagocytosis conditions (right panels). Cells were washed in PBS 0.1% Glucose and added to macrophages in a 4:1 ratio and incubated at 37°C for 1 hour. Samples were fixed and stained with in Phalloidin Texas-Red. (B) Exponentially growing C. glabrata skn7Δyap1Δ double mutant cells complemented with either the empty vector or pCgYAP1-CgSKN7 were washed with PBS supplemented with 0.1% glucose and added to macrophages in a 2:1 ratio and incubated at 37°C for 24 hours and 48 hours. The viability of the engulfed cells was assessed by liberating them by hypotonic lysis of the macrophages and quantification of colony formation by plating on rich medium. Assays were done in four independent experiments, three measurements each.

**Figure 8. Model for the oxidative stress response regulon mediated by CgSkn7 and CgYap1 in C. glabrata.** Both reactive oxygen and nitrogen species induce a similar stress regulon. One group of genes gets induced independently of CgSkn7 and CgYap1. Carbon starvation also leads to upregulation of this regulon. In addition, genes belonging to the SOD family are increasingly expressed during carbon starvation in C. glabrata. CgSOD1 appeared to be constitutively expressed in a CgSkn7/CgYap1 independent manner in C. glabrata.
Supplementary Figures:

Figure S1. Southern blot analysis of *C. glabrata* skn7Δ, yap1Δ, and skn7Δyap1Δ deletion strains. Both CgSKN7 and CgYAP1 were replaced by ScURA3. CgSKN7 was replaced by ScHIS3 in the *C. glabrata* double mutant skn7Δyap1Δ. Amplified and labelled probes were either binding 5´ or 3´ upstream of ORFs. Chromosomal restriction enzyme locations are indicated. Chromosomal DNA derived from *C. glabrata* skn7Δ digested with *Mfe*I and *C. glabrata* yap1Δ digested with *Bst*XI resulted in longer fragments. Digestion of chromosomal DNA from the *C. glabrata* skn7Δyap1Δ double mutant strain with *Msc*I led to shorter fragments, since CgSKN7 contains no *Msc*I site.

Figure S2. CgSkn7 and CgYap1 dependent genes share conserved *S. cerevisiae* consensus sites and *S. cerevisiae* Skn7-/Yap1-like sequences. Schematic illustration of 2kb upstream promoter regions of genes with discovered consensus-like sequences. Dark blue bars indicate CgYap1 consensus sites, red bars are CgSkn7 sites. Light blue bars indicate CgYap1-like sites, whereas pink bars mark CgSkn7-like sites, respectively.


Results


Transcriptional activation of metalloid tolerance genes in Saccharomyces cerevisiae requires the AP-1-like proteins Yap1p and Yap8p. Mol Biol Cell 15:2049-2060.
Figure 1

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Legend:
- A: 5x10^2 colonies
- B: 0.4mM H\(_2\)O\(_2\), 10mM H\(_2\)O\(_2\), O/N 0.4mM H\(_2\)O\(_2\)
Figure 2

wt wt (glu starv) wt/skn7 skn7∆ yap1∆ wt/yap1∆ skn7∆ yap1∆ Wt (glu starv)

>2X induced >2X repressed
Figure 3

A

Identified Consensus Sites

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<th>Group 3 (n=8)</th>
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- Number of genes in group 1-3
- Yap1-consensus within -1200bp
- Skn7-consensus within -1200bp

Yap1 consensus sequences

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B

Catalase U/mg/min

- 0.4mM H_2O_2
- No Stress

C. glabrata cta1Δ + CgCTA1

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CAGL0K10868g

- Yap1-consensus
- Yap1-like
- Skn7-like
### Figure 4

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0.4mM H$_2$O$_2$

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0.4mM H$_2$O$_2$

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glucose starvation

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*Note: The images show gel electrophoresis results for different conditions and treatments with annotations indicating the genes of interest.*
Figure 5

A

![Bar chart showing catalase activity for different strains: wt, cta1Δ, skn7Δ, yap1Δ, and skn7Δyap1Δ. The bars are colored according to conditions: o/n, no stress, and glucose starvation.]

B

![Bar chart showing catalase activity for different C. glabrata cta1Δ + CgCTA1 constructs: pCgC1500-CgCTA1, pCgC1200-CgCTA1, pCgC1100-CgCTA1, pCgC1000-CgCTA1, pCgC950-CgCTA1, pCgC925-CgCTA1, and pCgC900-CgCTA1. The bars are colored according to conditions: no stress and glucose starvation.]

C. glabrata cta1Δ + CgCTA1

-1500 bp
-1200 bp
-1050 bp
-1000 bp
-950 bp
-925 bp
-900 bp

pCgC1500-CgCTA1
pCgC1200-CgCTA1
pCgC1100-CgCTA1
pCgC1000-CgCTA1
pCgC950-CgCTA1
pCgC925-CgCTA1
pCgC900-CgCTA1
Figure 6

**CgYap1HA:**
- no tag
- yap1Δ + CgYap1HA
- skn7Δyap1Δ + CgYap1HA

**CgSkn7HA:**
- no tag
- skn7Δ + CgSkn7HA
- skn7Δyap1Δ + CgSkn7HA

**0.4mM H₂O₂ Treatment**

**CgTRR2 Promoter**

**CgGPX2 Promoter**

**CgSkn7HA**

**0.4mM H₂O₂ Treatment**

**CgTRR2 Promoter**

**CgGPX2 Promoter**
Figure 7

A

C. glabrata skn7Δ + CgSKN7-CFP

no stress
BF CFP

1h
BF CFP / Texas-Red

C. glabrata skn7Δ + CgSKN7-CFP

no stress
BF CFP / Texas-Red

C. glabrata yap1Δ + GFP-CgYAP1

no stress 0.4mM H2O2 2mM AsCl3
BF GFP

1h
BF GFP / Texas-Red

C. glabrata yap1Δ + GFP-CgYAP1

no stress 0.4mM H2O2 2mM AsCl3
BF GFP

1h
BF GFP / Texas-Red

B

% of cfu recovery of engulfed C. glabrata cells

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Legend:
- BF: Bright Field
- CFP: Cyan Fluorescent Protein
- GFP: Green Fluorescent Protein
- Texas-Red: Texas Red fluorophore
Figure 8

ROS/RNS inducible genes

ROS

RNS

CgSkn7

CgYap1

CgYap1

CgSOD1

CgSOD2

CgCTA1

CgTRR1

CgTSA1

CgHSP78

CgGCY1

CgZWF1

CgHSP31

CgGLO1

CgADH6

Y

Carbon Starvation
Figure S1

wt sknΔ

wt yapΔ

sknΔ

wt yapΔ

MfeI

probe

CgSKN7

Cagl0F

ScURA3

MfeI

2.5kb

2.3kb

MfeI

BstXI

BstXI

BstXI

MscI

MscI

probe

CgYAP1

Cagl0H

ScURA3

MscI

2.2kb

2.5kb

4.9kb

6.0kb

2.5kb
Major results obtained:

- The environmental stress response (ESR) pattern of *C. glabrata* is orthologous to *S. cerevisiae*.
  - *CgMsn2* and *CgMsn4* were identified as orthologous transcription factors and regulate at least part of the CgESR.

- The oxidative stress response regulon of *C. glabrata* is similar to *S. cerevisiae*, and gets partially induced during starvation conditions.
  - *CgYap1* and *CgSkn7* control the core response to oxidative stress, but are dispensable for prolonged survival in macrophages.

- Phagocytosis of *C. glabrata* cells leads to a switch to alternative carbon catabolism and induces autophagic processes.
  - *CgAtg11*, a protein essential for pexophagy, and *CgAtg17*, which is essential for non-selective autophagy, are crucial for survival of engulfed cells.
3. General Discussion:

*Candida glabrata* – a *S. cerevisiae*-like pathogen?

The aim of this work was to investigate the stress response of the human fungal pathogen *Candida glabrata*. *C. glabrata* is phylogenetically related to *S. cerevisiae* and displays a high degree of gene synteny but it is adapted to a mammalian host environment. This is reflected by adhesin-mediated adherence to surfaces and the absence of certain biosynthetic pathways and different physiology, for example an optimal growth temperature of 37°C [1].

In *S. cerevisiae*, the general or environmental stress response (ESR) comprises the up- and downregulation of many genes. In this work I explored the characteristics of related responses in *C. glabrata*. The identified environmental stress response pattern in *C. glabrata* (CgESR) underlined the close relation of *C. glabrata* to *S. cerevisiae*. In addition CgMsn2, the orthologue of the yeast transcription factor Msn2, which gets activated during various stress conditions, plays also an important role in the general stress response of *C. glabrata*. The environmental stress response seems to be highly conserved among the class of *Saccharomycetes*.

Furthermore, I focused especially on the oxidative stress response of *C. glabrata*, since the oxidative burst executed by phagocytic cells is one of the main chemical weapons against engulfed foreign cells. Similar to the environmental stress response, I observed that the oxidative stress response is highly conserved between *S. cerevisiae* and *C. glabrata* including the orthologues of the crucial transcription factors Skn7 and Yap1. Interestingly, many *C. glabrata* oxidative stress-associated genes were also upregulated during glucose starvation. To assess the importance of these stress response factors as virulence determinants, I used an *ex vivo* primary mouse macrophage infection model for *C. glabrata*. To my surprise, CgSkn7 and CgYap1 did not contribute to survival during phagocytosis. Since *C. glabrata* has to counteract the oxidative burst, oxidative stress-associated genes might be upregulated by starvation conditions which *C. glabrata* cells encounter inside of phagocytes.

The third part of this thesis dealt with the situation of fungal cells inside the phagosome. Similar to *S. cerevisiae*, *C. glabrata* is unable to form true hyphae. Pseudohyphae may facilitate the invading of tissues. We could not observe such a morphologic switch to promote the escape in the engulfed situation as do hyphae for *Candida albicans*, another opportunistic human fungal pathogen [2]. Therefore, *C.
*glabrata* developed other strategies to survive during engulfment. Here I demonstrated that engulfed cells are forced to break down internal resources in order to prolong their survival upon phagocytosis. One crucial process seems to be autophagy, the engulfment and degradation of large molecules and organelles. I found that factors involved in selective autophagy (CgAtg11) as well as in non-selective autophagic processes (CgAtg17) contribute to sustained survival of *C. glabrata* inside the phagosome.

In the following, I will summarize the discovered characteristics of *C. glabrata* and discuss how these findings make a contribution to our picture how a fungal opportunistic pathogen is adapted to a host environment.

**Conserved features of the environmental stress response (ESR) in *C. glabrata* versus *S. cerevisiae*.**

At first, I focused on the analysis of the environmental stress response of *C. glabrata*. Proliferation of cells depends on beneficial extracellular conditions. Rapid changes in the external environment always affect the internal milieu. One mechanism *S. cerevisiae* cells use to counteract effects of environmental variation is to initiate a broad gene expression program, referred to as the environmental stress response (ESR). It comprises ~900 genes, whose expression is similarly altered to diverse types of stress [3]. *C. glabrata* as a human commensal and occasional pathogen exists in a radically different environment compared to *S. cerevisiae* [4]. Mammalian mucosal areas entail nutrient shortage, osmotic changes, presence of other microorganisms and protective secretory mechanisms of the host [5-9]. Adaptation to this environment does most likely require adapted stress response patterns compared to those *S. cerevisiae* is exposed to.

Therefore, the genome wide environmental stress response (ESR) expression profile of *Candida glabrata* was determined and subsequently compared to expression profiles of baker’s yeast. In addition, known stress response patterns of the fission yeast *Schizosaccharomyces pombe* and the opportunistic fungal pathogen *Candida albicans* were included in this comparison. Strikingly, global transcript analysis of *C. glabrata* cells exposed to the generic stress types carbon starvation, heat, osmotic and oxidative stress revealed a transcription pattern highly related to *S. cerevisiae* and *S. pombe* [10, 11]. In contrast, a similar analysis of stress responses of *C. albicans* pointed to a different adaptation program [10, 12, 13]. *C. albicans* lacks a general
stressed response exhibited by *S. cerevisiae*. For example, in contrast to *S. cerevisiae* and *C. glabrata*, the use of alternative carbon sources like glycogen and trehalose in response to mild stresses was not observed in *C. albicans*. However, the patterns of induced genes during specific stresses in *C. albicans* are similar to the specific stress patterns in *S. cerevisiae* [12]. Beside stress specific transcriptional responses, a niche-specific core transcriptional response to stress has been identified in *C. albicans* [13]. Despite the fact that the fungal pathogens *C. glabrata* and *C. albicans* occupy similar host niches, the overall environmental stress response of *C. glabrata* shares more similarities with the ESR of its non-pathogenic, phylogenetically close relative *S. cerevisiae*.

Since environmental stress response patterns exist, the question arises, how are the main generic stress response pathways regulated? In all organisms, various signaling pathways sense and transmit environmental alterations. Among those, the best characterized are the highly conserved MAP kinase (MAPK) pathways [14-18]. For example, in mammalian cells, two MAPK pathways exist, that respond to stress conditions and result in the activation of the SAPKs/JNKs (Stress-activated protein kinases/Jun N-terminal kinases) and the p38 kinases [18, 19]. Similar stress-activated MAPK pathways have been characterized in fungi [20-22]. In *S. cerevisiae*, one important MAP kinase is Hog1, a key regulator of the osmoregulatory signal transduction cascade, which mediates the upregulation of ~600 genes by phosphorylating several transcription factors [23-25]. The Hog1 pathway also responds to heat, oxidative and specific weak acid stress in *S. cerevisiae* [26-29]. In *S. pombe*, the MAP kinase and fission yeast homologue of Hog1, Sty1 is responsible for transducing signals from various stimuli including oxidative, osmotic and heat stress [30-32]. Further, in *C. albicans*, CaHog1 mediates the core stress response to a variety of environmental conditions [20, 33]. In *C. glabrata*, it has been found that the HOG signaling pathway is highly similar to *S. cerevisiae*; in addition, this pathway mediates the response to a broader spectrum of stress conditions including different weak acids in *C. glabrata* [34].

However, in *S. cerevisiae*, many ScESR genes are not solely dependent on Hog1 but rely on induction by the general stress factors Msn2 and Msn4 [3]. Both transcription factors (Msn2 and Msn4) are activated by a variety of stress conditions and changing nutrients, such as the depletion of the preferred carbon source glucose [35-40]. In fact, Hog1 also regulates Msn2/4 during osmotic stress in *S. cerevisiae* [41]. In contrast, the *C. albicans* Msn2-like proteins (CaMsn4 and Mln1) do not play any role in stress response [42]. We identified putative Msn2/Msn4 orthologues in *C. glabrata*.
Therefore, we wanted to know whether *S. cerevisiae* has developed a unique stress response mechanism involving Msn2 and Msn4, and whether the *C. glabrata* stress response is governed by a *S. cerevisiae* or a *C. albicans*-like mechanism.

**How does CgMsn2 contribute to the general stress response (CgESR) in *C. glabrata***?

In *C. glabrata*, transcript profiles of CgMsn2 and CgMsn4 double mutants and CgMsn2 overexpression strains confirmed the important role of CgMsn2 and CgMsn4 as major regulators of a part of the CgESR. Genes functionally connected to stress response, such as *CgHSP12, CgHSP42, CgDDR48, CgGPH1, CgGDB1* or *CgPGM2* displayed a CgMsn2/4-associated upregulation. In addition, CgMsn2 is required for full resistance against severe osmotic stress and rapid and full induction of trehalose synthesis genes (*TPS1, TPS2*), indicating a strikingly similar situation as in *S. cerevisiae* [43, 44]. Further, similar to baker’s yeast [45], constitutive nuclear localisation of CgMsn2 seems to be detrimental for *C. glabrata*. Resulting constitutive expression of a large set of genes leads to an unbearable waste of energy. In *C. glabrata*, CgMsn2 is beneficial under extreme stress conditions, such as high osmolarity, indicating a condition-specific role in providing future stress protection similar to Msn2 in *S. cerevisiae* [44]. Interestingly, CgMsn2 does not seem to be involved in weak acid stress response in contrast to the situation in *S. cerevisiae* [3, 39, 46], indicating the absence of a CgMsn2-associated weak acid stress pathway, since *C. glabrata* does not display higher resistance to weak acids [34]. Instead CgHog1 seems to be responsible for weak acid driven signal transduction in *C. glabrata* [34]. Taken together, the orthologues CgMsn2/CgMsn4 play a relevant role for induction of the CgESR in *C. glabrata* similar to Msn2/4 in *S. cerevisiae*. From a phylogenetic point of view, both *C. glabrata* and *S. cerevisiae* belong to the same clade among the class of *Saccharomycetes*. Therefore, I explored, whether the stress-specific role of Msn2 and Msn4 is linked to this clade.

**Can the stress response-associated role of Msn2 and Msn4 be found throughout the class of *Saccharomycetes***? The most significant similarity between putative ScMsn2 orthologues apart from the DNA binding domain is a region designated as Msn2 homology domain 1 (HD1), which is embedded within the nuclear export sequence (NES), a domain necessary for a stress-regulated localization [47, 48]. Importantly, this motif (HD1) is not present in the Msn2-like proteins of *C. albicans* or its close relatives, including *Candida parapsilosis* and *Candida tropicalis*. Strikingly, the *C. glabrata* orthologues CgMsn2 and CgMsn4 contain the HD1 motif. Site directed mutagenesis of two conserved serines confirmed that nuclear export of CgMsn2 in *C. glabrata* requires
an intact HD1. Further, I observed that the region including the HD1 motif of Msn2/4 orthologues from *S. cerevisiae*, *C. glabrata* and two other related fungi present in this clade, *Kluyveromyces lactis* and *Ashbya gossypii*, is sufficient for nuclear export. Therefore, the HD1 motif and the stress-mediating role of Msn2 appeared after the separation of *C. albicans* from the lineage leading to *S. cerevisiae*. Accordingly, Msn2 orthologues are likely to be important stress-regulated factors in the pre-whole-genome duplication clade, including *S. cerevisiae*, *C. glabrata*, *K. lactis* and *A. gossypii*.

These results establish a *S. cerevisiae*-like general stress response in *C. glabrata*. Despite the preference for different niches, both *S. cerevisiae* and *C. glabrata* share a conserved environmental stress response. Although slight differences, such as the constant repression of *ERG* genes (involved in sterol biosynthesis), were discovered, the overall signal transducing machinery was closely related to its counterpart in *S. cerevisiae* [34].

**Are CgMsn2 and CgMsn4 virulence determinants for C. glabrata?** Comparison of expression levels of *C. glabrata* cells during macrophage engulfment revealed no significant overlaps to the CgMsn2-dependent part of the CgESR [49]. One exception was CgPNC1, encoding a nicotinamidase that converts nicotinamide to nicotinic acid as part of the NAD salvage pathway [Ghislain, 2002 #313; Lin, 2003 #315]. Accordingly, CgMsn2 seems to have no major impact on virulence. *C. glabrata* *msn2Δmsn4Δ* double mutants displayed no attenuated virulence in a *Drosophila melanogaster* infection model. Further, it has been recently shown, that CgMsn2 and CgMsn4 have only a minor impact on survival during oxidative stress [50]. Their relevance for the opportunistic lifestyle of *C. glabrata* still needs to be elucidated. In conclusion, rapid adaptation to changing environment inside the mammalian host requires rather a pathogen-specific response pattern than the general stress response mechanisms. One specific response, which might be crucial for adaptation inside the host, is the oxidative stress regulon, since phagocytic cells are able to induce an oxidative burst to attack engulfed cells.

**Oxidative stress during phagocytosis: a negligible threat for engulfed *C. glabrata* cells?**

Oxidative stress is caused by exposure to reactive oxygen species. It is thought that the selective release of ROS is one of the main weapons of phagocytic cells to kill engulfed pathogens, such as *C. glabrata* [51-53]. In the mature phagolysosome, the
NADPH oxidase complex mediates oxidative destruction of engulfed cells [54, 55]. It catalyzes the production of superoxide (O$_2^-$), serving as initial source for the production of ROS. A large number of reactive oxidants are produced, such as hypochlorous acid (HOCl), which is formed by the myeloperoxidase-catalyzed oxidation of Cl$^-$ by H$_2$O$_2$, hydroxyl radicals (OH$^*$), produced by the reduction of H$_2$O$_2$ by Fe$^{2+}$ or Cu$^+$, peroxynitrite (ONOO$^-$), formed by the reaction between O$_2^-$ and NO$^*$, and many others [56, 57].

To counteract emerging oxidative stress, a broad variety of enzymes and non-enzymatic substances exists, which are able to prevent the formation of ROS or can efficiently eliminate ROS in the cell. Antioxidant factors are under an elaborated control of expression [58]. In *S. cerevisiae*, the two transcription factors Skn7 and Yap1 control the oxidative stress response regulon: Yap1 controls at least 32 genes of the identified oxidative stress stimulon [Lee, 1999 #96; Inoue, 1999 #122]. 15 of these proteins require both Skn7 and Yap1 for a proper induction. Two distinct Yap1 regulons were defined in *S. cerevisiae*, one covering the oxidative stress response, the second involved in the metabolic pathways regenerating the main cellular reducing power, GSH and NADPH [59]. I tried to reveal the mechanisms of *C. glabrata* cells to overcome emerging oxidative stress in the mammalian host.

*How does C. glabrata mediate the oxidative stress response?* In *C. glabrata* we found 38 genes to be upregulated more than two-fold during oxidative stress. The oxidative stress response patterns of *C. glabrata* skn7$\Delta$, yap1$\Delta$ and skn7$\Delta$yap1$\Delta$ mutants were determined and aligned to discover putative dependencies on CgSkn7 and CgYap1. Similar to *S. cerevisiae*, 20 genes were dependent on CgYap1. One set of genes consisting of CgCTA1, CgTRR1/2, CgTSA1/2, CgTRX2, CgGPX2 and CgCCP1 was dependent on the presence of both, CgSkn7 and CgYap1, and was defined as core oxidative stress response (COR). A second CgYap1-associated group included proteins displaying aldo-keto reductase and oxidoreductase activity (*CgADH6, CgGRE2, CgSCS7 and CgOYE2*). In the upstream regions of CgYap1 dependent genes, known *S. cerevisiae* Yap1 consensus sequences were identified [60, 61]. However, corresponding consensus sites were also found in a set of 18 genes, which did not display a decreased upregulation in skn7$\Delta$ and yap1$\Delta$ mutants during oxidative stress. In addition, catalase activity measurements of CgCTA1 expressed by truncated promoter versions pointed to an altered recognition pattern of CgYap1, since the upstream region, which was crucial for full catalase activity, did not comprise *S. cerevisiae* Skn7 and Yap1 consensus sequences. Instead, *S. cerevisiae* Skn7- and Yap1-like sites, bearing one deviation of the known consensus sites, were found throughout the upstream regions of genes involved in oxidative stress response,
including the essential part of the CgCTA1 promoter. Further analysis of putative C.
glabrata-specific binding sites will be necessary to confirm my hypothesis about new
consensus sites for Skn7 and Yap1 orthologues in C. glabrata. Nevertheless, our
results suggest an oxidative stress regulon in C. glabrata similar to baker´s yeast.

How do CgSkn7 and CgYap1 cooperate to regulate the response to oxidative stress?
In S. cerevisiae, a direct interaction between Skn7 and Yap1 seems to be necessary
for functional induction of oxidative stress response genes [60, 62]. It is thought that
Skn7 undergoes a post-translational modification by phosphorylation following
exposure to ROS, which seems to be dependent on Yap1 itself [62]. Mutations
introduced in the receiver domain of Skn7 were found to compromise the association of
Yap1 and Skn7 to upstream regions of oxidative stress-associated genes [60, 63].
Activation of Yap1 during oxidative stress involves a C-terminal cysteine-rich domain,
which contains a NES [64, 65]. Yap1 becomes oxidized by the thiol peroxidase Gpx3
which catalyzes the formation of an intramolecular disulfide bond. This causes a
conformational change that drives Yap1 accumulation in the nucleus [65, 66]. Cysteine-
based redox sensing is highly conserved among all living cells and e.g. found in the
oxidative stress activation of the bacteria and mammals [67-69]. Therefore, I tried to
analyze the interdependence of CgSkn7 and CgYap1 to mediate the oxidative stress
response in C. glabrata.

To assess the interaction of CgSkn7 and CgYap1 in C. glabrata, I tested
whether binding of CgSkn7 and CgYap1 to oxidative stress gene promoters in C.
glabrata is dependent on the presence of the other transcription factor. For this part of
my thesis, I cooperated with my colleague Eva Klopff. We chose the upstream regions
of CgTRR2 and CgGPX2 to investigate the binding properties of CgSkn7 and CgYap1.
Northern blot analysis showed that expression of both was significantly decreased in
skn7Δ mutant cells. Further, CgGPX2 expression was severely decreased and delayed
in yap1Δ mutant cells, whereas CgTRR2 expression was completely stopped.
Chromatin immunoprecipitation data showed that a strict interdependence occurs at
the level of promoter recognition. In the presence of CgYap1, CgSkn7 was able to bind
to both upstream sequences, whereas in the absence of CgYap1, CgSkn7 could not
bind to the promoters. Similarly, CgYap1 bound only in the presence of CgSkn7 to the
tested upstream regions. Binding occurred immediately upon stress induction (< 3
minutes). Both factors stayed there for the same period of time (CgTRR2 < 10 minutes,
CgGPX2 > 10 minutes). This kind of interdependence was also observed in S.
cerevisiae: an electrophoretic mobility shift assay demonstrated in vitro the presence of
a Skn7-Yap1 complex at promoter DNA fragments of TSA1 [59]. However, the precise
mechanism by which these two factors co-activate oxidative stress response was not clear yet. Here, we showed the first time that, in \textit{C. glabrata}, CgSkn7 is dependent on CgYap1 and vice versa to bind to the upstream region of certain oxidative stress genes.

\textit{Does the loss of CgSkn7 and CgYap1 affect prolonged survival upon phagocytosis?} Since CgSkn7 and CgYap1 regulated the core oxidative stress response in \textit{C. glabrata}, the loss of these factors might also attenuate virulence. We found that \textit{C. glabrata yap1Δ} and \textit{skn7Δyap1Δ} mutant cells displayed a diminished recovery rate when exposed to hydrogen peroxide and peroxynitrite, whereas \textit{skn7Δ} single mutant cells did not show this phenotype. In contrast, upon exposure to menadione, which induces the production of superoxide, \textit{C. glabrata} wild type and mutant cells displayed a similar recovery rate. However, in our primary mouse macrophage model, the \textit{C. glabrata skn7Δyap1Δ} double mutant did not show a diminished survival rate compared to the wild type situation. The loss of the response coordinated by CgSkn7 and CgYap1 has no impact on survival of engulfed cells. Accordingly, it has been shown recently by Cuéllar-Cruz \textit{et al.}, that a low but significant number of exponentially growing \textit{skn7Δyap1Δ} double mutant cells can even survive 10mM H$_2$O$_2$, which is a much higher concentration than \textit{C. glabrata} cells experience inside the mammalian host [50, 70]. Since both factors were dispensable for survival in a macrophage infection model, I suggest either only a mild oxidative stress situation inside the phagosome of phagocytic cells or the involvement of other regulatory factors, which can induce a response protecting against oxidative stress. Microarray analysis of \textit{C. glabrata} cells internalized by macrophages, displayed a rather diverse oxidative stress response [49]: \textit{CgCTA1}, \textit{CgGDB1}, \textit{CgHEM15} and \textit{CgGLO1} displayed an increased expression, whereas \textit{CgTTR1/2} and \textit{CgTSA1/2} were downregulated during phagocytosis. Interestingly, it has been reported recently, that \textit{C. glabrata} cells can suppress ROS production upon internalization by macrophages [71]. Therefore, I conclude that \textit{C. glabrata} cells have developed several lines of defence to counteract the oxidative burst.

\textit{Do other regulatory mechanisms induce oxidative stress-associated genes in C. glabrata?} Microarray analysis revealed that among the highly oxidative stress induced genes, the largest group comprising 18 genes, appeared to be upregulated independently of CgSkn7 and CgYap1. \textit{E.g., CgZWF1} (encoding the glucose-6-
phosphate dehydrogenase, involved in oxidative stress adaptation [72]), and CgHSP78, encoding a mitochondrial chaperone [73], were expressed regardless of CgSkn7/CgYap1 presence. Notably, in S. cerevisiae, expression of both ZWF1 and HSP78 was found to be dependent on both, Skn7 and Yap1 [59], suggesting the existence of different regulatory mechanisms in C. glabrata adapted to the mammalian host. Furthermore, parallel upregulation of oxidative stress-associated genes via other signals might occur. Consistently, I found that among the oxidative stress regulon 26 genes were also upregulated during glucose starvation [74]. This group also includes COR genes such as CgCTA1, CgGPX2 and CgTRX2. Interestingly I also found genes encoding for superoxide dismutases CgSOD1 and CgSOD2 to be upregulated during glucose starvation. Genes involved in the oxidative stress response including superoxide dismutases were also found to be upregulated during nutrient starvation in C. albicans [75]. Northern blot analysis of the COR genes CgCTA1 and CgGPX2 revealed a CgSkn7/CgYap1 independent expression behaviour upon nutrient starvation in C. glabrata. Therefore, I suggest the existence of a crucial set of oxidative stress-associated genes, which can get induced by parallel pathways in C. glabrata.

This is consistent with our own unpublished results: a population of engulfed cells suffers transient oxidative stress and continuous carbon shortage inside the phagosome. Possible transcription factors, which can induce the oxidative stress regulon during other stresses, such as carbon starvation, still remain unknown. Existing C. glabrata knock out collections have to be screened for putative candidates. One approach to determine involved transcription factors, is based on the measurement of catalase activity, since the catalase CgCTA1, is also upregulated during glucose starvation. Among those stresses to overcome upon engulfment, it has been shown that nutrient shortage is a predominant issue for pathogenic yeasts [49, 76, 77]. Therefore, I tried to find out more about the nutrient situation of C. glabrata cells inside the phagosome.

**Nutrient starvation as a major impact on survival of phagocytosed C. glabrata cells.**

What are the specific fungicidal characteristics of the unique habitat inside of phagocytic cells? The phagolysosome is considered as oxidative, acidic and degradative milieu. As described above, the oxidative burst contributes to killing of engulfed microbes [55]. In addition, during maturation, the phagosome fuses with
lysosomes which implicates a decreased pH and activated hydrolytic enzymes [78, 79]. Furthermore, upon phagocytosis, engulfed cells are sealed from the environment and have to rely on endogenous resources or those acquired from the phagocytic cell. For example, *C. albicans* induces genes involved in non-fermentative carbon metabolism upon engulfment by macrophages [76]. During the early phase of phagocytosis, *C. albicans* cells shift from glycolysis to gluconeogenesis. Additionally, genes involved in conversion of fatty acids to glucose are induced [76, 80]. Similarly, *C. neoformans* displayed an altered transcriptional response upon phagocytosis [77]. Amongst others, genes involved in autophagy, peroxisome function, and lipid metabolism were induced. The metabolism of alternative carbon sources seems to be of significant importance for engulfed fungal pathogens.

In general, growth on non fermentable carbon sources, such as fatty acids or ethanol, requires β-oxidation and the glyoxylate cycle, which are mainly localized in peroxisomes in yeast. More precisely, the glyoxylate cycle permits the synthesis of glucose from lipids via acetate generated in fatty acid β-oxidation. The products of the glyoxylate cycle (malate and oxalacetate) can be converted to phosphoenolpyruvate, which can be used for gluconeogenesis. Interestingly, the glyoxylate cycle is a virulence determinant for *C. albicans* [80]: the first enzyme of the glyoxylate cycle, the isocitrate lyase (Icl1), which is localized in the peroxisomal matrix, is essential for pathogenicity. Fox2, the second enzyme of the β-oxidation pathway (3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase) is also required for *C. albicans* virulence [81]. In contrast, mutants defective for peroxisomal import (*pex5Δ*) are not compromised for virulence [81]. Therefore, in *C. albicans*, peroxisomes per se are no major determinants for virulence but rather the contained metabolic pathways which can function apparently to a sufficient extent in the cytosol [82, 83].

**What kind of stress response is prevailing in *C. glabrata* upon internalization?** Only little is known about acute stress responses in *C. glabrata* inside the phagosome. To visualize these responses of *C. glabrata* upon macrophage engulfment at a single cell resolution, I characterized fluorescent in vivo reporters. Therefore I established an ex vivo infection model: primary macrophages were isolated from the bone marrow of mice and infected with *C. glabrata* cells. This was done in cooperation with Nina Gratz and Pavel Kovarik (Max F. Perutz Laboratories, Department of Microbiology and Immunology).

The first reporter gene was the catalase of *C. glabrata*, CgCta1. Notably, it has been shown that the loss of CgCta1 did not attenuate virulence of *C. glabrata* [50]. I demonstrated in vitro that expression of CgCta1 is both oxidative stress and carbon
source regulated, whereas the intracellular localization depends on the presence of peroxisomes. Consistently, *S. cerevisiae* Cta1 localized to peroxisomes during growth on non-fermentative carbon sources [84, 85]. In engulfed *C. glabrata* cells, GFP-CgCta1 is induced during the earliest stages during phagocytosis; this could be due to oxidative stress or acute carbon source starvation. Intracellular localization of two other fluorescent reporters (CgYap1 and CgMig1) suggested rather low oxidative stress load and starvation for glucose of engulfed *C. glabrata* cells. Only a minority of engulfed *C. glabrata* cells displayed nuclear localization of the oxidative stress-associated transcription factor GFP-CgYap1. The glucose-dependent repressor CgMig1-CFP was found localized in the cytoplasm of all *C. glabrata* cells immediately upon engulfment. This is consistent with previous results observed in *C. albicans*, where only a small portion of cells derived from mouse kidneys displayed an acute oxidative stress response when examined for CaCTA1 expression [70]. Therefore, nutrient starvation may be considered a major constraint for engulfed pathogenic yeasts.

*Do peroxisomes have an impact on virulence of *C. glabrata***? Similar to peroxisome biogenesis in *S. cerevisiae* [86, 87], *C. glabrata* peroxisomes accumulate during growth on non fermentable carbon sources. Those organelles also appeared during engulfment of *C. glabrata* cells in macrophages. GFP-tagged catalase revealed that induction of peroxisomes appeared to be transient. Induction of peroxisomes occurred a few hours after internalization, whereas no peroxisomes could be observed after 24 hours. This demonstrates efficient carbon source deprivation of *C. glabrata* within the phagosome and corresponding metabolic adjustments. Interestingly, similar to *C. albicans* cells deleted for CaPex5, which are defective for peroxisomal import [81], I found that the survival of *C. glabrata* devoid of peroxisomes in a mutant lacking CgPex3 was not compromised in our macrophage infection model. However, this does not automatically exclude carbon starvation as major problem for engulfed *C. glabrata* cells: it has been observed that the main metabolic pathways (gloxylate cycle, β-oxidation) continue to function in the cytosol in the absence of peroxisomes in *S. cerevisiae* as well as in *C. albicans* [81, 82]. Accumulation of GFP-CgCta1 to peroxisomes was reversed at later time points in *C. glabrata*. Since peroxisomes are not known to export proteins, the observed cytosolic fluorescence is most probably due to peroxisome turnover [88]. To visualize the putative peroxisomal structures, we fused a generic peroxisomal targeting signal peptide (KNIESKL) derived from the *S. cerevisiae* citrate synthase to the C-terminus of YFP [89, 90]. YFP-KNIESKL staining shifted from punctual staining of peroxisomes to diffuse vacuolar staining. This
suggests strongly that \textit{C. glabrata} peroxisomes are degraded by pexophagy, a selective autophagy pathway during prolonged engulfment.

\textit{Do the autophagic processes contribute to virulence of \textit{C. glabrata}?} Since nutrient supply from within the phagosome of phagocytes seems not to be sufficient for prolonged survival \cite{76, 77}, engulfed yeast cells have to initiate degradation of their own cellular components. Importantly, cells have to maintain a balance between synthesis and recycling of intracellular resources. In general, this catabolic process is known as autophagy \cite{91-93}. Double-membrane autophagosomes sequester macromolecules or organelles and fuse with the vacuole (or lysosomes), where the degradation takes place. At least 30 autophagy-related (Atg) proteins involved in autophagy have been identified in \textit{S. cerevisiae} \cite{94}. Several subtypes of autophagy have been described \cite{95}. Selective mitophagy, pexophagy, reticulophagy and ribophagy seem to be important for a fast adaptation to regularly changing surroundings. To date, nothing is known about autophagy in \textit{C. glabrata}. The contribution of autophagy to virulence was already investigated in other fungal pathogens: \textit{C. neoformans} showed a markedly attenuated virulence in a mouse model of infection, upon knock down of Atg8 \cite{96}. In contrast, \textit{C. albicans} mutants deleted for \textit{CaATG9} are defective for autophagy but killed macrophages after engulfment due to stretching \cite{97}. The rapid filamentation response of \textit{C. albicans} might prevent manifestation of the autophagy defect of \textit{Caatg9\Delta} mutants, since prolonged starvation inside the phagosome is prevented by the escape through hyphal outgrowth. Consistently, the filamentous fungi \textit{A. fumigatus} lacking Atg1 also remained virulent \cite{98}. Thus the role of autophagy for fungal pathogens is dependent on their morphology and hence their niches \cite{92}.

In contrast, \textit{C. glabrata} is trapped inside the phagosome, and is cut off from nutrient supply. Therefore, I investigated the importance of autophagic processes upon internalization of \textit{C. glabrata} cells. I tested the role of pexophagy in engulfed \textit{C. glabrata} cells with mutants lacking CgAtg11. In \textit{S. cerevisiae}, Atg11 acts as an adapter protein, directing other factors to the phagophore assembly site (PAS) \cite{91, 99}. Pexophagy appears to involve the same machinery as nonspecific autophagy, while other pexophagy-specific components, except Atg11, have not been elucidated yet. The sequestering vesicles that form during pexophagy, the pexophagosome, enwrap only peroxisomes \cite{88}. Indeed, in \textit{atg11\Delta} mutants, the survival rate of engulfed \textit{C. glabrata} cells was significantly diminished. Non-selective autophagy also encompasses peroxisome degradation. In \textit{S. cerevisiae}, one specific factor essential for functional
bulk autophagy is Atg17, which is thought to act as a scaffold for other ATG genes during the formation of the autophagosome [100]. The loss of Atg17 resulted in fewer and smaller autophagosomes and a severe delay of pexophagy in *S. cerevisiae* [101]. I tested whether this non-selective autophagy pathway is beneficial for the survival of *C. glabrata* upon internalization. Similar to the situation of *atg11Δ*, *atg17Δ* mutants displayed a diminished survival rate. Both, selective and non-selective autophagy, contribute to prolonged survival of engulfed *C. glabrata* cells.

Furthermore, in *C. glabrata pex3Δatg11Δ* cells, I found the sensitivity of *atg11Δ* partially reversed. I suggest from this genetic observation, that autophagy of peroxisomes is beneficial for engulfed *C. glabrata* cells. In contrast, *C. glabrata pex3Δatg17Δ* cells did not display this phenotype. Although the lack of peroxisome biogenesis per se had no influence on survival (*pex3Δ*) and can partially diminish the *atg11Δ* phenotype (*pex3Δatg11Δ*), I argue that it did not outweigh the loss of non-selective autophagy (*pex3Δatg17Δ*). Strikingly, I observed the by far severest decrease of survival in the *C. glabrata* double mutant *atg11Δatg17Δ*. This is in line with results found by Cheong *et al* in *S. cerevisiae*: the *atg11Δatg17Δ* strain did not contain any detectable autophagic bodies [100]. Notably, homologues of proteins of the autophagy core machinery have been found from yeast to mammals, but both Atg11 and Atg17 are not conserved and are only found in yeast [94]. Selective pexophagy, which is affected in both *atg11Δ* and *atg17Δ* mutants, might help to mobilize intracellular resources during prolonged engulfment. Other selective autophagic processes might also contribute to counteract sustained internalization, but have not been topic of this work. Of course, further important autophagy factors, such as the cytosolic protein kinase Atg1, which is essential for the vesicle formation during autophagy [102], or Atg8, which is crucial for the maturation of the autophagosome [103], are likely to attenuate virulence of engulfed *C. glabrata* cells as well. Their importance to successfully counteract phagocytosis still needs to be confirmed. Taken together, I conclude that resisting permanent nutrient starvation is of predominant importance for *C. glabrata* to survive and subsequently escape from the phagosome.
Sleeping with the enemy – what do we know about *Candida glabrata*?

As a common commensal, *C. glabrata* is likely to be isolated from the mammalian mucosa (Figure 1A) [104]. Cells are able to attach to epithelial cells (and in addition to other artificial components such as central venous catheters) due to the expression of specific adhesins [4]. In healthy individuals, *C. glabrata* is unable to spread because of the functional immune system and the bacterial flora, which counteracts fungal dissemination through nutrient restriction and secretion of toxins [105, 106]. Occasionally fungal cells form biofilms, which can exhibit resistance against antifungal agents [107, 108]. Immunocompromised patients recovering from operations or treated with antibiotics are much more susceptible to bloodstream infection of *C. glabrata* cells (Figure 1B). Inside of blood vessels, the milieu is more stable and the nutrient supply is sufficient [109]. Nevertheless, patrolling phagocytes engulf invading pathogens. Cell-mediated immunity, which is based on efficient engulfment of pathogens by phagocytic cells, is crucial to counteract fungal infections (Figure 1C). If the immune system fails to respond to *C. glabrata* infection, cells are able to enter tissues and cause severe organ failure [110]. Notably, due to its opportunistic lifestyle, quantitative and qualitative abnormalities of phagocytes benefit systemic candidiasis [111]. Amongst others, the cytokine TNF-α initiates activation of phagocytes [112, 113], and has been found to be essential to prevent systemic *C. albicans* and *C. glabrata* infections, since lack of TNF-α activity results in enhanced growth in infected tissues in mice [114, 115]. Therefore, efficient phagocytosis by monocytes, macrophages and neutrophils is essential to counteract *C. glabrata* dissemination. The question how *C. glabrata* cells can escape engulfment to cause systemic infection, still needs to be answered.

Upon internalization by phagocytes, engulfed microbes have to sustain several life-threatening restrictions (Figure 1D). Phagocytic cells initiate the so-called oxidative burst through producing reactive oxygen and nitrogen species inside the phagosome to extinct internalized cells [53, 56]. Interestingly, it was observed that engulfed *C. albicans* cells experience only low oxidative stress in macrophages, which are localized in tissues, whereas phagocytosis done by neutrophils, which are found in the bloodstream, causes a severer oxidative stress situation in engulfed cells [70]. Notably, it has been reported recently, that *Candida* species can suppress production of reactive oxygen species in murine and human phagocytes [71]. In line with these results, analysis of the transcription pattern of *C. glabrata* cells during phagocytosis did not show a significant upregulation of oxidative stress-associated genes [49]. Accordingly, I found that the loss of essential oxidative stress transcription factors did not attenuate survival of *C. glabrata* cells in a macrophage infection model. However, I observed that the oxidative stress regulon could also be induced through glucose starvation, a
situation which is prevailing inside the phagosome. Therefore, other pathways might induce the oxidative stress regulon and hence promote resistance.

Engulfed cells are sealed from environmental nutrient supply, and have to deal with carbon source depletion, which leads to a slowdown of proliferation. It has been shown for Candida species and Cryptococcus neoformans, that upon phagocytosis, alternative carbon metabolism pathways are switched on [49, 76, 77]. Consistently, the glyoxylate cycle is a virulence determinant for C. albicans [80]. Internalized cells have to rely on intracellular resources for prolonged survival in the phagosome. Similar to the situation of engulfed C. neoformans cells [96], I discovered that C. glabrata cells are forced to induce autophagic processes, such as pexophagy, which are crucial for prolonged survival upon phagocytosis. Notably, both microorganisms cannot form hyphae to escape from the phagosome, in contrast to C. albicans, which can induce hyphal growth upon engulfment [92]. Autophagy, which makes intracellular resources available, is an essential contributor for dissemination of pathogenic yeasts which are likely to be trapped in the phagosome.

There are still many important questions to answer. E.g., the phagosome fuses with lysosomes in order to decrease the pH, which initiates enzymatic digestion [79, 116]. Since C. glabrata is unable to form hyphae, cells either have to avoid pH acidification or develop strategies to inhibit digestion by released enzymes. Further, for successful dissemination, C. glabrata has to escape from the phagolyso)some. For example, C. neoformans can escape from macrophages through extrusion of the phagosome, without killing the phagocyte [117]. C. glabrata might follow a similar strategy to spread and establish systemic infection. In this respect, the duration of survival of C. glabrata inside phagocytic cells might directly relate to its success as a commensal and pathogen. The quantitative contribution of the various processes requires further evaluation.
Figure 1. Schematic overview of the key points during infection of *Candida glabrata*. As a common commensal, *C. glabrata* is regularly found on mucosal areas. In immunocompromised persons, bloodstream infections and subsequent systemic infections can occur. Cell-mediated immunity, based on efficient engulfment of pathogens by phagocytic cells, is essential for containing fungal infections. During phagocytosis, *C. glabrata* has to overcome multiple stresses inside the phagosome. Nutrient limitation, oxidative stress and eventually pH acidification seem to be the major constraints upon engulfment. Interestingly, neutrophils located in the bloodstream can induce a higher oxidative stress in engulfed yeast cells, than macrophages, which are located in tissues. To sustain survival and proliferation, cells have to decompose endogenous carbon sources. Pexophagy, a subtype of autophagy, has a crucial impact on prolonged survival of *C. glabrata* inside the phagosome of macrophages. However, to date, nothing is known about the detailed strategies of *C. glabrata* to escape upon phagocytosis.
References


4. Appendix:

4.1. Summary

4.2. Zusammenfassung

4.3. Danksagung

4.4. Curriculum vitae
4.1. Summary:

The yeast *Candida glabrata* displays a two-faced fungal lifestyle. In healthy mammals it is part of the normal flora, whereas it can turn into an opportunistic human pathogen in immunocompromised persons. Strikingly, it is closely related to baker’s yeast, *Saccharomyces cerevisiae*. Therefore the question arises, which peculiarities make it a pathogen. The present work focuses on responses of *C. glabrata* to a variety of stresses and its impact on survival inside the host. To reveal similarities and discrepancies between *S. cerevisiae* and *C. glabrata*, a genome-wide transcriptional analysis upon glucose starvation, heat, oxidative and osmotic stress was performed and the involvement of according transcription factors was investigated. In *S. cerevisiae*, the transcription factor Msn2 plays an important role in the environmental stress response (ESR), a common gene expression program, which generally protects the cell during various stresses. Analysis of the stress-induced transcriptome revealed a striking similarity between *C. glabrata* and *S. cerevisiae*. Furthermore the orthologues CgMsn2 and CgMsn4 play a significant role in the general stress response of *C. glabrata*. Nevertheless, the loss of CgMsn2/CgMsn4 did not affect virulence in our ex vivo model. Therefore, other stress-associated mechanisms seem to be of predominant importance. The orthologue transcription factors CgSkn7 and CgYap1 induce – similar to *S. cerevisiae* – the majority of genes of the oxidative stress regulon. Since the so-called “oxidative burst” is a weapon of phagocytic cells to combat microbes, engulfed yeasts have to deal with oxidative stress inside the host. CgYap1 was required for protection against peroxides and peroxynitrite, but analysis of the localization of CgYap1 during phagocytosis suggested only a transient oxidative stress situation. In addition, the loss of CgSkn7 and CgYap1 had no impact on virulence in primary mouse macrophages. Finally, the nutritional status of engulfed *C. glabrata* cells was analyzed using *in vivo* reporter constructs, such as the catalase CgCta1 or the glucose-dependent repressor CgMig1, which indicated a permanent nutritional shortage inside the phagosome. Further, we observed a transient proliferation of peroxisomes, implying the urgent need of engulfed cells to metabolize alternative carbon sources. Intracellular resources seem to be last resort to overcome continuous shortage in carbon supply. One important process to recycle internal resources is autophagy. Intriguingly, degradation of peroxisomes (i.e. pexophagy) turned out to be a significant contributor to survival under these conditions. The autophagy-associated proteins CgAtg11 and CgAtg17 were found to be crucial factors to mediate this response. Taken together, in this work, general stress signaling pathways were characterized in the fungal pathogen *C. glabrata*.
4.2. Zusammenfassung:

Die Hefe *Candida glabrata* ist ein humaner opportunistischer Krankheitserreger, der vor allem für immunsupprimierte Menschen zur Gefahr werden kann. *C. glabrata* kommt ubiquitär in der mikrobiellen Flora der Säugetiere vor und ist mit der Bäckerhefe *Saccharomyces cerevisiae* nahe verwandt. Daraus ergibt sich die Frage, welche Eigenschaften *C. glabrata* zu einem pathogenen Organismus machen. Die hier präsentierten Arbeiten konzentrieren sich auf die Wahrnehmung von Umwelteinflüssen der pathogenen Hefe, und vergleichen sie mit homologen Mechanismen in *S. cerevisiae*. Um die Gemeinsamkeiten und Unterschiede zwischen *S. cerevisiae* und *C. glabrata* genauer zu erforschen, wurde das Transkriptom von *C. glabrata* bei Hitze, oxidativem und osmotischem Stress oder bei Entzug der Kohlenstoffquelle verglichen und die Rolle der zugehörigen Transkriptionsfaktoren untersucht. Msn2 ist in *S. cerevisiae* einer der Hauptakteure der sogenannten „Generellen Stressantwort“, also der Regulierung von Genen durch verschiedene Umweltstresse. Die Analyse des Stress-Transkriptoms zeigte eine überwältigende Übereinstimmung zwischen *S. cerevisiae* und *C. glabrata* und eine wichtige Rolle der Msn2 homologen Proteine CgMsn2 und CgMsn4. Die Virulenz von *C. glabrata* in einem *ex vivo* Modell wurde durch Abwesenheit von CgMsn2/4 nicht beeinträchtigt. Daraus kann auf weitere wichtige Stressresistenzmechanismen in *C. glabrata* geschlossen werden. Die Transkriptionsfaktoren CgSkn7 und CgYap1 regulieren - ähnlich wie in *S. cerevisiae* - auch viele homologe Gene in *C. glabrata* während des oxidativen Stresses. Eine Verteidigungsstrategie von phagozytierenden Zellen des Wirtes ist der sogenannte „oxidative burst“, wo eingeschlossene Mikroorganismen mittels reaktiver Sauerstoffmoleküle attackiert werden. CgYap1 wurde benötigt zum Schutz der Zellen gegen geringe Dosen von Wasserstoff Peroxid und Peroxynitrit, aber die Analyse der Lokalisation von CgYap1 während der Phagozytose deutete nur auf einen transienten oxidativen Stress. So hatte auch der Verlust von CgSkn7 und CgYap1 keinen Einfluss auf die Virulenz von *C. glabrata*. Andere Reportergene wie CgCta1 und CgMig1 deuteten auf eine andauernde Hungersituation der Zellen innerhalb des Phagosoms an. Wir beobachteten auch eine transientse Vermehrung von Peroxisomen, die auf den Versuch der Zellen alternative Kohlenstoffquellen zu benutzen hindeutete. Um den Nachschub von Kohlenstoff zu gewährleisten, muss die eingeschlossene Zelle andere intrazelluläre Quellen nützen. Eine Möglichkeit besteht in Autophagie, wobei Bestandteile der Zelle abgebaut werden. Durch die Ausschaltung von CgATG11 und CgATG17, welche zwei wichtige Signalmoleküle für den autophagozytischen Prozess kodieren, wurde die Überlebensrate von phagozytierten *C. glabrata* Zellen erheblich abgeschwächt.
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01/02 Analysis of A. thaliana gene AtMKP1 (Prof. Hirt).
03/02 Effect of temperature shift on expression of the S-layer protein SbsA of B. stearothermophilus (Prof. Kuen).
06/02 Experimental Immunology (Company Intercell AG, Vienna).
07/02 Biochemistry of viral proteins, HRV2 2A proteinase activity on eIF4G (Prof. Skern).
09/02 The Mg2+ influx system: Investigation of Alrp1 & Alrp2 localisation (Prof. Schweyen).
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Tutorials:
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06/06, 06/07, 10/07: practical course Molecular Biology I (MFPL, University of Vienna)
06/08, 11/08: practical course Biochemistry I (MFPL, University of Vienna)

Attended Meetings:
05/05: Nice - 1st FEBS Lecture Course Human Fungal Pathogens
  Functional conservation of the key transcription factor Msn2. Andreas Roetzer, Christa Gregori, Karl Kuchler, Gustav Ammerer and Christoph Schüller.
Appendix

09/05: Vienna – Life Sciences (Annual joint meeting of the ÖGBM, ÖGGGT, ÖGBT and ANGT)

11/06: Heidelberg - 8th International EMBL PhD Student Symposium (Biology of Disease)
Poster: Living on a hostile ground: key transcription factors mediate stress response of the pathogen Candida glabrata. Andreas Roetzer, Christa Gregori, Karl Kuchler, and Christoph Schüller.

05/07: Nice - 2nd FEBS Lecture Course Human Fungal Pathogens
Selected short talk: Living on a hostile ground: key transcription factors mediate stress response of Candida glabrata. Andreas Roetzer, Christa Gregori, Karl Kuchler, and Christoph Schüller.

09/07: Göttingen - 4th International PhD Student Symposium (Horizons in Molecular Biology)
Poster: Stress response in yeast: following the route of key transcription factors in Saccharomyces cerevisiae and Candida spp. Andreas Roetzer, Christa Gregori, Anne Marie Jennings, Geraldine Butler, Karl Kuchler, Christoph Schüller.

10/08: Heidelberg - EMBO Workshop on Evolutionary and Environmental Genomics of Yeast

Publications:


