**The Novel *Candida albicans* Transporter Dur31 Is a Multi-Stage Pathogenicity Factor**

François L. Mayer¹, Duncan Wilson¹, Ilse D. Jacobsen¹, Pedro Miramón¹, Katharina Große¹, Bernhard Hube¹,²

¹ Department of Microbial Pathogenicity Mechanisms, Hans-Knoell-Institute, Jena, Germany, ² Friedrich Schiller University, Jena, Germany

**Abstract**

*Candida albicans* is the most frequent cause of oral fungal infections. However, the exact pathogenicity mechanisms that this fungus employs are largely unknown and many of the genes expressed during oral infection are uncharacterized. In this study we sought to functionally characterize 12 previously unknown function genes associated with oral candidiasis. We generated homozygous knockout mutants for all 12 genes and analyzed their interaction with human oral epithelium *in vitro*. Eleven mutants caused significantly less epithelial damage and, of these, deletion of orf19.6656 (*DUR31*) elicited the strongest reduction in pathogenicity. Interestingly, *DUR31* was not only involved in oral epithelial damage, but in multiple stages of candidiasis, including surviving attack by human neutrophils, endothelial damage and virulence *in vivo*. In *silico* analysis indicated that *DUR31* encodes a sodium/substrate symporter with 13 transmembrane domains and no human homologue. We provide evidence that Dur31 transports histatin 5. This is one of the very first examples of microbial driven import of this highly cytotoxic antimicrobial peptide. Also, in contrast to wild type *C. albicans*, *dur31Δ/Δ* was unable to actively increase local environmental pH, suggesting that Dur31 lies in the extracellular alkalinization hyphal auto-induction pathway; and, indeed, *DUR31* was required for morphogenesis. In agreement with this observation, *dur31Δ/Δ* was unable to assimilate the polyamine spermidine.

**Citation:** Mayer FL, Wilson D, Jacobsen ID, Miramón P, Große K, et al. (2012) The Novel *Candida albicans* Transporter Dur31 Is a Multi-Stage Pathogenicity Factor. PLoS Pathog 8(3): e1002592. doi:10.1371/journal.ppat.1002592

**Editor:** Anita Sil, UCSF, United States of America

**Received** September 7, 2011; **Accepted** January 31, 2012; **Published** March 15, 2012

**Copyright:** © 2012 Mayer et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** FLM was supported by the International Leibniz Research School for Microbial and Biomolecular Interactions (ILRS) as part of the excellence graduate school Jena School for Microbial Communication (JSMC). DW received funding from the European Community’s Seventh Framework Programme under grant agreement no. PIEF-GA-2008-219406. PM was supported by the EU FINSysB Marie Curie Research Training Network project no. FP7-214004. IDJ and KG were supported by the Federal Ministry of Education and Health (BMBF). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: Bernhard.Hube@hki-jena.de

**Introduction**

The human oral cavity represents a discrete environmental niche, harboring a diverse and complex microbiome. In up to 80% of healthy individuals the fungus *Candida albicans*, and to a lesser extent other *Candida* spp. are part of this oral microbiome where they usually exist as harmless commensals [1–3]. However, disturbances in the human immune status and other predisposing factors can allow the fungus to switch from a commensal to a pathogen, causing oral infections (oral candidiasis). For example, denture wearing, reduced salivary flow and extremes of age are risk factors [2]. HIV/AIDS patients in particular are predisposed to oral candidiasis with as many as 80–90% suffering from recurrent infections [4–7]. In order to persist as part of the human oral microbiome and as a prerequisite for infection, *C. albicans* must adhere to other microbes and/or to epithelial host cells. Adherence is mediated mainly by adhesins, including the hyphal wall protein 1 (Hwp1) and members of the agglutinin-like sequence (Als) family [8]. The corresponding genes were found to be upregulated during oral candidiasis [9]. Besides adhesins, hydrophobicity and the interaction between pathogen-associated molecular patterns (PAMPs) and pattern recognition receptors (PRRs) on host cells also mediate adherence [10]. The initiation of oral infections is associated with the formation of elongated fungal filaments (hyphae) which can penetrate into the oral epithelium. This invasion process can occur via two distinct mechanisms, induced endocytosis and active penetration [9,11–13]. Induced endocytosis does not depend on fungal viability and is characterized by engulfment of the fungal cell by the host cell [8,9,11]. This process is initiated by binding of the host cell cadherins, N-cadherin (endothelial cells) and E-cadherin (epithelial cells), to the fungal invasins Als3 [14] and Ssa1 [15]. Active penetration is dependent upon fungal viability and involves direct penetration of *C. albicans* hyphae into host cells or at intercellular junctions [8,11]. This process is believed to be driven by mechanical pressure of the invading hyphal tip and the secretion of hydrolytic enzymes. Following these adhesion and invasion events, the fungus damages epithelial host cells, mediated by a combination of active penetration, hyphal extension and the expression of largely unknown virulence factors for deeper tissue invasion and further inter-epithelial invasion [12]. Importantly, we recently demonstrated that adhesion and invasion events alone do not result in host cell damage [12], suggesting that other, yet unidentified, activities play a role in tissue destruction. The morphogenetic switch is believed to be a key virulence factor because mutants which are impaired in filament-formation are avirulent [16]. Filamentation is induced by multiple environmental cues such as temperature, pH, CO₂, or contact to epithelial and endothelial
Author Summary

The normally commensal yeast, Candida albicans is the leading cause of both oral and vaginal thrush, as well as life-threatening disseminated infections in the nosocomial setting. Treatment of such infections is hampered by the limited number of suitable antimycotics. Therefore, understanding how this fungus causes disease is crucial for the development of more effective diagnostic tools and therapeutic strategies. Here we identified a novel C. albicans transporter that has no human homologue and demonstrate its role during multiple stages of candidiasis. We also show that this transporter is involved in modifying environmental pH and in filamentous growth. Additionally, deletion of DUR31 rendered cells unable to assimilate a polyamine (sperrmidine) as a nutrient source, but resistant to the antimicrobial peptide histatin 5. Therefore, Dur31 has multiple transport functions, which can be either beneficial or detrimental to the fungus.

host cells [9,12]. Recently, it has been shown that C. albicans is able to auto-induce filament formation by actively alkalinizing its extracellular environment [17]. In an acidic environment the fungus can raise the extracellular pH from 4 to >7 within 12 h, thereby triggering the yeast-to-hypha transition [17]. Alkalinization occurs in glucose-limited media and depends on the presence of exogenous amino acids [17]. By screening around 500 mutant strains and performing transcriptional profiling, the authors demonstrated that the amino acid permease regulator Stp2, the acetyl-coenzyme A hydrolase Aeh1, the urea amidolyase Dur1,2 and the putative ammonia exporter Ato5 are required for extracellular alkalinization [17]. The authors concluded that under nutrient limitation, C. albicans assimilates amino acids as carbon source, exports the amine group in the form of ammonia and thereby raises extracellular pH, which in turn results in the yeast-to-hyphal transition [17].

One of the key strategies that oral epithelial cells employ to defend themselves against C. albicans infections is the production of antimicrobial peptides like cathelicidins [18,19], defensins [20], and histatins [21,22]. Among the family of histatins, histatin 5 has the highest killing efficacy against C. albicans. Additionally, in deeper tissues and in blood, neutrophils exhibit key defense activities as part of the innate immune system and have been shown to be central in killing C. albicans [23]. Importantly, in certain high risk patients [24], C. albicans can cause life-threatening systemic infections [25]. Approximately 30–37% of patients suffering from systemic candidiasis die during the course of the infection [26,27]. Interestingly, HIV infection is not an independent risk factor for disseminated candidiasis, suggesting that the two diseases might develop independently from each other [8]. In a previous study we performed genome-wide transcriptional profiling of samples from HIV+ patients with oral candidiasis and of in vitro oral infection models, and identified genes encoding known and unknown-function proteins associated with oral infection [9]. These investigations led to the discovery of the novel infection-associated gene EEDI, which appears to encode a key regulator of hyphal extension [9,28].

The aim of the current study was to identify further novel oral infection-associated genes in C. albicans. We therefore selected a set of 12 previously uncharacterized genes, based on their transcriptional upregulation during oral infection [9] and in silico functional predictions. We generated knockout mutants for all 12 genes and show that 11 mutants were significantly attenuated in their capacity to damage oral host cells in vitro. We then focused our investigations on the characterization of the novel gene DUR31 which encodes a predicted plasma membrane localized sodium substrate transporter with no homologue in humans. We demonstrate a crucial role for Dur31 in host cell damage, resistance to neutrophils and virulence. Finally, we provide mechanistic insight into the role of Dur31 in polyamine assimilation, histatin 5 import and extracellular alkalinization induced hyphal formation.

Results

Identification of C. albicans genes associated with human oral infections

The first objective of this study was to identify novel and previously uncharacterized C. albicans genes, associated with oral candidiasis. We hypothesized that C. albicans genes specifically upregulated during oral infection represent promising candidates for novel, oral infection-associated virulence factors. Based on previously published transcriptional data of C. albicans during both infection of the oral cavity of HIV+ individuals and infection of reconstituted human oral epithelium [9], we identified 12 genes that were upregulated at least two-fold under one or both conditions (Table S1). We performed in silico analysis of all 12 genes and found nine to contain one or more transmembrane domain(s) in their predicted protein sequences (Table 1). Using a targeted gene deletion strategy we then generated homozygous knockout mutants for each of the twelve unknown function genes (Table 2).

Screening of a defined set of mutants for reduced oral epithelial cell damage capacity identifies a novel sodium substrate symporter

Oral candidiasis is characterized by C. albicans adherence to, invasion into and ultimate damage of oral epithelial cells. We therefore first investigated the capacity of each mutant to damage monolayers of oral epithelial cells in vitro.

Host cells were infected with the different C. albicans strains for 15 hours and the degree of oral epithelial cell damage was quantified by measuring lactate dehydrogenase (LDH) activity [29,30]. After 15 hours of infection only one mutant (orf19.3617AΔ/A, lacking a predicted mitochondrial protein with similarity to human Ras-related GTP-binding protein A) caused similar damage as the wild type (Figure 1A). All 11 other mutants were significantly reduced in their capacity to damage these host cells (Figure 1A and Table 1).

Strain orf19.6656Δ/A, lacking a predicted plasma membrane localized sodium/substrate transporter with 13 TMs, was most attenuated and caused 65% less epithelial damage in comparison to the wild type. We therefore focused our further investigations on orf19.6656Δ/A. Since attenuated damage may be due to reduced adhesion and invasion [12], we tested the epithelial adhesion and invasion capacities of orf19.6656Δ/A. Interestingly, adhesion and invasion levels were comparable to the wild type (Figure S1), suggesting stage specific functions at later stages during interaction with epithelial cells.

According to the Candida Genome Database (www.candidagenome.org), the gene orf19.6656 has the common name DUR3, based on sequence similarities to the Saccharomyces cerevisiae DUR3 gene. S. cerevisiae DUR3 encodes a transporter for urea and polyamines [31–33]. However, a nomenclature conflict exists for orf19.6656, as the common name DUR3 has also been used to refer to orf19.781 [34]. We therefore performed alignments using the EMBOSS Needle analysis tool (www.ebi.ac.uk/Tools/psa/emboss_needle) and found the orf19.781 predicted protein
sequence to be 52.5% identical and 68.5% similar to \textit{S. cerevisiae} Dur3 while \textit{orf19.6656} encodes a predicted protein with only 14.7% identity and 29.9% similarity with \textit{ScDur3}. Therefore, in comparison to \textit{orf19.781}, \textit{orf19.6656} is only distantly related to \textit{ScDur3}.

We performed a BLASTp analysis of the \textit{orf19.6656} protein sequence and found the highest sequence similarities for proteins of unknown function in \textit{Candida dubliniensis} (CD36_53230, 95.6% identity, 98.3% similarity) and \textit{Candida tropicalis} (CTRG_05438, 80.1% identity, 88.2% similarity). Interestingly, \textit{orf19.6656} is 81.2% identical and 90.5% similar to \textit{Pichia stipitis} \textit{DUR3}. However, \textit{DUR3} has not yet been functionally characterized in \textit{P. stipitis}. Analysis with the SMART (www.smart.embl-heidelberg.de), ExPaSy (www.expasy.ch/prosite) and Wolf PSORT (www.wolfpsort.org) databases suggested that \textit{C. albicans} \textit{orf19.6656} encodes a plasma membrane localized sodium substrate-symporter of the sodium substrate symporter family (SSSF) containing 13 transmembrane (TM) domains (Figure 1B, Table 1). We furthermore identified amino acids in TM9 which have been shown to be critical for ion binding in \textit{E. coli} PutP, a sodium proline symporter belonging to the SSSF. Based on a recent publication [35], we refer to the \textit{C. albicans} gene \textit{orf19.6656} as \textit{DUR31} (degradation of urica). For further analysis, we next constructed a \textit{dur31Δ::DUR31} complemented strain.

**Deletion of \textit{DUR31} affects cell wall integrity in \textit{C. albicans}**

Due to the predicted localization of \textit{DUR31} in the plasma membrane (Table 1 and Figure 1), we investigated the effect of different cell wall and plasma membrane disturbing agents and stresses on growth of the \textit{dur31Δ/Δ} mutant. Deletion of \textit{DUR31} did not affect growth of \textit{C. albicans} on SD agar at 37°C and 42°C (Figure 2A). However, the \textit{dur31Δ/Δ} mutant was more sensitive to cell wall stress induced by 450 μg ml⁻¹ Congo red. Complementation of the mutant with \textit{DUR31} restored growth under these stress conditions. Western blot analysis of strains grown under conditions of cell wall stress revealed higher levels of phosphorylated Mek1, a marker for cell wall perturbances [36], in the \textit{dura31Δ/Δ} mutant in comparison to the wild type and complemented strains (Figure 2C). Heavy metal stress, induced by 0.75 mM silver nitrate, led to 100- to 1000-fold reduction in growth of the \textit{dur31Δ/Δ} mutant in comparison to the wild type and \textit{dur31Δ/Δ::DUR31} complemented strain. Osmotic stress (1.5 M NaCl) had no effect on growth of the mutant (data not shown). The \textit{dur31Δ/Δ} mutant was moderately more tolerant to UV-(C)-stress than the wild type and complemented strain (Figure 2B). We furthermore investigated the effect of the membrane potential-disrupting ionophore nigericin (Fluka) on growth of the \textit{dur31Δ/Δ} mutant but did not detect a difference in susceptibility in comparison to the wild type (data not shown). Collectively, these results indicate that deletion of \textit{DUR31} affects cell wall integrity in \textit{C. albicans}.

**A \textit{dura31Δ/Δ} mutant displays defective colony and microcolony formation**

Amongst the range of putative SSSF substrates, polyamines and certain amino acids such as arginine have been demonstrated to induce filamentation [37,38]. Since the production of filaments is one of the major virulence traits of \textit{C. albicans} [39], we next investigated the morphology of \textit{dura31Δ/Δ} under various hypha-inducing conditions.

We first analyzed filamentation of \textit{dura31Δ/Δ} on a single cell level on a plastic surface in liquid hyphae inducing media (RPMI or 10% serum, Figure S2). By 3 h, the \textit{dura31Δ/Δ} mutant formed filaments of similar length and morphology to the wild type, indicating that \textit{DUR31} is dispensable for the early stages of hyphal formation in response to liquid serum or RPMI. Early stage (3 h) hyphal formation on human-derived oral epithelial cell monolayers was also comparable to that of the wild type (Figure S2B). However, when we extended the incubation time from 3 to 24 h, the \textit{dura31Δ/Δ} mutant formed significantly smaller microcolonies than the wild type and complemented strains (Figure 3A and 3B). Therefore, although dispensable for initial hyphal growth, \textit{DUR31} appears to be required for further filamentation and the production of regular microcolonies.

We therefore next grew \textit{dura31Δ/Δ} on agar containing 10% serum or 5% RPMI, on Spider or SLAD agar or by embedding in
endothelial damage and immune evasion

filamentous structures such as colonies.

required for the initiation of hyphal formation in liquid media, this
formed aberrant colonies which lacked the peripheral, invasive

colonies. Under each condition tested, the
attenuation in epithelial damage, led us to postulate that Dur31
functions during blood and liver infection [9,23,40]. However, the stress
was exclusively upregulated during oral infection, and
traverse the endothelial lining of blood vessels in order to access
must resist attacks by the
must adhere to and
reactive oxygen species (ROS) plays an important role in
restored wild type (71.2%) survival rates. The generation of
neutrophils was due to increased oxidative stress sensitivity.

Models of systemic infection: **DUR31 is required for endothelial damage and immune evasion**

**DUR31** was exclusively upregulated during oral infection, and not during blood and liver infection [9,23,40]. However, the stress resistance- and hyphal formation- defects, together with the strong attenuation in epithelial damage, led us to postulate that Dur31 may also function during other forms of candidiasis.

During systemic infections, *C. albicans* must adhere to and traverse the endothelial lining of blood vessels in order to access internal organs. Moreover, *C. albicans* must resist attacks by the immune system. We therefore next investigated whether **DUR31** is required for damage of endothelial cells and tolerance to killing activities by human neutrophils (Figure 4). We used monolayers of HUVEC endothelial cells for infection with the different *C. albicans* strains in vitro. Following a 15 or 24 hour co-incubation we determined host cell damage by measuring LDH release. The **dura/L** mutant caused significantly reduced damage to endothelial cells after 15 and 24 hours of infection (Figure 4A). The mutant caused 83% less damage in comparison to the wild type after 15 hours and 35% less damage after 24 hours post infection. These results indicate that Dur31 is not only required for damage of epithelial cells, but also for endothelial cell damage and therefore may play a role during systemic infections.

Neutrophils play a key role in host defense against *C. albicans* infections [23,41]. We therefore investigated survival of the **dura/L** mutant in the presence of these phagocytes. While 73.6% of wild type cells survived exposure to neutrophils, only 43.2% of **dura/L** cells remained viable following co-incubation with these phagocytes (Figure 4B). Complementation with **DUR31** restored wild type (71.2%) survival rates. The generation of reactive oxygen species (ROS) plays an important role in neutrophil-killing of microbes [41,42]. We therefore investigated whether the higher susceptibility of the **dura/L** mutant to neutrophils was due to increased oxidative stress sensitivity.

---

**Table 2. C. albicans strains used in this study.**

| Strain | Genotype | Reference |
|--------|----------|-----------|
| SCP314 | *Candida albicans* wild type | [75] |
| BWP17  | ura3::IMM434/ura3::3 IMM434 arg4-5 hisG::arg4-5 hisG::his1::hisG his1::hisG::hisG | [66] |
| BWP17+Clp30 | ura3::IMM434/ura3::3 IMM434 arg4-5 hisG::arg4-5 hisG::his1::hisG::his1::hisG+::Clp30 | [9] |
| dura/L | orf19.6656::ARG4/ORF19.6656 | This study |
| dura/L/ura | orf19.6656::ARG4/orf19.6656::HIS1 | This study |
| dura/L/Clp10 (URA3) | orf19.6656::ARG4/orf19.6656::HIS1+::Clp10 (URA3) | This study |
| dura/L/Clp10 (ORF19.6656, URA3) | orf19.6656::ARG4/orf19.6656::HIS1+::Clp10 (ORF19.6656, URA3) | This study |
| mkl/CIp10 | orf19.7523::HIS1/orf19.7523::ARG4+::K70 | [73] |
| orf19.1150 | orf19.1150::ARG4/ORF19.1150 | This study |
| orf19.1150/L | orf19.1150::ARG4/orf19.1150::HIS1+::Clp10 (URA3) | This study |
| orf19.1353 | orf19.1353::ARG4/ORF19.1353 | This study |
| orf19.1353/L | orf19.1353::ARG4/orf19.1353::HIS1+::Clp10 (URA3) | This study |
| orf19.2959 | orf19.2959::ARG4/ORF19.2959.1 | This study |
| orf19.2959/L | orf19.2959.1::ARG4/orf19.2959.1::HIS1+::Clp10 (URA3) | This study |
| orf19.3617 | orf19.3617::ARG4/ORF19.3617 | This study |
| orf19.3617/L | orf19.3617::ARG4/orf19.3617::HIS1+::Clp10 (URA3) | This study |
| orf19.3872 | orf19.3872::ARG4/ORF19.3872 | This study |
| orf19.3872/L | orf19.3872::ARG4/orf19.3872::HIS1+::Clp10 (URA3) | This study |
| orf19.5443 | orf19.5443::ARG4/ORF19.5443 | This study |
| orf19.5443/L | orf19.5443::ARG4/orf19.5443::HIS1+::Clp10 (URA3) | This study |
| orf19.548 | orf19.548::ARG4/ORF19.548 | This study |
| orf19.548/L | orf19.548::ARG4/orf19.548::HIS1+::Clp10 (URA3) | This study |
| orf19.6200 | orf19.6200::ARG4/ORF19.6200 | This study |
| orf19.6200/L | orf19.6200::ARG4/orf19.6200::HIS1+::Clp10 (URA3) | This study |
| orf19.6847 | orf19.6847::ARG4/ORF19.6847 | This study |
| orf19.6847/L | orf19.6847::ARG4/orf19.6847::HIS1+::Clp10 (URA3) | This study |
| orf19.7670 | orf19.7670::ARG4/ORF19.7670 | This study |
| orf19.7670/L | orf19.7670::ARG4/orf19.7670::HIS1+::Clp10 (URA3) | This study |
| orf19.988 | orf19.988::ARG4/ORF19.988 | This study |
| orf19.988/L | orf19.988::ARG4/orf19.988::HIS1+::Clp10 (URA3) | This study |

doi:10.1371/journal.ppat.1002592.t002
However, using a spot dilution assay with SD medium containing 2 mM H$_2$O$_2$ or 0.4 mM menadione, no growth defect was observed for the mutant in comparison to the wild type (data not shown). Alongside neutrophils, macrophages also play an important role in host defense. We thus tested survival of the mutant in the presence of macrophages. Here, although a trend towards reduced survival of the $dur31^{Δ/Δ}$ mutant was observed, the difference was not significant (data not shown).

The reduced endothelial damage capacity of $dus31^{Δ/Δ}$ together with reduced survival in the presence of neutrophils strongly implied that $DUR31$ may play a role during systemic candidiasis.

Deletion of $DUR31$ attenuates virulence in a mouse model of hematogenously disseminated candidiasis

We determined the virulence of the $dur31^{Δ/Δ}$ mutant using a murine model of hematogenously disseminated candidiasis.
Survival of mice infected with the wild type or the dur31D/Δ::DUR31 complemented strain showed no significant difference (Figure 5A). The majority of mice infected with these strains died within 5 to 8 days post infection (p.i.). In contrast, all mice infected with the dur31D/Δ mutant strain were still alive at day 6 p.i.. Starting at day 7 p.i., however, mice began to succumb to infection and died between day 7 and day 13 p.i.. Therefore, DUR31 is required for the rapid onset of symptomatic disseminated candidiasis.

To investigate whether the dur31D/Δ mutant also exhibited morphological defects in vivo, we performed histological analysis of the kidneys of end-point mice. Interestingly, despite delayed mortality of dur31Δ/Δ-infected mice, upon death, the kidneys exhibited similar pathologies to wild type-infected mice with the presence of fungal filaments and neutrophil infiltrates (Figure 5B).

Given the importance of Dur31 for different stages of pathogenesis and its proposed role as a transporter, we next sought to identify its substrate(s).

Dur31 facilitates spermidine utilization and histatin 5 susceptibility

Members of the sodium substrate symporter family have been shown to transport a variety of different substrates, including urea, sugars, amino acids, polyamines, vitamins, ions and water [43].
Because *DUR31* was originally annotated as a urea transporter (*DUR3*, above) we investigated growth of the *dur31Δ/Δ* mutant on urea as sole carbon and nitrogen source, but did not find a difference in growth in comparison to the wild type (data not shown). Additionally, transcriptional data from a recent publication [34] support the view that Dur31 probably does not transport urea. We therefore investigated growth of the *dur31Δ/Δ* mutant in the presence of different sugars and amino acids as sole carbon or nitrogen sources, but again did not find differences in growth between the mutant and the wild type (data not shown). When we investigated growth of the mutant with the polyamine spermidine as sole carbon source, only moderate growth of the wild type and complemented strains was observed (Figure 6A), however the *dur31Δ/Δ* mutant did not grow at all, indicating that Dur31 might transport this polyamine or a related amine-containing compound. Indeed, in a parallel study, the laboratory of Mira Edgerton demonstrated that Dur3, and to a lesser extent also Dur31, function as spermidine transporters [35]. These results are in agreement with our own data that Dur31 facilitates spermidine import.

One of the key mechanisms which prevent oral *C. albicans* infections in healthy individuals is the production of antimicrobial peptides such as the histidine-rich histatins. Among the histatins, salivary histatin 5 is the most effective candidacidal peptide [44]. Interestingly, HIV patients often exhibit lower levels of histatin 5 [45]. Since *DUR31* was specifically upregulated in *C. albicans*...
samples from HIV patients with oral candidiasis (low histatin 5), we postulated that Dur31 might function in the absence of histatin 5 in vivo. Strikingly, the mutant was significantly more resistant to killing by this antimicrobial peptide in comparison to the susceptible wild type and the dur31Δ::DUR31 complemented strain (Figure 6B). We used FITC-labeled histatin 5 to monitor uptake by the dur31ΔΔ mutant (Figure 6C). Cells were incubated for 15 min at 30°C with 30 μM FITC-histatin 5 and immediately visualized with fluorescence microscopy. FITC-histatin 5 was detected intracellularly, as previously described [46]. However, in comparison to the wild type and complemented strain, fewer mutant cells had taken up FITC-histatin 5. We quantified the

Figure 4. Dur31 is required for damage of human endothelial cells and for immune evasion. (A) Monolayers of HUVEC endothelial cells were infected with C. albicans wild type (Wt) and dur31ΔΔ mutant strains for 15 or 24 h. Damage of host cells was determined by measuring lactate dehydrogenase (LDH) levels. Results are the mean ± SD of at least three independent experiments, each performed in triplicate. *P<0.05 and **P<0.01 compared to the wild type strain. (B) A dur31ΔΔ mutant is more susceptible to killing by human neutrophils. Wild type (Wt), dur31ΔΔ mutant and dur31ΔΔ::DUR31 complemented mutant cells were exposed to human neutrophils for three hours and viability was determined by plating on YPD agar. Experiments were performed three times. The result values are indicated by circles, triangles or rectangles. The bar represents the mean of these single values. *P<0.01 compared with the wild type and dur31ΔΔ::DUR31 complemented strain.

doi:10.1371/journal.ppat.1002592.g004

Figure 5. DUR31 deletion attenuated virulence in a mouse model of hematogenously disseminated candidiasis. (A) Female Balb/C mice (n = 10 mice per C. albicans strain) were challenged intravenously with either the wild type (Wt), the dur31ΔΔ mutant or the dur31ΔΔ::DUR31 complemented strain via the lateral tail vein and survival followed for up to 21 days. *P<0.0001 compared with mice either infected with the wild type or dur31ΔΔ::DUR31 complemented strain. (B) Periodic acid Schiff staining of kidney sections from mice infected with the wild type, the dur31ΔΔ mutant or the dur31ΔΔ::DUR31 complemented strain at the indicated time points post infection. Pictures were taken at 63× magnification. Arrows point to C. albicans filaments within the tissue.

doi:10.1371/journal.ppat.1002592.g005

C. albicans Dur31 and Virulence
fluorescence intensities of approximately 100 cells of each strain and found the \textit{dur31Δ/Δ} mutant to have 32% reduced levels of FITC-histatin 5 in comparison to the wild type (Figure 6D). Therefore the increased survival of the \textit{dur31Δ/Δ} mutant in the presence of histatin 5 correlates with decreased internalization of this antimicrobial peptide.

Histatin 5 binding to the \textit{C. albicans} cell surface is mediated by Ssa1 and Ssa2 \cite{47,48}. To ensure that the reduced histatin 5-uptake and increased survival of the \textit{dur31Δ/Δ} mutant was not due to altered levels of Ssa1/2 on the cell surface, we performed indirect immunofluorescence with a mouse anti-Hsp70 monoclonal antibody, which detects Ssa1/Ssa2 (Figure S3A). The \textit{dur31Δ/Δ} mutant had comparably reduced levels of Hsp70 proteins on the cell surface (Figure S3B). In addition, we performed western blotting with the anti-Hsp70 antibody on cell wall extracts of the wild type and \textit{dur31Δ/Δ} mutant. Comparable levels of cell surface-associated Ssa1/2 were detected in both the wild type and \textit{dur31Δ/Δ} mutant (Figure S3C). These data are in agreement with the recent report of Kumar et al. \cite{35}, who also provided evidence that Dur31 is a histatin 5 transporter.
Together, these results indicate that Dur31 is likely to transport both, the polyamine spermidine and the antimicrobial peptide histatin 5.

**Dur31 mediates extracellular alkalinization**

In a recent publication it has been elegantly demonstrated that *C. albicans* is able to actively alkalinize its environment, thereby auto-inducing hyphal formation [17]. In the current study we have shown that the *dur31Δ/Δ* mutant is defective for hyphal formation and that *DUR31* encodes a predicted sodium substrate transporter. Given its proposed role in transporting amine-containing molecules (necessary for environmental alkalinization/hyphal auto-induction [17]), we hypothesized that Dur31 may mediate extracellular alkalinization, thereby mediating hyphal auto-induction.

We used two approaches for visualization of extracellular alkalinization: solid GM medium supplemented with bromocresol green as pH indicator and liquid medium 199 with phenol red as pH indicator. Both media were initially adjusted with HCl to pH 4. The wild type and *dur31Δ/Δ::DUR31* complemented strain alkalinized solid GM-BCG medium within three to four days, indicated by a change of medium colour from green to blue (Figure 7A). This colour change indicates a shift in pH from 4 to >5.4. In contrast, the *dur31Δ/Δ* mutant did not alkalinize the medium as efficiently. After three days of incubation, the medium colour surrounding *dur31Δ/Δ* colonies remained green, indicating that the extracellular medium was still in the range of pH 4 at that time. Following more extensive incubation times (10 and 14 days), *dur31Δ/Δ* was eventually capable of alkalinizing the surrounding medium (Figure 7B); however, by this time point, the wild type and *dur31Δ/Δ::DUR31* colonies formed a strong blue, whilst *dur31Δ/Δ* colonies remained white.

In liquid medium 199, alkalinization is indicated by a colour change from yellow (pH 4) to red (pH 6–7). Similar to the results
obtained on solid GM-BCG medium, the dur31Δ/Δ mutant again displayed defective alkalinization (Figure 7C). To confirm this observation, we adjusted medium pH 190 to pH 4, grew the strains for 24 h and directly measured alkalinization with a pH meter. Within this time, wild type C. albicans raised the medium pH from 4 to pH 6 (Figure 7D). In contrast, the dur31Δ/Δ mutant alkalinized the surrounding medium to only pH 4.5. Complementation of the mutant with a single copy of DUR31 significantly increased medium alkalinization. Together these data demonstrate that Dur31 is involved in extracellular alkalinization.

Discussion

Novel oral infection-associated C. albicans genes

Of the 6203 predicted C. albicans open reading frames (ORFs), around 75% (4672 ORFs) are still uncharacterized (Candida Genome Database) [49]. The high number of uncharacterized genes within the genome of C. albicans makes it highly likely that important fungal virulence factors have yet to be discovered [50]. We therefore characterized the role of 12 novel C. albicans genes that we predicted to be involved in oral infection. These genes were selected based on their expression during oral infection [9] and in silico predicted structural/functional features. Strikingly, 11 mutants were significantly reduced in their capacity to damage oral epithelium, suggesting effective selection criteria. We thereafter focused our analysis on orf19.6656 (DUR31) as a mutant lacking this gene had the strongest defect in damage. In order to cause epithelial damage, C. albicans first has to adhere to and subsequently invade into host cells [9,12,13,30,51–53]. Despite the strong reduction in epithelial damage observed upon DUR31 deletion, the mutant adhered to and invaded epithelial cells at levels comparable to the wild type. This is most likely the result of wild type rates of germ tube formation by dur31Δ/Δ as initial hyphal formation is generally sufficient to allow epithelial adhesion and invasion [12]. Consequently, the attenuation in epithelial damage must be due to other fungal activities (see below).

DUR31 mediates multiple stages of candidiasis

DUR31 was observed to be exclusively upregulated by C. albicans during clinical oral candidiasis and was required for oral epithelial damage. However, we found DUR31 to be involved in multiple interactions associated with systemic candidiasis and dur31Δ/Δ exhibited significantly reduced virulence in vivo. If C. albicans gains access to the bloodstream it can infect virtually every internal organ and kill the host. First, however, the fungus must survive the hostile milieu of the blood and then traverse the endothelial lining of blood vessels. Within the blood, neutrophils are believed to kill C. albicans via a combination of acute nutrient starvation, oxidative stress and the action of antimicrobial peptides. Although dur31Δ/Δ grew normally in the presence of reactive oxygen species, increased sensitivity to cell wall stress may partially account for the higher killing rate of dur31Δ/Δ compared to the wild type. Interestingly, the neutrophil-associated antimicrobial peptide, defensin-1 kills C. albicans via a pathway related to histatin 5-mediated killing [54]. As Dur31 likely mediates histatin 5 import (below), it is possible that deletion of DUR31 also altered sensitivity to other antimicrobial peptides, which are present in neutrophils. C. albicans cells which survive attack by blood components can next penetrate the endothelial lining of blood vessels to access other organs. During traversal of the endothelial cell layer, the fungus damages these host cells [51,55]. Therefore, the reduced endothelial damage potential of dur31Δ/Δ may reflect an impaired capacity to disseminate through blood vessels during systemic candidiasis. Indeed, C. albicans mutants with reduced capacity to damage endothelial cells in vitro, often display reduced virulence in mice models of hematogenously disseminated candidiasis [56].

Deletion of DUR31 also resulted in aberrant filamentous growth in vitro, a phenotype often associated with reduced virulence [16,29,51]. However, histological examination of the kidneys of endpoint dur31Δ/Δ-infected mice revealed filamentous fungal foci comparable to the wild type. It would appear, therefore, that deletion of DUR31 delayed the maturation of larger fungal lesions, rather than preventing their formation completely. This view fits with the eventual fate of dur31Δ/Δ-infected mice: delayed, yet ultimately complete mortality. Therefore, Dur31 is involved in the rapid onset of symptomatic systemic candidiasis but not its final conclusion. However, in the hospital setting, following the onset of hypotension, each hour of delay in starting antifungal treatment increases the mortality of candidaemia by around 5% [24]. Therefore, the contribution of Dur31 to virulence may be clinically relevant.

In summary, a combination of increased sensitivity to neutrophils, together with the reduced endothelial damage capacity and morphological defects of dur31Δ/Δ cells, likely contributes to the delayed virulence of this strain.

DUR31 mediates polyamine utilization, histatin 5-sensitivity and extracellular alkalinization

In silico analysis suggested that DUR31 encodes a plasma membrane localized protein with 13 transmembrane domains, belonging to the sodium/substrate symporter family (SSSF). Indeed, the common topological motif of SSSF transporters has been defined as an arrangement of 13 transmembrane domains with an extracellular N-terminus and cytoplasmic C-terminus [43]. Each of these topological features was present in the Dur31 sequence. Moreover we identified the amino acids serine and threonine in the transmembrane domain TM9 of C. albicans Dur31, a feature which has been shown to be critical for ion binding in Escherichia coli PutP, a sodium proline symporter of the SSSF [43]. Therefore, in silico analysis suggests that DUR31 encodes a sodium substrate transporter.

Members of the SSSF transport a wide range of substrates, including sugars, amino acids (e.g. proline), vitamins (e.g. pantothenate), ions, urea and water [43]. As these substrates are often transported against a concentration gradient, cells use a sodium motive force to fuel substrate uptake. The sodium motive force is built up by sodium pumps or sodium/proton antiporters. Most of the SSSF transporters are involved in the acquisition of their respective substrate as carbon or nitrogen sources [43]. Interestingly, the Staphylococcus aureus orthologue of E. coli PutP is a virulence factor: a S. aureus putP mutant had approximately 10-fold reduced virulence in wound and murine abscess infection models compared to a wild type control [57]. Based on these data and the fact that C. albicans Dur31 was required for normal virulence in a murine infection model, we reasoned that DUR31 might encode a sodium/proline symporter; however, we did not find evidence of Dur31-mediated proline uptake by C. albicans (data not shown). Based on apparent sequence similarities to S. cerevisiae DUR3, C. albicans DUR31 (orf19.6656) was originally designated the common name, DUR3 (CGD). However, orf19.781 is also named DUR3. In S. cerevisiae, DUR3 encodes a urea and polyamine transporter. A recent study demonstrated that orf19.781 is the major urea transporter in C. albicans [34]. This finding was supported by our in silico analyses which indicated that orf19.781, and not orf19.6656, is the true orthologue of ScDUR3. Indeed phylogenetic analysis revealed that ScDUR3 and CaDUR3 (orf19.781) cluster together with Dur3 orthologues from other fungal species such as A. fumigatus.
fumigatus, Aspergillus terreus, Magnaportha oryzae, Schizosaccharomyces pombe and Cryptococcus gattii (Figure S4). GaDur31 (orf19.6656) on the other hand, belonged to an independent cluster with proteins of unknown function from species of the CUG-clade and non-CUG-clade fungal species including Candida glabrata, Rhoryomyces lactis, Coccioides immitis, Neurospora crassa, Malacozia globosa and Ustilago maydis. No DUR31 orthologues were detected in S. cerevisiae or A. fumigatus. We therefore propose that C. albicans DUR31 has evolved separately or diverged from DUR3 and is present in a limited yet diverse range of fungal species, including many pathogenic species. Therefore, together with the results of Navarathna et al. [34], we conclude that DUR31 is unlikely to encode a urea transporter.

In order to determine which substrate or substrate family Dur31 might transport, we systematically analyzed the growth of the dur31Δ/Δ mutant in the presence of known SSSF-substrates as sole C- or N-source. The mutant displayed no growth defects with sugars, amino acids or the vitamin pantothenate as nutrient sources (data not shown). Importantly however, dur31Δ/Δ failed to utilize the polyamine spermidine as sole C-source. This indicates that Dur31 may transport the polyamine spermidine. Polyamines are essential for cell growth and for modulating the function of nucleic acids and ATP [58]. In S. cerevisiae, polyamine uptake is mainly catalyzed by Dur3 and Sam3 [33]. In C. albicans, the role of Dur3, and another transporter, Dur3', in polyamine transport has been independently investigated by the laboratory of Mira Edgerton [35]. These authors demonstrated that Dur3 and Dur3' share overlapping functions in transporting spermidine in C. albicans. Interestingly, polyamines have also been implicated in filament formation [37], providing a potential link between defective polyamine transport in the dur31Δ/Δ mutant and the resulting filament formation defect on semi-solid media.

Therefore, expression of DUR31 likely benefits C. albicans by allowing the fungus to utilize distinct amine-containing substrates and facilitating hyphal growth. Interestingly, we also found a role for Dur31 that may benefit the infected host, rather than the fungus itself. The cationic antimicrobial peptide histatin 5 protects the host from microbial infections of the oral cavity and has been shown to efficiently kill C. albicans [59]. Opposed to other cationic antimicrobial peptides, histatin 5 does not induce disruption of the fungal cell membrane but rather exerts its killing activity intracellularly. Therefore, the transport of histatin 5 into the cell has been defined as an essential process for its antifungal activity [59]. The laboratory of Mira Edgerton has demonstrated that the two cell wall localized heat shock protein 70 family members Ssa1 and Ssa2 bind histatin 5 [47,48]. However, the mechanism of internalization has, until very recently, remained unknown [35,60]. We initially investigated susceptibility of dur31Δ/Δ to killing by histatin 5 based on the specific transcriptional upregulation of DUR31 in samples from HIV+/AIDS patients with oral candidiasis – patients in which histatin 5 levels are much lower than in healthy individuals [45,61]. Indeed, we found that Dur31 mediates killing of C. albicans by histatin 5. Similarly, deletion of the histatin 5 receptor encoding gene (Ssa2) also led to enhanced C. albicans survival in the presence of this antimicrobial peptide, with ssa2Δ/Δ survival values comparable to that of the dur31Δ/Δ mutant [59]. Together with the work of the Edgerton laboratory, our data support a model whereby histatin 5 binds Ssa1/2 on the surface of C. albicans and is then transported, via Dur31, into the cell, subsequently killing the fungus (Figure 8). The interaction of histatin 5 with Dur31 likely represents an example of co-evolution, whereby the fungus expresses a transporter to acquire a nutrient – in this case polyamines – and the host expresses a cytotoxic substrate for this very transporter. The fact that DUR31 is expressed by C. albicans infecting HIV+ patients (where histatin 5 expression is impaired) supports this view. In the future, it will be intriguing to investigate DUR31 expression levels by C. albicans colonizing the oral cavity of humans with normal levels of histatin 5 expression. Moreover, elucidating the molecular mechanism of histatin 5 import by Dur31 will provide invaluable insight into how the host immune system “tricks” microorganisms into taking up this highly cytotoxic compound.

Finally we provide a mechanism of Dur31-mediated filamentation. It has recently been shown that C. albicans actively alkalinizes its surrounding environment, thereby auto-inducing filamentation [17]. Indeed, these authors propose that nutrient-starved C. albicans cells import and catabolize amino acids, whereby the amine groups are removed during substrate entry to the tricarboxylic acid cycle, converted to volatile ammonia and excreted, thereby raising the environment pH. As Dur31 was required for polyamine utilization, extracellular alkalization and hyphal morphogenesis, we propose that this transporter is involved in the import of amine-containing substrates (such as spermidine), which feed into the hyphal auto-induction pathway described by Vykova et al. [17] (Figure 8). These authors identified 10 C. albicans proteins encoded by the ATO (ammonia transport outward, [62]) family, of which Ato5 appears to be critical for extracellular alkalization, as deletion of ATO5 renders C. albicans cells unable to neutralize their environment [17]. We propose that Dur31 contributes to the capacity of C. albicans to actively alkalize its environment under nutrient starvation by importing amine-containing substrates (e.g. spermidine) and thereby contributing to the intracellular production of ammonium which is then released as volatile ammonia by the Ato proteins (Figure 8). In line with its alkalization function, expression of DUR31 has been shown to be down-regulated at alkaline pH in vitro [63]. However, it is unlikely that this gene is constitutively repressed at alkaline pH, as Dur31 was required for filamentous growth in RPMI medium, which is neutral/alkaline. Indeed, we originally identified DUR31 as being transcriptionally upregulated during oral candidiasis and Dur31 was required for oral epithelial damage, suggesting that this transporter also functions at neutral/alkaline pH.

In summary, we have characterized the roles of 12 previously unknown function genes in oral infection. For one gene (DUR31) we provide evidence for multiple cellular and pathogenic functions including histatin 5 import, polyamine utilization, environmental alkalization, hyphal morphogenesis, epithelial and endothelial destruction, immune evasion and virulence (Figure 8).

Materials and Methods

Ethics statement

All animal experiments were in compliance with the German animal protection law and were approved (permit no. 03-007/07) by the responsible Federal State authority (Thüringer Landesamt für Lebensmittelsicherheit und Verbraucherschutz) and ethics committee (beratende Kommission nach § 15 Abs. 1 Tierschutzgesetz). The use of human primary cells in this study was conducted according to the principles expressed in the Declaration of Helsinki. All protocols used in this study were approved by the local ethics committee of the University of Jena under the permit no. 2207-01/08. Written informed consent was provided by all study participants.

Strains and culture conditions

All C. albicans strains used in this study are listed in Table 2. The triple-auxotrophic strain BWP17 complemented with plasmid...
CIp30 [9] was used as wild type control in all experiments. Strains were routinely cultivated on YPD agar [1% yeast extract, 2% bacto-peptone, 2% D-glucose, 2% agar] or SD minimal medium agar [2% dextrose, 0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% agar]. Liquid overnight cultures were grown in YPD or SD medium in a shaking incubator at 30°C and 180 rpm. For selection purposes fungal cells were spread on SD agar supplemented with 20 µg ml⁻¹ arginine, histidine and/or uridine as required. E. coli was grown on LB agar [1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, 2% agar]. Overnight liquid cultures of E. coli were cultivated in a shaking incubator at 37°C and 210 rpm. For growth curves of C. albicans strains, overnight YPD cultures grown at 30°C were diluted to an OD₆₀₀ of 0.1 in a 200 µl final volume of the desired medium. Growth of the strains was then recorded by measurement of the OD₆₀₀ at 30 min interval for up to 50 hours in an ELISA reader (Infinite M200, Tecan) [64]. Experiments were performed at least twice in triplicate.

Strain construction

The dur31Δ/Δ homozygous null mutant was constructed using a PCR-based gene disruption technique [65]. Using the Arg-, His- and Ura-auxotrophic strain BWP17 [66] as background, the complete open reading frames (ORFs) of both DUR31 alleles were replaced with polymerase chain reaction (PCR)-amplified ARG4 and HIS1 disruption cassettes flanked by 104 base pairs of target homology region. Two sequential transformations using the improved lithium-acetate method [67] were applied for both disruption cassettes. Primers DUR31-FG and DUR31-RG (Table S2) were used for generation of the deletion cassettes with the pFA-ARG4 and pFA-HIS1 plasmids as templates. Resultant deletion cassettes were used to sequentially delete both copies of DUR31 (orf19.6656). The resultant Ura-auxotrophic mutant was rendered prototrophic for uridine by transformation with the NcoI-linearized plasmid CIp10, which harbours the URA3 gene and stably integrates at the RPS10 locus [68]. The correct deletion of both alleles and integration of CIp10 was verified by colony PCR using target gene and disruption/integration cassette flanking and internal primers: DUR31-F1, DUR31-R1, ARG4-F1, ARG4-R1, HIS1-F1, HIS1-R1, URA3-F2 and RPF-F1 (Table S2), respectively. An identical strategy with relevant gene-specific primers was employed for deletion and confirmation of orf19.1150, orf19.1353, orf19.2959.1, orf19.3617, orf19.3872, orf19.5443, orf19.5848, orf19.6200, orf19.6847, orf19.7670 and orf19.988.

Additionally, Southern blot analysis (Figure S5) using a 354 base-pair PCR product, generated with the primers DUR31-F2 and DUR31-R2 (Table S2) from C. albicans SC5314 genomic DNA, as a probe on HindII-digested genomic DNA was used to confirm deletion of DUR31 (orf19.6656).

For construction of a dur31Δ/Δ::DUR31-reconstituted strain, the open reading frame of DUR31 as well as 304 base pairs of target

---

**Figure 8. Proposed model for C. albicans Dur31.** Dur31 functions as a transporter of both amine-containing substrates and the antimicrobial peptide histatin 5 in C. albicans. Histatin 5 (red rectangles) is transported into the cell by Dur31 which leads to cell death. Dur31 also transports an amine-containing substrate (green circles) which is beneficial for the cell, as it promotes growth, alkalinization of the extracellular environment through amine exporters (Ato) and concomitant filament formation required for full virulence. The Ato transporter has been included into the model based on [17].

doi:10.1371/journal.ppat.1002592.g008
upstream and 460 base pairs of downstream sequence were amplified from SC5314 genomic DNA with the Phusion High-Fidelity DNA Polymerase Kit (Finnzymes) using the HindIII restriction site containing primers DUR31-rec-F1 and DUR31-rec-R1 (Table S2). The PCR product was first digested with HindIII and then further purified with the QIAquick PCR Purification Kit (Qiagen). In parallel 0.3 μg ml⁻¹ of plasmid Ctp10 was digested with HindIII and the restriction enzyme then heat inactivated by incubation at 65°C for 20 min. The linearized plasmid was dephosphorylated with calf intestinal alkaline phosphatase (New England BioLabs) and gel extracted using the QIAquick Gel Extraction Kit (Qiagen). The DUR31 insert and Ctp10 vector were then ligated for 30 min at 22°C using the Rapid DNA Ligation Kit (Fermentas). Five μl of ligation product was used for the transformation of E. coli DH5α and positive clones were selected on LB agar plates supplemented with 50 μg ml⁻¹ Ampicillin. Plasmid Ctp10 carrying DUR31 was re-isolated using plasmid miniprep (peqlab) and midiprep (Qiagen) kits and confirmed by control digestions with HindIII, SacI and Spel. The final plasmid was then digested with Ncol prior to transformation into the uridine auxotrophic C. albicans strain dur31A/Δura⁻ (Table 2). Positive clones were selected on SD agar plates without amino acids. Correct integration was verified by PCR on whole yeast colonies using primers RPF-F1 and URA3-F2 (Table S2).

Stress susceptibility

Aliquots ofYPD overnight cultures were washed twice in phosphate buffered saline (PBS) and 10-fold serial dilutions in 5 μl (covering a range of 10⁶–10⁷ cells) were spotted onto SD agar containing 0.4 μM menadione (Sigma), 2 mM H₂O₂ (Appli-Chem), 1.5 M NaCl (Roth), 0.75 mM silver nitrate (Roth) or 450 μg ml⁻¹ Congo red (Sigma) and incubated at 37°C for 3–4 days. Plates incubated at 42°C were photographed after 4–6 days. UV-stress was induced by exposure of 10-fold serial dilutions on YPD agar to 0, 2, 4 or 8 mJ/cm² UV-C light with a wavelength of 254 nm using a UV-crosslinker (Bio-Link, Vilber-Lourmat). Plates were then incubated for 2 days at 37°C. Each experiment was repeated at least twice. Representative pictures are shown.

Histatin 5 fungicidal assay

Sensitivities of C. albicans strains to histatin 5 were investigated by microdilution assay as previously described [59,69].

FITC-labeling of histatin 5

Labeling of histatin 5 (Sigma) was performed as previously described [70,71]. Briefly, 1.25 μl of freshly dissolved FITC (1 mg/ml) in DMSO was added to 500 μl of histatin 5 (65 μM) in 50 mM potassium phosphate buffer. Following 16 h incubation at 4°C in darkness, residual FITC was inactivated by incubation with 10 μl 1 M NH₄Cl for 2 h at 4°C in darkness. Fifty μl aliquots of FITC-labeled histatin 5 (FITC-histatin 5) were stored at −20°C until use.

Histatin 5 localization study

To investigate intracellular localization of FITC-histatin 5, strains were grown overnight in YPD medium, washed twice with 10 mM sodium phosphate buffer (NaPB) and cell numbers were adjusted to 10⁶ cells ml⁻¹ in 10 mM NaPB. Cells were incubated with 30 μM FITC-histatin 5 for 15 min at 30°C with shaking (300 rpm). Cells were then immediately mounted on glass coverslips and analyzed by fluorescence microscopy. Experiments were performed twice in duplicate. For quantification, the mean fluorescent intensities of at least 80 cells per strain were determined.

Filamentation

Filamentation was investigated on solid water agar supplemented with 10% fetal bovine serum or 5% RPMI, on SLAD agar, on solid Spider medium [72] or by embedding in YPS agar [1% yeast extract, 2% bacto-peptone, 2% D(+)-saccharose, 2% agar]. RPMI agar and SLAD agar plates were incubated for 4 days, serum agar plates for 2 days, and Spider agar plates for 10 days at 37°C. Embedded plates were incubated at 25°C for 5 days. Experiments were performed twice in duplicate yielding similar results. Representative pictures are shown.

For analysis of filament formation on a single cell level, fungal cells were grown overnight to stationary phase in SD medium, washed twice with water, and resuspended in water. Cell numbers were adjusted to 10⁵ cells per well in a 24-well cell culture plate in RPMI1640 or water supplemented with 10% serum, and incubated at 37°C for 4 hours in the presence of 5% CO₂. Experiments were performed in duplicate and repeated twice. Representative pictures are shown.

For induction of filamentation using host cells was performed by preparation of a monolayer of oral epithelial cells (TR146) as described previously [12] and infecting it with 10⁵ C. albicans cells. Monolayers were incubated at 37°C for three hours in a 5% CO₂ atmosphere and hyphal cells were then differentially stained according to the invasion assay protocol described below. Representative pictures are shown.

For induction of hyphal microcolonies, fungal cells were grown overnight to stationary phase in YPD medium, washed twice with PBS, and resuspended in PBS. Cell numbers were adjusted to 10⁴ cells per ml and 10 μl (100 cells) were inoculated in 500 μl RPMI medium without serum per well in a 24-well cell culture plate and incubated at 37°C for 24 hours in presence of 5% CO₂ in an incubator (Binder). Experiments were performed in quadruplicate on two different occasions. The dimensions of 40 cells per strain and experiment were determined using an inverse microscope (Leica DMIL) and the software LAS (Leica Application Suite). Representative pictures taken at 40× magnification are shown.

Extracellular alkalination assays

Investigation of extracellular alkalination by C. albicans was performed as previously described [17], with minor modifications. Briefly, for alkalination on solid media, strains were grown on GM-BGC (1% yeast extract, 30 mM CaCl₂, 3% glycerol, 0.01% bromocresol green, 4% agar) without glucose. Alkalination assays in liquid media were conducted using medium 199 with Earle’s salts (PAA) and supplemented with sodium hydrogen carbonate (PAA), according to the manufacturer’s instructions. All media were adjusted to pH 4 using HCl and NaOH. For assays on solid GM-BGC media, 12- or 6-well plates were used, in which 2 or 5 ml of molten GM-BGC agar, without glucose, were added and allowed to solidify. C. albicans strains were grown to stationary phase overnight in YPD, centrifuged, washed once in water and diluted to an OD₆₀₀ of 1.0 in water. Seven μl of this dilution were then pipetted onto the agar, with one strain per well. Plates were incubated at 30°C and alkalization was followed daily for up to 14 days as a progressive change of the medium colour from green to blue. Experiments were performed at least in duplicate and repeated three times. Representative pictures are shown. Alkalization assays in liquid medium 199 (M199) were performed in 96-well plates. Overnight YPD fungal cultures were harvested by centrifugation, washed once in water and adjusted to an OD₆₀₀ of 1.0 in pH 4-adjusted medium 199. Serial five-fold dilutions were prepared and plates were incubated at 37°C for 24 h. Alkalization of the medium was observed by a change of color from yellow to red. Experiments were performed in duplicate on three different
occasions. Representative pictures are shown. In order to directly quantify the alkalization capacity of the different strains, overnight YPD cultures were adjusted to OD 0.1 in M199 pH 4 and 8 ml were added per well to a 4-well cell culture plate and incubated at 37°C for 24 hours. The cells were then resuspended and the pH and OD_{600} was measured.

Western blot analysis

Western blot analysis for detection of phosphorylated Mkc1 was performed as previously described [73], with some modifications. Briefly, overnight YPD cultures of the BWP17::Clp30 wild type and the Δmke1Δ/Δ, Δmke1Δ/Δ:ΔCIP30 and mke1Δ/Δ mutant strains were adjusted to OD 0.5 in 10 ml final volume and grown under the following conditions for 4 hours at 30°C with shaking (180 rpm): (i) SD minimal medium, and (ii) SD minimal medium with 450 μg/ml Congo red. Cells were collected by centrifugation at 4°C and washed twice with cold lysis buffer containing 1× PBS, 3 mM KCl, 2.5 mM MgCl₂, 0.1% Triton X-100, 50 mM NaF, 2 mM Na₃VO₄. Cell pellets were resuspended in cold lysis buffer (see above) containing a protease inhibitor cocktail (Roche). Cells were then mechanically disrupted by adding acid-washed glass beads and bead beating in a Precellys 24 homogenizer (peqlab). Protein concentrations were determined by BCA Protein Assay (Pierce). Protein samples (80 μg) were mixed with one-fourth volume of 4× sample buffer containing 125 mM Tris-HCl (pH 6.8), 50% glycerol, 4% SDS, 2.5% β-mercaptoethanol, and 0.02% bromophenol blue for SDS-PAGE. Samples were heated at 95°C for 5 min and then separated by SDS-PAGE using 12% acrylamide gels. Proteins were electro-transferred to Protran B85 nitrocellulose membranes (Whatman) and blocked with 5% BSA (Serva) in PBS with 0.05% tween. Blots were then probed with primary anti-phospho-p44/42 MAP kinase antibody (1:1000, Cell Signaling Technology) and secondary goat anti-rabbit-horseradish peroxidase (HRP)-conjugated antibody (1:2500, Santa Cruz), and developed using the Enhanced Chemiluminescent (ECL) SuperSignal West Dura kit (Thermo Scientific) according to the manufacturers’ instructions. Membranes were then stripped for 30 min at 50°C using a buffer containing 2% SDS, 125 mM Tris-HCl (pH 6.8) and 0.7% β-mercaptoethanol. Stripped membranes were then blocked with 5% BSA (Serva) in PBS with 0.05% tween and re-probed for α-tubulin (loading control) by using a primary rat anti-α-tubulin antibody (1:1000, AbD Serotec) and a secondary goat anti-rat HRP-conjugated antibody (1:2000, Santa Cruz), and developed as described above. Experiments were performed twice.

Western blot analysis for detection of Ssa1/2 levels was performed as described above, with minor modifications. The phosphatase inhibitors NaF and Na₃VO₄ were omitted from the lysis buffer (see above). Blots were probed with primary mouse anti-Hsp70 monoclonal antibody (1:1000, Stressgen) and secondary goat anti-mouse HRP-conjugated antibody (1:2500, Santa Cruz).

Surface expression of Ssa1/2

The assessment of surface expression of Ssa1/2 was performed based on a previously described method [15], with some modifications. Briefly, to analyze the surface distribution of Ssa1/2 on the different strains, 10⁷ yeast cells in 1 ml RPMI 1640 medium with L-glutamine and HEPEs were added to 12 mm diameter glass cover slips in a 24-well cell culture plate. Following a 90 min incubation in 5% CO₂ at 37°C, the resulting germ tubes were washed three times with PBS and fixed with 4% paraformaldehyde for 1 hour at room temperature (RT), washed again twice with PBS, and blocked with 2% BSA for 30 min at RT. Following blocking, the germ tubes were then washed again three times in PBS, and then stained with a mouse anti-Hsp70 monoclonal antibody (1:100, Stressgen) and Alexa Fluor 555-conjugated goat anti-mouse IgG (1:500, Invitrogen) as the secondary antibody. Next, the germ tubes were counter stained with Alexa Fluor 647-conjugated concanavalin A (1:500, Invitrogen) to label the cell surface. The glass coverslips were mounted inverted on microscope slides and imaged by fluorescence microscopy. Experiments were performed twice in duplicate.

Endothelial and oral epithelial cells

The human buccal carcinoma epithelial cell line TR-146 (Cancer Research Technology, London) [74] and the human umbilical vein endothelial cell line HUVEC (ATCC CRL-1730, LGC Standards, Promocell) were cultured and passaged in Dulbecco Modified Eagles Medium (DMEM) with 2 mM L-glutamine (PAA) supplemented with 10% heat inactivated (56°C, 10 min) fetal bovine serum (PBS, PAA). For experiments, TR-146 cells were used during passage 10–20 and HUVEC cells during passage 10–40. Both cell lines were cultured in an incubator at 37°C with 5% CO₂ atmosphere. Cultivation medium was replaced by fresh medium every second day and accutase (PAA) was used for detaching cells after confluency had reached about 80–100%.

Quantification of adherence to host cells

C. albicans adherence studies were performed using ihidi µ-Slides VI 0.4 with six channels per slide. For adherence assays with human host cells, 1.8×10⁴ endothelial or epithelial cells were seeded per µ-slide channel and incubated for 3 days at 37°C and 5% CO₂ with medium changed daily. Confluent monolayers were infected with 1.5×10⁵ C. albicans cells per channel for 45 min. Monolayers were then thoroughly washed with PBS to remove un-adhered fungal cells and fixed with 4% paraformaldehyde. C. albicans cells were subsequently stained with calcofluor white for 30 min in the dark and visualized by fluorescence microscopy (Leica DM500B, Leica DFC360 FX). The number of adhered cells was determined by counting at least 50 high power fields of 200 μm×200 μm size. Experiments were performed in duplicate on two separate occasions.

Quantification of invasion into host cells

The invasion capacity of the different C. albicans strains was determined as previously described [30]. Briefly, epithelial TR-146 cells were grown to confluency on 15 mm diameter glass coverslips for 2–3 days. Monolayers were washed with PBS and infected with 10⁴ C. albicans yeast cells for 3 hours at 37°C and 5% CO₂. Next, epithelial cells were washed twice with PBS and fixed with 4% paraformaldehyde (Roth). Fungal cells were then stained for 45 min with fluorescein-conjugated concanavalin A (Con A) (Invitrogen). After washing with PBS, epithelial cells were permeabilized in 1% Triton X-100 for 15 min. Next, fungal cells were stained with calcofluor white for 30 min. All incubation steps were carried out in the dark. Coverslips were then rinsed three times with water and mounted with the cells upside down on microscope slides with ProLong Gold Antifade Reagent. Fluorescence microscopy was performed (Leica DM5500B, Leica DFC360 FX) using appropriate filter sets for detection of fluorescein-conjugated Con A and calcofluor white. At least 100 C. albicans cells were examined for each strain and the invasion rate was expressed as percentage of invaded cells divided by the number of invaded plus non-invaded cells. Representative pictures were taken for each strain. All experiments were performed in duplicate on two separate occasions.
Quantification of damage to host cells

The extent of damage caused to host cells by the C. albicans strains was quantified by measuring lactate dehydrogenase (LDH) activity with the Cytotoxicity Detection Kit (Roche Applied Science). TR146 or HUVEC cells were adjusted to 10^5 cells ml^{-1} in DMEM with 10% FBS and 200 μl were seeded per well in 96 well plates (TTP). Plates were incubated at 37°C and 5% CO₂ for 2 days until confluency had been reached. Cells were then washed twice with PBS and 100 μl DMEM with 2% FBS were added per well. For the C. albicans strains, aliquots of overnight YPD cultures were washed twice in PBS, diluted to 5×10^5 cells ml^{-1} in DMEM without FBS and 100 μl used for infection of host cells. Controls included a medium only control, a low control with uninfected host cells and a high control with uninfected host cells and medium supplemented with 1% Triton X-100 prior to measurement. Incubation was carried out at 37°C and 5% CO₂ for 15 or 24 h. Measurement of LDH activity with the Cytotoxicity Detection Kit was performed according to the manufacturer’s manual. Absorbance of the samples was measured at 490 nm. Medium only and low control values were subtracted from all sample values. Damage was expressed as percentage of the high control, which was set to 100%. Each experiment was performed at least twice in triplicate.

Susceptibility to killing by neutrophils

Neutrophils were isolated from blood of healthy human donors by a density gradient centrifugation using Histopaque 1077 and 1119 (Sigma, MO, USA) following the manufacturer’s instructions. Polymorphonuclear cells (PMNs) were obtained after a centrifugation step at 700 g for 30 min at room temperature and then transferred to PBS. The remaining erythrocytes were lysed in a lysis buffer (0.83% NH₄Cl, 10 mM HEPES, pH 7.0), the PMNs washed once in PBS and resuspended in 1 ml RPMI1640+5% FBS. For investigating susceptibility of C. albicans to killing by neutrophils, 100 μl of fungal overnight cultures were collected and washed twice with PBS. C. albicans cells were opsonized with 50% human serum for 30 min at 37°C. Following centrifugation and resuspension in PBS, 10^5 cells ml^{-1} were inoculated into RPMI1640+5% FBS. Neutrophils and fungal cells were then mixed in a ratio of 10:1 (final volume: 400 μl) and incubated for 3 hours at 37°C in the presence of 5% CO₂. Neutrophils were lysed by the addition of 100 μl 0.25% SDS at 30°C in order to release phagocytosed C. albicans cells. After addition of 900 μl cold water and 20 U of DNase-1, cells were incubated for 15 min at 30°C. Following preparation of appropriate dilutions, aliquots were spread in duplicate on YPD and incubated for 24 hours at 37°C. Three independent experiments were performed.

Murine model of hematogenously disseminated candidiasis

Five to six weeks old female Balb/C mice (Mus musculus) (18–20 g; Charles River, Germany) were used for the experiments. The animals were housed in groups of five in individually ventilated cages and cared for in strict accordance with the principles outlined in the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (http://conventions.coe.int/Treaty/en/Treaties/Html/123.htm). Mice were challenged intravenously on day 0 with 5×10^5 cfu in 200 μl PBS via the lateral tail vein. The health status of the mice was examined at least twice a day by a veterinarian. Body surface temperature and body weight were recorded once a day. Mice showing severe signs of illness like isolation from the group, apathy, hypothermia and drastic weight loss, were anaesthetized by application of 200 μl ketamine hydrochloride (50 mg ml^{-1}) prior to blood collection by heart puncture. Gross pathological alterations were recorded during necropsy. For histology, left kidneys were collected and fixed with buffered formalin and paraffin-embedded sections were stained with Periodic acid-Schiff (PAS) according to standard protocols.

Statistical analysis

Differences in damage of endothelial and oral epithelial cells were compared by two-tailed Student’s t-test. The statistical analysis for the susceptibility of C. albicans strains to killing by neutrophils was performed using Turkey’s Multiple Comparison test. Differences in survival of mice were evaluated by Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests. P-values<0.05 were considered to be statistically significant. All statistical tests were performed using GraphPad Prism version 5.00.

Accession numbers for genes and proteins mentioned in the text (NCBI Entrez Gene ID number)

| Gene Name | Accession Number |
|-----------|------------------|
| C. albicans | orf19.1150 (3645322); orf19.1333 (3648169); orf19.2959.1 (no data); GTR1 (3643914); BRAH (3640528); orf19.5848 (3647594); orf19.6200 (3639533); DUR31 (3646965); orf19.6847 (3641499); orf19.7670 (3638948); orf19.938 (3647183); DUR3 (3644760); SSAI (3636229); SXL4 (3644711); MKCI (3639710); AT05 (3643652). |

Supporting Information

**Figure S1** dur31Δ/Δ has normal adherence and invasion properties upon contact with oral epithelial cells.

(A) Adherence assays were performed using ibidi μ-Slides VI0.4. Confluent epithelial cell monolayers were infected with 1.5×10^5 C. albicans cells for 45 min. Monolayers were then thoroughly washed 3× with PBS to remove non-adhered fungal cells and fixed with 4% paraformaldehyde. C. albicans cells were subsequently stained with calcein amalgam and quantified by fluorescence microscopy. The number of adhered cells was determined by counting at least 50 high power fields of 200 μm×200 μm size. Results are the mean ± SEM of two independent experiments, each performed in duplicate. (B) Invasion of dur31Δ/Δ mutant cells into human-derived oral epithelial cells is comparable to that of the wild type. Monolayers of confluent epithelial cells were infected with 10^5 C. albicans yeast cells and incubated for 3 hours at 37°C and 5% CO₂. After washing with PBS, cells were fixed with 4% paraformaldehyde. Fungal cells were then stained for 45 min with fluorescein-conjugated concanavalin A (ConA). Epithelial cells were then permeabilized with 1% Triton X-100. Next, fungal cells were stained with calcein amalgam. Fluorescence microscopy was performed using appropriate filter sets for detection of Con A (stains only the extracellular, non-invaded fungal elements) and calcein amalgam (stains both invaded and non-invaded fungal elements). At least 100 C. albicans cells were examined for each strain and the invasion rate was expressed as percentage of invaded cells divided by the number of invaded plus non-invaded cells. Results are the mean ± SEM of two independent experiments, each performed in duplicate. (TIF)

**Figure S2** dur31Δ/Δ exhibits normal filament formation in liquid media and upon contact with oral epithelial cells. (A) Wild type (Wt), dur31Δ/Δ and dur31Δ/Δ::DUR31 filament formation in liquid RPMI101 and 10% serum. Overnight cultures were diluted 1:2500 into RPMI1640, and water supplemented with 10% fetal bovine serum in 24-well cell
culture plates and incubated at 37°C in presence of 5% CO2. Cells were photographed after 4 h. Scale bar: 20 μm. (B) Filament formation on epithelial monolayers. TR146 epithelial cells were cultured to confluency and infected with C. albicans wild type (Wt) and dur3Δ/Δ mutant cells were grown in RPMI medium at 37°C and 5% CO2 for 90 min, washed, fixed and then stained with a mouse anti-Hsp70 monoclonal antibody (primary) and an Alexa Fluor 555-conjugated goat anti-mouse antibody (secondary). Cells were counterstained with Alexa Fluor 647-conjugated concanavalin A to label the cell surface. (A) Fluorescent microscopic images of anti-Hsp70 antibody and anti-C. albicans cell surface antibody. The merged images show co-localization (yellow) of the anti-Hsp70 antibody and anti-C. albicans cell surface antibody. (B) Fluorescent intensity of different cross sections of the filaments shown in the merged images in panel (A). The green lines represent the fluorescent intensity of the anti-Hsp70 antibody and the red lines represent the fluorescent intensity of the anti-C. albicans cell surface antibody. The letters (a-d) denote the positions of the cross sections. (B) Graph A at which the fluorescent intensities were measured. (C) Western blotting with an anti-Hsp70 antibody for detection of Ssa1/2 (two bands) in cell wall extracts of the wild type (Wt) and dur3Δ/Δ mutant. The blot was stripped and re-probed for α-tubulin as a loading control. (TIF)

Figure S3 Expression levels of Ssa1/2 on the surface of dur3Δ/Δ are comparable to those of the wild type. Wild type (Wt) and dur3Δ/Δ mutant cells were grown in RPMI medium at 37°C and 5% CO2 for 90 min, washed, fixed and then stained with a mouse anti-Hsp70 monoclonal antibody (primary) and an Alexa Fluor 555-conjugated goat anti-mouse antibody (secondary). Cells were counterstained with Alexa Fluor 647-conjugated concanavalin A to label the cell surface. (A) Fluorescent microscopic images of anti-Hsp70 antibody and anti-C. albicans cell surface antibody. The merged images show co-localization (yellow) of the anti-Hsp70 antibody and anti-C. albicans cell surface antibody. (B) Fluorescent intensity of different cross sections of the filaments shown in the merged images in panel (A). The green lines represent the fluorescent intensity of the anti-Hsp70 antibody and the red lines represent the fluorescent intensity of the anti-C. albicans cell surface antibody. The letters (a-d) denote the positions of the cross sections. (B) Graph A at which the fluorescent intensities were measured. (C) Western blotting with an anti-Hsp70 antibody for detection of Ssa1/2 (two bands) in cell wall extracts of the wild type (Wt) and dur3Δ/Δ mutant. The blot was stripped and re-probed for α-tubulin as a loading control. (TIF)

Figure S4 Phylogenetic relatedness of C. albicans orf19.6656 (Dur3) with other orthologous proteins and the urea transporter Dur3. The tree was generated using the Clustal W method in the DNAStar Lasergene MegAlign sequence analysis software. All sequences were retrieved from CGD's (Candida Genome Database) Multi-Genome Search database and SGD's (Saccharomyces Genome Database) Fungal Genomes Search database using C. albicans orf19.6656 (Dur3) or orf19.781 (Dur3) as protein query sequence. Selected fungal species encoding Dur3 are shown. C. dubliniensis CDS6_53230; C. albicans orf19.6656; C. tropicalis CTRG_05438; C. parapsilosis CFPG_05452; L. elongisporus LELO_03888; D. hansenii DEHA2E22396g; P. stipitus PICST_06304; C. boudieri CLIU_04732; C. glabrata CAGL000613g; K. lactis KLA0011913g; C. inomitis CIMG_00418; N. crassa NCU01977.1; M. gulosus MGL_0550; U. maydis UM02953.1. Asterisks indicate putative C. albicans Dur3 orthologues. A. nidulans AN0418; A. terreus ATEG_06209; A. niger An0103790; F. magniatu ureA (Dur3); A. clavatus ACLA_029800; M. oryzae MGCH7_ch7g2226; S. pombe SPBC225G7.13c; C. gattii CGB_J2070C. Dur31 orthologues of CUG-clade species are marked in red. Species marked in green were selected amongst the best hits to the C. albicans Dur3 (orf19.781) protein sequence. C. albicans Dur31 is marked in bold. (TIF)

Figure S5 Deletion of both DUR31 (orf19.6656) alleles in C. albicans. The correct deletion of DUR31 was confirmed by Southern blot analysis. Strains BW17 (Wt), dur3Δ1, dur3/Δ1 and dur3Δ/Δ were analyzed. A 354 base-pair (bp) PCR product, with C. albicans SC5314 genomic DNA as template, was used as a probe on HindIII-digested genomic DNA. (A) Expected band sizes are: 2927 bp (wild type DUR31), 1692 bp (ARG4-deletion-cassette) and 2173 bp (HIS1-deletion-cassette), (B) Southern blot. (TIF)

Table S1 Transcriptional upregulation of C. albicans unknown function genes during oral in vivo and in vitro infections. (DOC)

Table S2 Primers used in this study. (DOC)

Acknowledgments

We would like to thank Ursula Stockel, Birgit Weber and Silvia Slesiona for assistance with the murine infection experiments. Furthermore, we would like to thank Hans-Martin Dahse and Peter Zipfel for providing endothelial cells and Cancer Research Technology for providing the TR146 cell line. We thank Carol Kumamoto for providing the mka1Δ/Δ mutant strain. Finally, we would like to acknowledge Sascha Brunke, Lydia Schild, Anja Lautich, Jessica Voigt and Ronny Martin for helpful discussions.

Author Contributions

Conceived and designed the experiments: FLM DW IDJ KM. Performed the experiments: FLM DW IDJ KG KM. Analyzed the data: FLM DW IDJ KM. Wrote the paper: FLM DW BH.
45. Torres SR, Garzino-Demo A, Meller TF, Meeks V, Jbara-Rizk MA (2009) The fungal Ras-protein kinase A pathway in governing epithelial cell interactions of Candida albicans with endothelial cells. PLoS Pathog 5: e1000100.

46.トルバおよびC. albicans表面の非伝統的なATP結合部位を特定。Mol Microbiol 70: 1246–1260.

47. Ruppink HT, Rowlett C, Lane EB, Steele JT, Treplisowicz LK, et al. (2015) Characteristics of Four new human cell lines derived from squamous cell carcinoma. J Cell Physiol 230: 2335–2345.

48. Itoh S, Matsueda T, Takeda T, Kato M, Nakanishi M, et al. (2009) Candida albicans cell wall sa proteins bind and facilitate import of salivary histatin 5 required for toxicity. J Biol Chem 284: 22453–22463.

49. Sreekumar MS, Armau CD, Costanzo MC, Inglis DO, Shah P, et al. (2010) New tools at the Candida Genome Database: biochemical pathways and full-text literature database. Nucleic Acids Res 38: D428–D432.

50. Wilson D, Thesew S, Zakikhany K, Fradin C, Albrecht A, et al. (2009) Identifying infection-associated genes of Candida albicans in the postgenomic era. FEMS Yeast Res 9: 198–210.

51. Chang LY, Sheppard DC, Bruno VM, Mitchell AP, Edwards JE, Jr., et al. (2007) Candida albicans protein kinase CK2 governs virulence during oropharyngeal candidiasis. Cell Microbiol 9: 233–243.

52. Filler SG, Sheppard DC (2006) Fungal invasion of normally non-phagocytic host cells. PLoS Pathog 2: e129.

53. Fradin C, De Groot P, MacCallum D, Schaller M, Klis F, et al. (2005) Granulocytes govern the transcriptional response, morphology and proliferation of Candida albicans in human blood. Mol Microbiol 56: 397–415.

54. Perlroth J, Choi B, Spellberg B (2007) Nosocomial fungal infections—epidemiology, diagnosis, and treatment. Med Mycol 45: 321–346.

55. Calderone RA (2002) Candida and Candidiasis. Washington D.C.: ASM Press.

56. Pfaller MA, Jones RN, Messer SA, Edmond MB, Wenzel RP (1998) National surveillance of nosocomial bloodstream infection due to Candida albicans: frequency of occurrence and antifungal susceptibility in the SCOPe Program. Diagn Microbiol Infect Dis 31: 327–332.

57. Wieglinghoff H, Bischoff T, Talleau SM, Seifert H, Wenzel RP, et al. (2004) Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin Infect Dis 39: 309–317.

58. Natan C, Moriyama NP, Jacobson ID, Heyken A, Donney J, et al. (2011) The Candida albicans-specific gene EED1 encodes a key regulator of hyphal extension. PLoS One 6: e18394.

59. Pham QT, Belanger PH, Filler SG (2000) Role of hyphal formation in interactions of Candida albicans with endothelial cells. Infect Immun 68: 3485–3490.

60. Park H, Myers CL, Sheppard DC, Pham QT, Sanchez AA, et al. (2005) Role of the fungal Ras-protein kinase A pathway in governing epithelial cell interactions during oropharyngeal candidiasis. Cell Microbiol 7: 499–510.

61. Sumrada R, Gorski M, Cooper T (1976) Urea transport-defective strains of Saccharomyces cerevisiae. J Bacteriol 175: 4681–4698.

62. ElBerry HM, Majumdar ML, Cunningham TS, Sumrada RA, Cooper TG (1993) Regulation of the urea active transporter gene (DUR3) in Saccharomyces cerevisiae. FEBS Lett 315: 105–106.

63. Uemura T, Kashiwagi K, Igarsi K (2007) Polymine uptake by DUR3 and SAM3 in Saccharomyces cerevisiae. J Biol Chem 282: 7374–7381.

64. Navarathna DH, Das A, Morschhaeuser J, Roberts DD, Nickerson KW, et al. (2010) Antimicrobial peptide histatin-5 causes a spatially restricted disruption on the Candida albicans surface, allowing rapid entry of the yeast into the host. PLoS Pathog 6: e1000800.

65. Kumamoto CA (2005) A contact-activated kinase signals Candida albicans internalization by translocation, but not endocytosis, is required for fungicidal activity in Candida albicans. Mol Microbiol 77: 354–370.

66. Saito MN, et al. (1993) Regulation of the urea active transporter gene (DUR3) in Saccharomyces cerevisiae. J Bacteriol 175: 4681–4698.

67. Visconto A, Wendland J (2003) An improved transformation protocol for the Saccharomyces cerevisiae genomic library. FEMS Yeast Res 10: 579–586.

68. Mochon AB, Lopez MC, Garcia S, Schmidt A, Spalmann F, et al. (1999) Control of filament formation in Candida albicans by polyanine levels. Infect Immun 67: 4870–4878.

69. Ghosh S, Navaratna DH, Roberts DD, Cooper JT, Atkin AL, et al. (2009) Arginine-induced germ tube formation in Candida albicans is essential for Candida albicans Ssa1/2p is the cell envelope binding protein for human salivary histatin 5. J Biol Chem 284: 20533–20561.