For submission to *Eukaryotic Cell*

Candida albicans specific genes:

distinct roles in host-pathogen interactions

Duncan Wilson[1,¤,#], François L. Mayer[1,§], Pedro Miramón[1,*], Francesco Citiulo,[a] Silvia Slesiona[2], Ilse D. Jacobsen[1,2], and Bernhard Hube[1,3,4]

[1] *Department of Microbial Pathogenicity Mechanisms, Hans-Knoell-Institute, Jena, Germany*

[2] *Research Group Microbial Immunology, Hans-Knoell-Institute, Jena, Germany*

[3] *Center for Sepsis Control and Care, Universitätsklinikum, Jena, Germany*

[4] *Friedrich Schiller University, Jena, Germany*

[¤] *Current address: The Institute of Medical Sciences, Foresterhill, Aberdeen, AB25 2ZD*

[§] *Current address: Michael Smith Laboratories, Vancouver Campus, 2185 East Mall, Vancouver, BC Canada*

[*] *Current address: Department of Microbiology & Molecular Genetics, University of Texas-Houston Medical School, 6431 Fannin Street, MSB 1.209, Houston, Texas 77030*

Running title: *C. albicans* specific genes

[#]Corresponding author

Duncan Wilson

Hans-Knoell-Institute, Microbial Pathogenicity Mechanisms,

Beutenbergstr. 11a, 07743, Jena, Germany

Phone: (+49-3641) 532 1401; Fax: (+49-3641) 532-0810,

Email: Duncan.Wilson@abdn.ac.uk

Abstract

Human fungal pathogens are distributed throughout their kingdom, suggesting that pathogenic potential evolved independently. *Candida albicans* is the most virulent member of the CUG clade of yeasts and a common cause of both superficial and invasive infections. We therefore hypothesised that *C. albicans* possesses distinct pathogenicity mechanisms. *In silico* genome subtraction and comparative transcriptional analysis identified a total of 65 *C. albicans* specific genes (*ASGs*) expressed during infection. Phenotypic characterisation of six *ASG*-null mutants demonstrated that these genes are dispensable for *in vitro* growth, but play defined roles in host pathogen interactions. Based on these analyses, we investigated two *ASGs* in greater detail. An orf19.6688Δ mutant was found to be fully virulent in a mouse model of disseminated candidiasis and to induce higher levels of the proinflammatory cytokine, IL-1β, following incubation with murine macrophages. A *pga16*Δ mutant, on the other hand exhibited attenuated virulence. Moreover we provide evidence that secondary filamentation events (multiple hyphae emerging from a mother cell and hyphal branching) contribute to pathogenicity: *PGA16* deletion did not influence primary hypha formation or extension following contact with epithelial cells; however, multiple hyphae and hyphal branching were strongly reduced. Significantly, these hyphae failed to damage host cells as effectively as the multiple hyphae structures formed by wild type *C. albicans* cells. Together, our data show that species-specific genes of a eukaryotic pathogen can play important roles in pathogenicity.

Introduction

The fungal kingdom is predicted to consists of up to 5.1 million species (1), over 100,000 of which have been confirmed (2). Of these species, few (600) have been reported to infect humans (3) and only a fraction (~10) of those are prevalent aetiological agents (4). One such fungal species, *Candida albicans*, displays a remarkable range of interactions with humans. Primarily, *C. albicans* is a commensal member of the microbial flora of mucosal surfaces; however, it also causes infections at these sites extremely frequently: 75% of women experience at least one episode of vulvovaginal candidiasis, with *C. albicans* being the most dominant species; *C. albicans* is also a leading cause of diaper rash in infants and 90% of untreated HIV positive individuals suffer from oral candidiasis (4, 5). Importantly, *Candida* species are also the third most common cause of nosocomial bloodstream infections and these invasive infections have mortality rates of over 40% (6).

Although a number of *C. albicans* virulence factors, such as the yeast to hypha transition, secretion of extracellular hydrolases and expression of cell surface-associated adhesins have been described (7), their precise roles during the different types and stages of infection remain a hotly debated subject.

Other major fungal pathogens of humans, such as *Cryptococcus neoformans* or *Aspergillus fumigatus*, are distantly related to *C. albicans* and to each other. That is, human pathogens are distributed throughout the fungal kingdom (8). This has lead to the concept that the virulence potential of these species has evolved independently (9). This is supported by the fact that certain virulence-associated factors, such as the capsule of *C. neoformans* and gliotoxin synthesis by *A. fumigatus*, are not shared amongst pathogenic species.

This model of independent virulence evolution led us to explore the possibility that pathogenic fungal species possess unique genes which set them apart from their closer,

less-pathogenic relatives. Using comparative genomic and transcriptomic analyses, we describe 65 genes expressed during infection and unique to *C. albicans*. Further molecular analysis of six of these *C. albicans* specific genes demonstrated that they are indeed involved in host-pathogen interactions, including epithelial and endothelial damage and immune modulation. Surprisingly, all six investigated genes were required for at least one stage of infection.

Materials and methods

Ethics statement

All animal experiments were in accordance 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 Komission nach § 15 Abs. 1 Tierschutzgesetz). The use of human primary cells in this study was conducted in compliance with the principles expressed in the Declaration of Helsinki. All protocols used 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 growth conditions

C. albicans strains used in this study are listed in **Table S2**. The triple-auxotrophic strain BWP17 complemented with plasmid CIp30 was used as wild type control in all experiments. Strains were grown 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. Transformants were selected on SD agar supplemented with arginine, histidine and/or uridine (each 20 µg ml^{-1}) as required. *E. coli* was grown on LB agar [1% bacto-tryptone, 0.5% yeast extract, 1% NaCl, 2% agar] and overnight *E. coli* cultures were cultivated in a shaking incubator at 37°C and 210 rpm. For selection purposes 50 µg/ml ampicillin were added to solid or liquid LB medium.

Strain construction

For homozygous mutant construction, the BWP17 genetic background was used and alleles were sequentially deleted with PCR products based on pFA-HIS1 and pFA-ARG4 plasmids (10). Forward primers were designed to have 104 bp homology to the immediate upstream region of the gene of interest, followed by a 22 bp sequence, which has homology to the pFA plasmids, immediately upstream of the respective selective marker. Similarly, reverse primers were designed to have 104 bp homology to the immediate downstream region of the gene of interest (reverse complemented) followed by 24 bp sequence with homology to the pFA plasmids, immediately downstream of the respective selective marker (**Table S3**). Using these long primers, and either pFA-HIS1 or pFA-ARG4 as template, HIS1 and ARG4 deletion cassettes were generated for each gene of interest. Both copies of each gene were sequentially deleted using these constructs according to the improved *C. albicans* transformation protocol of Walther and Wendland (11) and selecting for either arginine or histidine prototrophy. In each case, correct integration was determined using gene-specific upstream and downstream primers, lying outside the site of homologous recombination to determine absence of wild type copy and presence of ::*HIS1* and ::*ARG4* alleles, as well as *HIS1* and *ARG4* specific internal primers to ensure correct integration of selective markers at

both 5’ and 3’. Resulting uridine auxotrophs with homozygous deletions of the genes of interest were finally transformed with *Nco*I-linearised CIp10 (12) to restore *URA3* to the *RP10* locus.

In the cases of orf19.6688 and *PGA16*, wild type alleles containing the entire upstream intergenic region, coding sequence and either 446 bp (orf19.6688) or 339 bp (*PGA16*) downstream sequence were amplified from SC5314 genomic DNA with PHUSION high fidelity DNA polymerase (FINNZYMES, New England Biolabs) and cloned into *Mlu*I / *Sal*I – digested CIp10. Resulting plasmids were sequenced, linearised with *Nco*I and used to transform the respective of19.6688Δ and *pga16*Δ uridine auxotrophs. To generate the CIp20/*PGA16* plasmid, the *PGA16* sequence was excised from CIp10/*PGA16* with *Mlu*I / *Sal*I and cloned into CIp20 cut with the same restriction enzymes.

PGA16 Heterozygote Reconstruction and Complementation

A *PGA16*-deletion cassette was generated by amplifying *URA3* from pFA-URA3 (10) using primers PGA16-FG and PGA16-RG. The resultant product was used to transform CAI4 and uridine prototrophs selected on SD medium. A resulting *PGA16*/*pga16*::*URA3* heterozygote was transformed with a wild type *PGA16* fragment excised from plasmid CIp10/*PGA16* via digestion with *Mlu*I / *Sal*I, yielding a *PGA16*/*pga16*Δ+*PGA16* complemented strain.

Susceptibility to stressors

Aliquots of overnight SD cultures were washed twice in phosphate buffered saline (PBS) and 10-fold serial dilutions in 5 µl (covering a range of 10^6 – 10^1 cells) were spotted onto SD agar containing 0.4 mM menadione (Sigma), 1.5 M NaCl 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. Each experiment was performed at least twice. Representative pictures are shown.

Endothelial and oral epithelial cells

The human buccal carcinoma derived epithelial cell line TR-146 (Cancer Research Technology, London) and the human umbilical vein derived 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 for 10 min) fetal bovine serum (FBS, PAA). For experiments, TR146 cells were used during passage 10-20 and HUVEC cells during passage 10-40. Both cell lines were cultured in a humidified incubator at 37°C with 5% CO_2 atmosphere. Cultivation medium was replaced by fresh medium every second day and accutase (PAA) was used for detaching cells after confluency had reached approximately 80-100%.

Damage assay

Standard damage assays were performed by measuring the activity of lactate dehydrogenase (LDH) following our previously published protocol (13). Each experiment was performed at least three times in triplicate. For damage kinetics, 12 well plates were seeded with 2×10^5 TR146 cells and incubated at 37°C, 5% CO_2 for 1 day. Monolayers were washed $3\times$ with PBS, the medium replaced with DMEM + 1% FBS and infected with 1×10^5 *C. albicans* cells. Samples were taken at indicated time points and damage assessed by LDH, normalised (fold increase in damage) against time-matched uninfected control wells. The damage kinetics experiment was performed twice in quadruplicate.

Hyphal ramification

To generate *C. albicans* microcolonies, TR146 cells were seeded onto glass coverslips in 12 well plates and incubated at 37°C, 5% CO_2 until confluence. *C. albicans* cells were grown for at least 20 h in YPD to generate predominantly single yeast cells, 50 cells per well were used to infect the monolayers for 16 h. Monolayers were subsequently fixed and fluorescently stained as described previously (14). To quantify earlier hyphal ramification events (multiple hyphae per mother cell and hyphal branching), monolayers were infected in the same manner, but with 1×10^4 *C. albicans* cells for 6 h.

Macrophage killing assay

To analyse killing of *C. albicans* strains by macrophages, human acute monocytic leukemia cells (THP-1) were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (PAA). Monocytes were differentiated into macrophages by addition of 5 µl phorbol 12-myristate 13-acetate (PMA; Enzo Life Sciences) for 24 hours at 37°C and 5% CO_2. Subsequently, a 24-well plate was seeded with 4×10^5 macrophages per well and incubated for 24 hours at 37°C and 5% CO_2. Twenty *C. albicans* cells per well were then added to the macrophages and incubated for two days. Experiments were performed in dodecaplicate on three independent occasions. *C. albicans* cells in medium only served as positive control.

Cytokine measurements

To determine cytokine stimulation by *C. albicans*, the murine peritoneal macrophage J774.2 cell line was used. 1×10^6 J774.2 cells were seeded into 6 well plates in DMEM + 10% heat-inactivated FBS and incubated at 37°C, 5% CO_2 for 1 day. The cells were then washed 2 ×

with PBS and the medium replaced with DMEM + 1% FBS. The macrophages were then infected with 1×10^6 *C. albicans* cells per well, incubated for a further 24 h and supernatants used for determining cytokine release. Cytokine (IL-1β, TNF-α and GM-CSF) measurements were performed by ELISA (eBioScience). The experiment was performed three times in triplicate.

Mouse model of hematogenously disseminated candidiasis

Six to eight weeks old female Balb/C mice (*Mus musculus*) (18-20 g; Charles River, Germany) were used for infection experiments. The mice 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). Animals were challenged intravenously on day 0 with 2.5×10^4 cfu/g body weight 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 daily. Mice displaying severe signs of illness such as 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. Left kidneys were collected for histology and fixed with buffered formalin. Paraffin-embedded sections were stained with Periodic acid-Schiff (PAS) according to standard protocols.

Statistics

Differences in damage of host cells by the different *C. albicans* strains were compared by two-tailed, type three Student's t-test. The statistical analysis for the susceptibility of *C. albicans* strains to killing by macrophages was performed using one-way ANOVA followed by Tukey's test. Differences in survival of mice infected with the different *C. albicans* strains 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.

Results

Identification of *C. albicans* specific genes by comparative genome subtraction

Based on the hypothesis that pathogenic potential evolved independently in fungal pathogens, we reasoned that genes unique to the major human pathogenic yeast, *C. albicans* may be important for infection. Although *C. albicans* belongs to the CUG clade, which comprises several other human pathogens (*Candida tropicalis*, *Candida parapsilosis, Candida guilliermondii* and *Candida lusitaniae*), the generally lower pathogenic potential of these other species suggests that *C. albicans*-specific factors may contribute to its higher virulence. *C. dubliniensis* on the other hand, although less virulent than *C. albicans* in murine infection models, is, genetically, very closely related, and the two species share many important phenotypic attributes, such as the ability to undergo the yeast-to-hypha transition. Due to the fundamental importance of morphogenesis in *C. albicans* biology, we decided to define *C. albicans* specific genes as those lacking orthologues in any other sequenced organism, with the exception of *C. dubliniensis*.

230 "Orthologous genes" were strictly defined as those with a BLASTp score of >40 in any other
231 sequenced organism. First, a comparative genomic subtraction was performed using
232 FindTarget software at CandidaDB ((15) http://genodb.pasteur.fr/cgi-
233 bin/WebObjects/CandidaDB). *C. albicans* SC5314 was set as the query genome and
234 *C. albicans* WO-1 as a reference genome. *Candida tropicalis*, *Candida lusitaniae*, *Candida*
235 *guilliermondii*, *Lodderomyces elongisporus*, *Debaryomyces hansenii*, *Pichia stipitis* and
236 *Saccharomyces cerevisiae* were set as the exclusion genome list. Both the selection and
237 exclusion criteria were set as 40 (score). The resulting genome subtraction yielded 254 genes
238 without sequence similarity in the non-*albicans* species. The predicted protein sequences of
239 these genes were batch downloaded from the *Candida* Genome Database ((16) CGD -
240 http://www.candidagenome.org/) and subsequently compared to fungal genomes held by
241 the BROAD Institute (http://www.broadinstitute.org/annotation/fungi/fgi/). Eleven of the
242 254 genes displayed a BLASTp score of >40 in other fungal species and were manually
243 removed. The remaining 243 genes were then analysed using BLASTp at NCBI
244 (http://blast.ncbi.nlm.nih.gov/Blast.cgi), yielding no hits to other species. We term these
245 *ASGs* for *C. albicans* specific genes. It should be noted that this set of genes may share
246 orthologues in species which have not yet been sequenced.
247

248 ## *C. albicans* specific genes are expressed during infection

249 According to Molecular Koch's postulates, virulence factors must be expressed during
250 infection (17). The expression profile of the 243 *ASGs* during models of oral (18) and liver
251 (19) infection was therefore analysed using GeneSpring software. Expression data was
252 present for only 65 of the 243 *ASGs*. Although it is likely that some of the *ASGs* with no
253 detectable expression were simply not expressed in these models or were absent for

technical reasons, we could not rule out that some of them may be mis-annotated open reading frames and did not represent *bone fide* genes. We therefore focused our analysis on the 65 *ASGs* which were expressed. Interestingly, one third of the *ASGs* exhibited statistically significant ($P < 0.05$) induction (> 1.5-fold up-regulation) during infection; amongst these, 14 genes were up-regulated > 2-fold (**Table S1**). This suggested that these *ASGs* may play roles during *C. albicans*-host interactions.

In silico prediction of sub-cellular localisation

Because of the very nature of their identification, we did not expect to identify conserved functional domains in the *ASG* sequences. Nevertheless the predicted protein sequences of the 65 expressed *ASGs* were scrutinised with a range of bioinformatic tools. Initial sub-cellular localisation prediction using WoLF PSORT (http://wolfpsort.org/) suggested the presence of two cytosolic-, 14 extracellular-, one golgi-, 11 mitochondrial-, 29 nuclear-, and six plasma membrane- localised proteins encoded amongst the *C. albicans* specific genes (**Table 1**). Only one gene had a predicted functional motif; this was orf19.3738/*PGA22* which possesses a putative aspartyl protease motif.

Amongst the 14 extracellular proteins, five possessed predicted GPI-anchors. As the *C. albicans* genome (6524 genes) encodes 104 predicted GPI-anchored proteins (20), this represents a significant enrichment of GPI-anchored proteins amongst the *ASG* set ($P = 0.0054$ by Fisher's exact test). This enrichment in cell surface-localised protein encoding genes amongst the *ASGs* fits with the concept of the microbial cell surface as a more rapidly evolving cellular structure and may promote cell surface diversity (21).

Genomic location

In *A. fumigatus*, infection-associated genes have been shown to cluster at sub-telomeric regions of the genome (22). All 65 *C. albicans* specific genes were therefore plotted as a function of genomic location. As shown in **Fig. 1**, *ASGs* were not restricted to any particular genomic locale, but distributed throughout the eight chromosomes with evidence of four clusters (indicated by asterisks). Interestingly, 13 (20%) were situated on chromosome 5, representing a significant enrichment of *ASGs* on this chromosome (P = 0.0085 by Fisher's exact test).

C. albicans specific gene families

Many *C. albicans* genes which are associated with infection belong to families, such as the *ALS* (agglutinin like sequence), *SAP* (secreted aspartic protease) and *LIP* (lipase) gene families (7, 23). We reasoned that if *C. albicans* specific genes were under positive selection in the ecological niche of the warm-blooded host, they may be amplified by gene duplication events. All 65 predicted protein sequences were subjected to multiple alignment using ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Seven genes yielded alignment scores of >25 and clustered into three groups: (i) orf19.3908, orf19.4691 and orf19.3906; (ii) orf19.3376 and orf19.3378; (iii) orf19.1266 and orf19.5246. These seven genes were individually analysed by BLAST at the *Candida* Genome Database and aligned using ClustalW2 (**Fig. 2**), confirming sequence similarity. Groups (i) and (ii) encode predicted extracellular proteins and group (iii) encodes a predicted nuclear (orf19.5246) and cytoplasmic (orf19.1266) protein (**Table 1**). The presence of these paralogues suggests that some of the *C. albicans* specific genes have undergone duplication and may therefore be under positive selection.

Six *C. albicans* specific genes are dispensable for *in vitro* growth

In order to characterise the *ASGs* in greater detail, 8 genes, which displayed differential expression patterns during infection (see above and **Table S1**) were selected for further molecular analysis. These were: orf19.3908, orf19.4055, orf19.4691, orf19.5057, orf19.6534, orf19.6688, orf19.7170 and orf19.848 (*PGA16*).

Homozygous mutants lacking six genes (attempts to delete the second copy of orf19.4055 and orf19.5057 were unsuccessful) were successfully generated and tested under a variety of *in vitro* growth conditions on agar-containing media. All strains grew at rates similar to the wild type control (BWP17 + CIp30) on minimal (SD) and rich (YPD) media at 37°C. All mutants also exhibited wild type levels of growth under oxidative, osmotic, cell wall and thermal stress, with the exception of *pga16*Δ which was moderately more sensitive to cell wall and thermal stress. Furthermore, all mutants formed normal filamentous colonies following growth at 37°C on agar containing 10% foetal bovine serum, Spider medium, Lee's medium and embedded in YP-sucrose agar and in RPMI in contact with a plastic surface (data not shown). These data suggest that in general, *ASGs* are not required for normal growth under standard *in vitro* conditions.

C. albicans specific genes are required for host-interactions

As far as it is known, *C. albicans* spends its entire natural lifecycle in association with warm-blooded animals, normally as a member of the commensal microbial flora. Under certain pre-disposing conditions however, the fungus is able to overgrow the microbiota, causing superficial infections on skin or mucosal surfaces and damaging the local tissue. Furthermore, if *C. albicans* reaches the bloodstream of susceptible individuals, life-

threatening disseminated candidiasis can occur. During both superficial and invasive infections, the fungus must also be capable of resisting local immune responses. As an initial assessment of the roles of *C. albicans* specific genes during these different stages of candidiasis, wild type and *ASG*-null mutant strains were tested using *in vitro* infection models reflecting oral infection, endothelial disruption (representing a key stage of disseminated candidiasis) and immune cell-interaction.

As a superficial infection model, 2×10^4 wild type or *ASG*-null mutant cells were used to infect monolayers of human oral epithelial cells (TR146) in 96 well plates and epithelial damage was assessed following 15 h infection as compared to 100% lysis (uninfected monolayer treated with 0.1% Triton X-100). Following infection, wild type *C. albicans* caused damage equivalent to lysis of 44.2% of the epithelial monolayer. Deletion of the paralogous genes, orf19.3908 or orf19.4691, encoding predicted integral membrane proteins, caused a moderate but significant reduction in damage. Deletion of orf19.6688 (encoding the predicted nuclear protein) and orf19.848 (*PGA16*) (encoding a predicted cell surface protein) elicited even stronger reductions in epithelial damage (**Fig. 3A**).

To investigate the epithelial damage potential of orf19.6688Δ and *pga16*Δ in more detail, a time course infection was performed. For these experiments, TR146 cells were grown in 12 well plates and infected with 10^5 *C. albicans* cells; samples were taken at 2 hour intervals and damage assessed by measuring LDH release. As shown in **Fig. 3B**, infection with wild type cells resulted in an effectively linear increase in epithelial damage during this time course. Strikingly, both orf19.6688Δ and *pga16*Δ caused substantially less epithelial damage, even after extended (24 h) infection.

The ability of *C. albicans* to grow as filamentous hyphae is thought to contribute to pathogenesis, as mutants with morphological defects often display attenuated virulence. Epithelial monolayers were infected with low numbers of wild type, orf19.6688Δ or *pga16*Δ cells (< 100 cells per 12 well plate monolayer (24)) and microcolony development visualised after 15 h incubation by fluorescent microscopy. Both wild type and orf19.6688Δ formed symmetrical microcolonies with radial hyphal growth patterns of similar appearance. Moreover, the radial hyphae of both wild type and orf19.6688Δ strains invaded the epithelium, as demonstrated by a differential staining protocol (14). *pga16*Δ cells, on the other hand formed aberrant, asymmetrical microcolonies (**Fig. 3C**).

Following fungal access to, and dispersal throughout the bloodstream, *C. albicans* must next traverse the endothelial lining of blood vessels to infect deep-seated organs. To assess the roles of the *ASGs* in this stage of disseminated candidiasis, wild type and mutant cells were used to infect HUVEC endothelial cells and endothelial damage was assayed following 24 h infection. Deletion of either orf19.3908 or orf19.7170 caused a moderate but significant reduction in endothelial damage (**Fig. 4**). As was the case for oral epithelial cells, both orf19.6688Δ and *pga16*Δ caused substantially less endothelial damage compared to the wild type.

Taken together, these data demonstrate an important role for *ASGs* for damage of host cells: with the exception of orf19.6534, all analysed genes were required for optimal epithelial and/or endothelial damage. Deletion of orf19.6688 and *PGA16* resulted in particularly strong defects in host cell damage.

Although sometimes associated with immune deficiencies (e.g. neutropenia), the majority (80%) of patients who develop disseminated candidiasis are not immuno-suppressed in the classical sense (6). *In vivo* therefore, *C. albicans* must be capable of resisting attack by immune cells such as macrophages and neutrophils. Therefore, the ability of mutants to survive interaction with THP-1 macrophages was determined. The killing potential of THP-1 macrophages was found to be relatively low and variable (up to 30% killing for wild type). Despite this, both orf19.6534Δ and orf19.6688Δ exhibited reproducibly reduced survival following co-incubation with macrophages compared to the wild type control (**Fig. 5**) and survival of orf19.6688Δ was significantly lower ($P = 0.0224$).

Together these data suggest that the *C. albicans* specific genes tested here are not generally required for growth under many standard laboratory conditions, but may play key roles during host pathogen interactions. Indeed, all six analysed genes were required for wild type behaviour in at least one of the infection models used here. Notably, deletion of orf19.6688 and *PGA16* resulted in particularly strong alterations in host-pathogen interactions.

orf19.6688 and *PGA16* play differential roles during disseminated candidiasis

Because of their mutant phenotypes during host pathogen interactions, we decided to further investigate the roles of orf19.6688 and *PGA16* during systemic infection. orf19.6688Δ+orf19.6688 and *pga16+PGA16* complemented strains were therefore constructed by reinserting a single copy of the gene of interest to the RP10 locus of the respective homozygous mutants using CIp10 (12).

The virulence of both orf19.6688Δ and *pga16*Δ mutants was assessed in an intravenous murine model of acute haematogenous disseminated candidiasis. As disseminated candidiasis can result in a febrile state, growth at 39.1°C in YPD was first tested to ensure that all strains were capable of growing at potentially elevated *in vivo* temperatures. All strains had a similar generation time at this temperature (data not shown).

Ten female BALB/c mice (5-6 week old; 18-20 g; Charles River, Germany) per strain were infected intravenously with 2.5×10^4 colony forming units (cfu)/g body weight as described in *Materials and Methods* and survival monitored. Surprisingly, despite exhibiting reduced *in vitro* damage of epithelium and endothelium, and reduced survival in the presence of macrophages, the orf19.6688Δ mutant was fully virulent in the mouse model of disseminated candidiasis. Indeed, although the mean survival times of mice infected with wild type and orf19.6688Δ strains were similar (**Fig. 6A**), post-mortem examination revealed more severe kidney pathologies in mice infected with orf19.6688Δ (**Fig. 6B**) and the orf19.6688Δ foci of infection were associated with high levels of granulocyte infiltration. These pathological observations suggest that orf19.6688 may actually have a negative impact on virulence during systemic candidiasis, possibly by dampening inflammation.

In contrast, mice infected with *pga16*Δ cells survived for longer than those infected with wild type cells. Indeed, 50% of *pga16*Δ-infected mice survived to the end of the experiment (21 days post infection). Complementation of *pga16*Δ with a single wild type allele significantly increased virulence, but not to wild type levels (**Fig. 7A**). These data indicate that *C. albicans* requires both copies of *PGA16* for full virulence. Histological analysis of the kidneys of *pga16*Δ-infected mice indicted the presence of aberrant filamentous morphologies (**Fig. 7B**).

orf19.6688Δ modulates macrophage IL-1β expression

In vivo, orf19.6688Δ cells attracted high levels of granulocyte infiltrates (**Fig. 6B**). As polymorphonuclear cells are known to play an important role during *C. albicans* infection, we sought to determine whether orf19.6688 influenced *C. albicans* interaction with neutrophils. Fungal survival was therefore assessed following 3 h co-incubation with human neutrophils (25). However, wild type, orf19.6688Δ and orf19.6688Δ+ orf19.6688 strains all exhibited similar survival (data not shown).

Following intravenous infection with virulent *C. albicans* strains, mice die of progressive sepsis (26), a condition associated with a severe proinflammatory cytokine response (27). Monocyte-dependent cytokines, such as IL-1 and TNFα, are predominantly responsible for inflammation during disseminated candidiasis (28). Similarly, *in vitro* macrophage recognition and subsequent killing of *C. albicans* is mediated by proinflammatory cytokine production (29). orf19.6688Δ exhibited reduced survival following co-incubation with a human macrophage cell line (**Fig. 5**) and appeared to cause more severe kidney pathology following intravenous murine infection (**Fig. 6B**).

We therefore hypothesised that orf19.6688 may play a role in immune interaction. Specifically, we predicted that deletion of orf19.6688 may result in elevated production of proinflammatory cytokines. A murine macrophage-like cell line was chosen to investigate this hypothesis to reflect the situation during experimental systemic candidiasis. Three cytokines were selected for analysis. These were TNFα, IL-1β and GM-CSF.

All strains induced high levels of TNFα, similar to an LPS positive control. GM-CSF stimulation by all three strains, on the other hand, was very low (around the detection limit). This was

not due to an inability of the macrophage-like cells to produce GM-CSF because stimulation with 1 µg LPS resulted in the release of 23.2±3.2 pg/ml GM-CSF (data not shown).

Fig. 8 shows that while wild type *C. albicans* elicited release of 2.8 pg/ml IL-1β, orf19.6688Δ stimulated the release of 9.0 pg/ml. Complementation of orf19.6688Δ with a single wild type copy of orf19.6688 reduced IL-1β production to 6.5 pg/ml.

Therefore, orf19.6688 deletion results in significantly higher activation of IL-1β, but does not appear to elicit a universal, non-specific up-regulation of proinflammatory cytokines and does not influence killing by neutrophils. Taken together, our data suggests that orf19.6688 may be involve in immune modulation.

PGA16 mediates hyphal ramification

As *PGA16* was required for damage of human cells *in vitro* and virulence in mice, we sought to characterise the *pga16*Δ mutant in greater detail. Although dispensable for initial hypha formation (data not shown), *pga16*Δ formed aberrant hyphal microcolonies on epithelial monolayers (**Fig. 3C**). Indeed, the microcolonies formed by *pga16*Δ were asymmetrical and the progenitor mother cell was often visible, distal from the colony centre. Given these microcolony characteristics, we hypothesised that Pga16 may be involved in the ramification of microcolonies. In order to assess this quantitatively, we incubated wild type or *pga16*Δ cells on epithelial monolayers (or under identical conditions without human cells) for 6 h and assessed the numbers of hyphae emerging from each mother cell (2° hyphae) and the numbers of hyphae emerging from each primary hypha (branches). Under control conditions (without epithelial cells), the majority of wild type and *pga16*Δ cells formed a single primary hypha with very few branches (**Fig. 9A,B**). Interestingly, incubation of wild type *C. albicans* in the presence of epithelial cells stimulated the production of secondary and tertiary hyphae

466 from mother cells and increased the degree of hyphal branching. In contrast, the presence of
467 epithelium did not stimulate such secondary filamentation events by *pga16*Δ cells. These
468 cells grew predominantly as a single primary hypha, with low numbers of secondary hyphae
469 emerging from mother cells and a lesser degree of hyphal branching (**Fig. 9A,B**).
470 We propose that reduced ramification events (secondary hyphae and branches) account for
471 the aberrant colony morphology and reduced epithelial damage caused by the *pga16*Δ
472 mutant (**Fig. 3**). However, analysis of the *pga16*Δ+*PGA16* revertant strain revealed similar
473 low levels of secondary hyphae and branches. Indeed, *pga16*Δ+*PGA16* also caused the same
474 (low) level of epithelial damage as the *pga16*Δ homozygous mutant (data not shown). We
475 therefore transformed the *PGA16*/*pga16*Δ heterozygous mutant with plasmid CIp20 (to
476 restore outstanding uridine and histidine auxotrophies) and tested the epithelial damage
477 potential of this heterozygous strain. *PGA16*/*pga16*Δ+CIp20 failed to form secondary hyphae
478 and exhibited the same epithelial damage as the *pga16*Δ mutant (data not shown),
479 indicating that *C. albicans* may require two copies of *PGA16* to damage epithelial cells. We
480 therefore transformed *PGA16*/*pga16*Δ with the CIp10/*PGA16* complementation plasmid and
481 assessed epithelial interactions. Again, this strain failed to form secondary hyphae and
482 caused the same degree of epithelial damage as the *pga16*Δ homozygous strain. We
483 postulated that the outstanding histidine auxotrophy of *PGA16*/*pga16*Δ+CIp10/*PGA16* may
484 influence interactions with epithelial cells. We therefore sub-cloned *PGA16* into CIp20
485 (which restores both uridine and histidine auxotrophies) and complemented the
486 *PGA16*/*pga16*Δ heterozygous strain. Again, this heterozygous complemented strain
487 exhibited similar low levels of secondary hyphae formation and epithelial damage (data not
488 shown).
489

Ectopic integration of a gene may impair transcriptional regulation and therefore function. We therefore designed a strategy to generate a heterozygous complemented strain with both copies of *PGA16* expressed from its native loci. We deleted a single copy of *PGA16* in the CAI-4 (uridine auxotrophic) genetic background with a *URA3*-based deletion cassette. Reassuringly, this strain exhibited defective hyphal ramification and reduced epithelial damage. We then replaced the *pga16*::*URA3* allele via transformation with a wild type copy of *PGA16* and selection with 5-fluoroorotic acid (5-FOA). Reconstitution with a second copy of *PGA16* to the *pga16*Δ locus restored epithelial damage to wild type levels (**Fig. 10**), indicating that two copies of *PGA16* are indeed required for epithelial damage and that these two copies are required to be at the native locus for this phenotype.

Discussion

In this study we hypothesised that *C. albicans* possesses distinct pathogenicity mechanisms represented by the presence of genes unique to this fungus. We describe 65 such species-specific genes, which are expressed during infection (**Table 1 and S1**). Of the subset we analysed, several of these genes were required for optimal host pathogen interactions, but dispensable for *in vitro* growth. For orf19.6688, which is predicted to encode a nuclear-localised protein, we demonstrate a role in modulating the proinflammatory response of macrophages. For Pga16, a predicted GPI-anchored protein, we demonstrate roles in secondary filamentation events, microcolony ramification, epithelial damage and virulence *in vivo*.

With the advent of genome sequencing of numerous pathogenic and, importantly, non-pathogenic fungal species, comparative genomics can now be employed to identify

514 pathogen-specific factors (30, 31). In the current study we have taken advantage of these
515 recently released genomes to characterise the role of *C. albicans* specific genes during
516 infection.
517 Here we defined *C. albicans* specific genes using both comparative genomic and
518 transcriptomics criteria as: (1) genes without sequence similarity in any other species, with
519 the exception of *C. dubliniensis*; (2) expressed during intraperitoneal mouse infection or oral
520 infection (RHE and/or samples from patients suffering from oral candidiasis) (18, 19).
521 *C. dubliniensis* was not included in the exclusion criteria because this species can, like
522 *C. albicans*, form hyphae. *C. albicans* hyphae are the dominant invasive morphology and
523 hypha formation is widely believed to be an important virulence attribute (32, 33).
524
525 On the sequence level, our *C. albicans* specific gene set displayed a number of interesting
526 features. The entire *C. albicans* genome (>6500 genes) encodes 283 predicted soluble
527 secreted proteins (34) and 104 predicted GPI proteins (20). Of the *C. albicans* specific gene
528 set, fourteen (21.5%) encode proteins with predicted signal peptides. Five of these likely
529 remain attached to the cell surface via a GPI anchor and two may be retained in the
530 membrane as they possesses more than one transmembrane helix. The remaining seven are
531 likely secreted to the extracellular space as they contain no retention motifs (**Table 1**). An
532 additional four genes encode predicted plasma membrane proteins, but lack signal peptides.
533 This indicates that a large proportion of these unique genes encode proteins which may
534 directly interact with the [host] environment of *C. albicans*. This hypothesis is supported by
535 our functional analysis: of the six mutants analysed in this study, five lacked genes encoding
536 predicted secreted or cell surface proteins: all five mutants displayed defective interactions
537 in at least one infection model.

538 Interestingly, almost half (29) of the *ASGs* encode predicted nuclear proteins, suggesting that
539 *C. albicans* may possess a number of unique proteins with regulatory roles, possibly fine-
540 tuning transcriptional responses. In the current study, one such predicted nuclear protein-
541 encoding gene was identified and characterised (orf19.6688). Initial infection model
542 screening suggested a role for orf19.6688 in both tissue damage (**Fig. 3 and 4**) and immune
543 evasion (**Fig. 5**). Despite these *in vitro* phenotypes, *in vivo*, deletion of orf19.6688 did not
544 attenuate virulence in a murine model of systemic infection. Further dissection revealed that
545 although orf19.6688Δ was killed by neutrophils normally, the mutant elicited significantly
546 higher release of the proinflammatory cytokine, IL-1β from murine macrophages.
547
548 Recognition of *C. albicans* by macrophages is mediated by an array of receptors (29)
549 including Dectin-1 (35) and Dectin-2 receptors (36), as well as TLR-2 and TLR-4 (37, 38),
550 which recognise fungal pattern associated molecular patterns (PAMPs) such as β-1,3-glucan
551 (35) – although the exact roles of Toll-like receptors remains debatable (39). Following
552 recognition, IL-1β is synthesised via the NLRP3 inflammasome (40). Although β-1,3-glucan
553 represents a major PAMP, other fungal factors also stimulate cytokine production – for
554 example, the secreted aspartic proteases, Sap1,2,3 and 6 have recently been demonstrated
555 to induce IL-1β production by human monocytes (41). The observed up-regulation of IL-1β
556 by macrophages exposed to orf19.6688Δ indicates that orf19.6688 may play a role in
557 down-regulating the expression, exposure or secretion of antigenic fungal components. Such
558 a strategy would be advantageous for a commensal organism and its host, where neither
559 party would benefit from potentially destructive inflammation. In this respect, it will be
560 intriguing to evaluate the role of unique genes, such as orf19.6688, during commensal
561 carriage of *C. albicans*.

Pga16, on the other hand was required for epithelial and endothelial damage, as well as virulence in a mouse model of systemic candidiasis. Although the molecular function of Pga16 remains unclear at this stage and will require further investigation, we show that this protein is required for epithelial-induced hyphal ramification.

We have recently reported that the presence of an epithelial monolayer stimulates *C. albicans* to form multiple hyphae per mother cell (42). Here we provide further evidence that epithelial cells promote the emergence of multiple hyphae per mother cell and hyphal branching (processes which we collectively term hyphal ramification).

We propose that coordinated hyphal ramification by *C. albicans* is necessary for the foraging behaviour of this fungus on one of its natural substrates (human epithelia) and that such behaviour contributes to pathogenicity. This hypothesis is supported by molecular data: deletion of *PGA16* strongly reduced multiple filamentation events at earlier time points (6 h) and this led to both aberrant microcolony development and epithelial damage potential at later time points (16-24 h). Whether the hyphal ramification defect of *pga16*Δ was also responsible for the attenuated virulence of this strain *in vivo* remains unclear; however, histological analysis did reveal atypical filamentous growth of *pga16*Δ in the kidneys of infected mice. In this context, we note that Vam3/Pep12 is required for normal hyphal branching (43) and virulence (44). In contrast, *rsr1*Δ and *bud2*Δ mutants, which exhibit defective hyper-branching phenotypes (45) also exhibit defective epithelial damage and attenuated virulence (46). Therefore, it would appear that the ability of *C. albicans* to correctly coordinate hyphal branching and mycelial ramification is an important element of its pathogenic lifestyle.

Our molecular analysis of *PGA16* revealed some potentially interesting aspects of *C. albicans* genetics. Firstly, complementation of the *pga16*Δ homozygous mutant with a single copy of *PGA16* to the RP10 locus was sufficient to increase virulence in the mouse model of disseminated candidiasis, but did not increase pathogenicity in an *in vitro* epithelial infection model. The adhesion molecule, Als3, has been shown to be absolutely required for biofilm formation *in vitro*, yet the *als3*Δ mutant can form biofilms *in vivo* (47). These authors suggested that [additional, or stronger] *in vivo* signals are sufficient to activate the expression of compensatory adhesins, thus bypassing the need for *ALS3*. In our case, *in vivo* signals, which might be absent, or reduced *in vitro*, may drive sufficient expression of *PGA16* in the *pga16*Δ+*PGA16* strain, to restore virulence.

Our second observation was that reintegration of a second copy of *PGA16* to the RP10 locus in a *pga16*Δ/*PGA16* heterozygous mutant did not restore pathogenicity in our *in vitro* epithelial model. In contrast, reintroduction of a second copy of *PGA16* to its native locus restored the wild type phenotype. *C. albicans* is a diploid organism, and examples of haploinsufficiency are numerous (48-50). Similarly, integration of genes at ectopic chormosomal locations can result in altered expression patterns and can have serious phenotypic consequences – the most famous (or infamous) example, in *C. albicans*, being *URA3* (51-54). Our own study on *PGA16* suggests that both haploinsufficiency and positional effects can occur for the same gene.

In summary, the current study suggests that the *C. albicans* specific genes analysed here are largely dispensable for growth in standard laboratory media, but can play distinct roles in

609 pathogen-host interactions. Our findings support the view that the pathogenic potential of
610 human fungal pathogens has arisen independently, multiple times during evolution.
611

612 ## Acknowledgments

613 We would like to thank Ursula Stöckel, Birgit Weber, and Katharina Große for assistance with
614 the mouse infection experiments. Furthermore we would like to acknowledge Hans-Martin
615 Dahse and Peter Zipfel for providing endothelial cells and Cancer Research Technology for
616 providing the TR146 cell line.
617 DW and BH were supported by the ERA-NET PathoGenoMics Program (Candicol; BMBF
618 0315901 B). DW received funding from the European Community's Seventh Framework
619 Programme under grant agreement no. PIEF-GA-2008-219406. FLM was supported by the
620 International Leibniz Research School for Microbial and Biomolecular Interactions (ILRS) as
621 part of the excellence graduate school Jena School for Microbial Communication (JSMC). PM
622 was supported by the EU FINSysB Marie Curie Research Training Network project no. FP7-
623 214004. BH was also supported by the Center for Sepsis Control and Care (CSCC; BMBF
624 01EO1002). IDJ was supported by the Federal Ministry of Education and Health (BMBF
625 0314108).
626

627 ## Figures Legends

628 **Figure 1. Chromosomal map of *C. albicans* specific genes**. Chromosomal coordinates for all
629 65 expressed *C. albicans* specific genes were downloaded from CGD and plotted as a
630 function of chromosome location. Asterisks denote clusters of genes at the same loci; ~
631 indicates the presence of a gene at the telomere. Note that a higher than expected

percentage of genes clusters on chromosome 5 (P = 0.0085 by Fisher's exact test). Chr., chromosome.

Figure 2. Three families of *C. albicans* specific genes. Alignments of the predicted protein sequences of the three unique gene families and *P* values of sequence similarity across the entire predicted protein length.

Figure 3. *C. albicans* specific genes are required for epithelial damage. Epithelial cell (TR146) monolayers were infected with indicated *C. albicans* strains for 15 h (A) or as indicated (B) and damage determined by measuring lactate dehydrogenase (LDH) release as a percentage of 100% lysis (A) or in ng/ml (B); *P < 0.05 and **P < 0.01 compared to the wild type (Wt) strain. (C) *C. albicans* microcolony formation on epithelial monolayers.

Figure 4. *C. albicans* specific genes are required for endothelial damage. Endothelial (HUVEC) monolayers were infected with indicated *C. albicans* strains for 24 h and damage determined by measuring lactate dehydrogenase (LDH) release as a percentage of 100% lysis. *P < 0.05, **P < 0.01 and ***P < 0.001 compared to the wild type (Wt) strain.

Figure 5. orf19.6688 is required for resisting killing by macrophages. Indicated *C. albicans* strains were incubated in the presence or absence of PMA-activated THP-1 macrophages for 48 h. Wild type (Wt), *P < 0.05.

Figure 6. orf19.6688Δ is hypervirulent in a mouse model of haematogenously disseminated candidiasis. Immune competent female BALB/c mice (10 per strain) were infected with the

wild type (Wt), orf19.6688Δ mutant or orf19.6688Δ+orf19.6688 complemented strain and survival monitored (A). Histological analysis of kidney sections (B).

Figure 7. *PGA16* is required for full virulence in a mouse model of haematogenously disseminated candidiasis. Immune competent female BALB/c mice (10 per strain) were infected with the wild type (Wt), *pga16*Δ mutant or *pga16*Δ+*PGA16* complemented strain and survival monitored (A). Histological analysis of kidney sections (B). The lower panels depict magnified views of the white-boxed areas shown in the upper pannels. **P* < 0.05 compared with mice either infected with the Wt or *pga16*Δ+*PGA16* complemented strain.

Figure 8. orf19.6688Δ elicits hyper activation of IL-1β by murine macrophages. Indicated *C. albicans* strains were incubated with the J774.2 murine macrophage-like cell line for 24 h and IL-1β release measured by ELISA. IL-1β, interleukin-1β; n.s., not significant. **P* < 0.05 and ***P* < 0.01.

Figure 9. *PGA16* mediates hyphal ramification. The number of hyphae per mother cell (A) and the number of branches per primary (1°) hypha (B) were quantified for the indicated *C. albicans* strains without (-) or following exposure to epithelial cells (+TR146) for 6 h. Data shown are the results of two independent experiments. In each experiment 100 cells per condition were analysed. Wild type (Wt).

Figure 10. Reintroduction of *PGA16* to the *pga16*Δ locus in a *PGA16*/*pga16*Δ-heterozygote restores epithelial damage. Epithelial cell (TR146) monolayers were infected with indicated *C. albicans* strains for 24 h and damage determined by measuring lactate dehydrogenase

(LDH) release as a percentage of 100% lysis. **$P < 0.01$ compared to the wild type (Wt) strain and *PGA16*-complemented heterozygote.

703 # Tables

704 **Table 1. *In silico* analysis of *C. albicans* specific genes.** Abbreviations: nucl, nuclear; mito,
705 mitochondria; extr, extracellular; cyto, cytosol; plas, plasma membrane; golg, Golgi
706 apparatus

707

Gene name	Common name	Chr.	Motif	Localis-ation
orf19.3712		R		nucl
orf19.3713		R	Transmembrane helices (4)	mito
orf19.1677		3		nucl
orf19.3738	*PGA22*	R	Signal peptide; aspartyl protease motif; GPI anchor	extr
orf19.6302	*PGA39*	R	Signal peptide; GPI anchor	extr
orf19.6350		1		cyto
orf19.2475	*PGA26*	1	Signal peptide; GPI anchor	extr
orf19.5057		1		nucl
orf19.4439		1	Signal peptide; transmembrane helices (2)	extr
orf19.1109		5	Bipartite nuclear localization signal	mito
orf19.1116		5		nucl
orf19.6920		7	Transmembrane helices (2)	mito
orf19.7028				nucl
orf19.3851		R		nucl
orf19.4241		5		nucl
orf19.3134		4		nucl
orf19.6493		7		nucl
orf19.1958		5	Transmembrane helices (2)	mito

orf19.5190		7		nucl
orf19.1999		2		nucl
orf19.848	*PGA16*	2	Signal peptide; GPI anchor	extr
orf19.3906		5	Signal peptide; transmembrane helices (4)	plas
orf19.3908		5	Transmembrane helices (4)	extr
orf19.1258		4	EF-hand calcium-binding domain	nucl
orf19.1266		4		cyto
orf19.7170		7	Signal peptide	extr
orf19.6534		7	Transmembrane helices (3)	extr
orf19.3210		5		cyto_nucl
orf19.4280		5		nucl
orf19.5246		1	Leucine zipper pattern	nucl
orf19.5262		1		nucl
orf19.937		5	Transmembrane helices (3)	mito
orf19.951		5		nucl
orf19.994		1		nucl
orf19.4691		4	Transmembrane helices (4)	extr
orf19.3336		1	Transmembrane helices (3)	plas
orf19.6688		7	Bipartite nuclear localization signal	nucl
orf19.3376		4	Signal peptide	extr
orf19.3378		4	Signal peptide	extr
orf19.5306		4	Signal peptide	nucl
orf19.7356		3	Signal peptide	extr
orf19.322		3	Transmembrane helices (3)	plas
orf19.344		3		mito
orf19.4055		5		nucl

orf19.4069		2		nucl
orf19.3413	*FGR37*	6	Transmembrane helices (1)	mito
orf19.3427		6	Transmembrane helices (1)	mito
orf19.4085		2	Transmembrane helices (1)	nucl
orf19.6777		3	Signal peptide	golg
orf19.3435		6		nucl
orf19.6021	*IHD2*	1		nucl
orf19.6030		1		nucl
orf19.2833	*PGA34*	R	Signal peptide; GPI anchor	extr
orf19.4149		5	Transmembrane helices (3)	plas
orf19.3543		2		mito
orf19.5549		6	Transmembrane helices (1)	mito
orf19.4936		1	Transmembrane helices (2)	plas
orf19.69		1		nucl
orf19.7608		R	Signal peptide	extr
orf19.1724		3		cyto_nucl
orf19.1735		R		nucl
orf19.7553		R		nucl
orf19.4321		5	Transmembrane helices (2)	plas
orf19.635		R		nucl
orf19.4214		6	Transmembrane helices (3)	mito

708

709

710

References

1. **Blackwell M.** 2011. The fungi: 1, 2, 3 ... 5.1 million species? American journal of botany **98:**426-438.

2. **Mora C, Tittensor DP, Adl S, Simpson AG, Worm B.** 2011. How many species are there on Earth and in the ocean? PLoS biology **9:**e1001127.

3. **Brown GD, Denning DW, Levitz SM.** 2012. Tackling human fungal infections. Science **336:**647.

4. **Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC.** 2012. Hidden killers: human fungal infections. Science translational medicine **4:**165rv113.

5. **Ruhnke M.** 2002. Skin and mucous membrane infections. In *Candida* and Candidiasis , R. Calderone, ed. (Washington, DC, USA: ASM Press)**:**pp. 307-325.

6. **Perlroth J, Choi B, Spellberg B.** 2007. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. Medical mycology : official publication of the International Society for Human and Animal Mycology **45:**321-346.

7. **Mayer FL, Wilson D, Hube B.** 2013. *Candida albicans* pathogenicity mechanisms. Virulence **4**.

8. **Fitzpatrick DA, Logue ME, Stajich JE, Butler G.** 2006. A fungal phylogeny based on 42 complete genomes derived from supertree and combined gene analysis. BMC evolutionary biology **6:**99.

9. **Bowman BH, Taylor JW, White TJ.** 1992. Molecular evolution of the fungi: human pathogens. Molecular biology and evolution **9:**893-904.

10. **Gola S, Martin R, Walther A, Dunkler A, Wendland J.** 2003. New modules for PCR-based gene targeting in *Candida albicans*: rapid and efficient gene targeting using 100 bp of flanking homology region. Yeast **20:**1339-1347.

11. **Walther A, Wendland J.** 2003. An improved transformation protocol for the human fungal pathogen *Candida albicans*. Current genetics **42:**339-343.

12. **Murad AM, Lee PR, Broadbent ID, Barelle CJ, Brown AJ.** 2000. CIp10, an efficient and convenient integrating vector for *Candida albicans*. Yeast **16:**325-327.

13. **Mayer FL, Wilson D, Jacobsen ID, Miramon P, Grosse K, Hube B.** 2012. The novel *Candida albicans* transporter Dur31 Is a multi-stage pathogenicity factor. PLoS pathogens **8:**e1002592.

14. **Wachtler B, Wilson D, Haedicke K, Dalle F, Hube B.** 2011. From attachment to damage: defined genes of *Candida albicans* mediate adhesion, invasion and damage during interaction with oral epithelial cells. PloS one **6:**e17046.

15. **Rossignol T, Lechat P, Cuomo C, Zeng Q, Moszer I, d'Enfert C.** 2008. CandidaDB: a multi-genome database for *Candida* species and related Saccharomycotina. Nucleic acids research **36:**D557-561.

16. **Inglis DO, Arnaud MB, Binkley J, Shah P, Skrzypek MS, Wymore F, Binkley G, Miyasato SR, Simison M, Sherlock G.** 2012. The *Candida* genome database incorporates multiple *Candida* species: multispecies search and analysis tools with curated gene and protein information for *Candida albicans* and *Candida glabrata*. Nucleic acids research **40:**D667-674.

17. **Falkow S.** 1988. Molecular Koch's postulates applied to microbial pathogenicity. Reviews of infectious diseases **10 Suppl 2:**S274-276.

18. **Zakikhany K, Naglik JR, Schmidt-Westhausen A, Holland G, Schaller M, Hube B.** 2007. In vivo transcript profiling of *Candida albicans* identifies a gene essential for interepithelial dissemination. Cellular microbiology **9:**2938-2954.

19. **Thewes S, Kretschmar M, Park H, Schaller M, Filler SG, Hube B.** 2007. *In vivo* and *ex vivo* comparative transcriptional profiling of invasive and non-invasive *Candida albicans* isolates identifies genes associated with tissue invasion. Molecular microbiology **63:**1606-1628.

20. **De Groot PW, Hellingwerf KJ, Klis FM.** 2003. Genome-wide identification of fungal GPI proteins. Yeast **20:**781-796.

21. **Nather K, Munro CA.** 2008. Generating cell surface diversity in *Candida albicans* and other fungal pathogens. FEMS microbiology letters **285:**137-145.

22. **McDonagh A, Fedorova ND, Crabtree J, Yu Y, Kim S, Chen D, Loss O, Cairns T, Goldman G, Armstrong-James D, Haynes K, Haas H, Schrettl M, May G, Nierman WC, Bignell E.** 2008. Sub-telomere directed gene expression during initiation of invasive aspergillosis. PLoS pathogens **4:**e1000154.

23. **Kumamoto CA.** 2008. Niche-specific gene expression during C. albicans infection. Current opinion in microbiology **11:**325-330.

24. **Southern P, Horbul J, Maher D, Davis DA.** 2008. *C. albicans* colonization of human mucosal surfaces. PloS one **3:**e2067.

25. **Miramon P, Dunker C, Windecker H, Bohovych IM, Brown AJ, Kurzai O, Hube B.** 2012. Cellular Responses of *Candida albicans* to Phagocytosis and the Extracellular Activities of Neutrophils Are Critical to Counteract Carbohydrate Starvation, Oxidative and Nitrosative Stress. PloS one **7:**e52850.

26. **Spellberg B, Ibrahim AS, Edwards JE, Jr., Filler SG.** 2005. Mice with disseminated candidiasis die of progressive sepsis. The Journal of infectious diseases **192:**336-343.

27. **Cai B, Deitch EA, Ulloa L.** 2010. Novel insights for systemic inflammation in sepsis and hemorrhage. Mediators of inflammation **2010:**642462.

28. **van de Veerdonk FL, Kullberg BJ, Netea MG.** Pathogenesis of invasive candidiasis. Current opinion in critical care **16:**453-459.

29. **Netea MG, Brown GD, Kullberg BJ, Gow NA.** 2008. An integrated model of the recognition of Candida albicans by the innate immune system. Nature reviews **6:**67-78.

30. **Butler G, Rasmussen MD, Lin MF, Santos MA, Sakthikumar S, Munro CA, Rheinbay E, Grabherr M, Forche A, Reedy JL, Agrafioti I, Arnaud MB, Bates S, Brown AJ, Brunke S, Costanzo MC, Fitzpatrick DA, de Groot PW, Harris D, Hoyer LL, Hube B, Klis FM, Kodira C, Lennard N, Logue ME, Martin R, Neiman AM, Nikolaou E, Quail MA, Quinn J, Santos MC, Schmitzberger FF, Sherlock G, Shah P, Silverstein KA, Skrzypek MS, Soll D, Staggs R, Stansfield I, Stumpf MP, Sudbery PE, Srikantha T, Zeng Q, Berman J, Berriman M, Heitman J, Gow NA, Lorenz MC, Birren BW, Kellis M, Cuomo CA.** 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida genomes*. Nature **459:**657-662.

31. **Noble SM, French S, Kohn LA, Chen V, Johnson AD.** 2010. Systematic screens of a *Candida albicans* homozygous deletion library decouple morphogenetic switching and pathogenicity. Nature genetics **42:**590-598.

32. **Kumamoto CA, Vinces MD.** 2005. Contributions of hyphae and hypha-co-regulated genes to Candida albicans virulence. Cellular microbiology **7:**1546-1554.

33. **Jacobsen ID, Wilson D, Wachtler B, Brunke S, Naglik JR, Hube B.** 2012. *Candida albicans* dimorphism as a therapeutic target. Expert review of anti-infective therapy **10:**85-93.

34. **Lee SA, Wormsley S, Kamoun S, Lee AF, Joiner K, Wong B.** 2003. An analysis of the Candida albicans genome database for soluble secreted proteins using computer-based prediction algorithms. Yeast (Chichester, England) **20:**595-610.

35. **Brown GD, Gordon S.** 2001. Immune recognition. A new receptor for beta-glucans. Nature **413:**36-37.

36. **Saijo S, Ikeda S, Yamabe K, Kakuta S, Ishigame H, Akitsu A, Fujikado N, Kusaka T, Kubo S, Chung SH, Komatsu R, Miura N, Adachi Y, Ohno N, Shibuya K, Yamamoto N, Kawakami K, Yamasaki S, Saito T, Akira S, Iwakura Y.** 2010. Dectin-2 recognition of alpha-mannans and induction of Th17 cell differentiation is essential for host defense against Candida albicans. Immunity **32:**681-691.

37. **Gasparoto TH, Tessarolli V, Garlet TP, Torres SA, Garlet GP, Da Silva JS, Campanelli AP.** 2010. Absence of functional TLR4 impairs response of macrophages after Candida albicans infection. Med Mycol.

38. **Netea MG, Van Der Graaf CA, Vonk AG, Verschueren I, Van Der Meer JW, Kullberg BJ.** 2002. The role of toll-like receptor (TLR) 2 and TLR4 in the host defense against disseminated candidiasis. The Journal of infectious diseases **185:**1483-1489.

39. **Netea MG, Gow NA, Joosten LA, Verschueren I, van der Meer JW, Kullberg BJ.** 2010. Variable recognition of Candida albicans strains by TLR4 and lectin recognition receptors. Med Mycol **48:**897-903.

40. **Kumar H, Kumagai Y, Tsuchida T, Koenig PA, Satoh T, Guo Z, Jang MH, Saitoh T, Akira S, Kawai T.** 2009. Involvement of the NLRP3 inflammasome in innate and humoral adaptive immune responses to fungal beta-glucan. J Immunol **183:**8061-8067.

41. **Pietrella D, Rachini A, Pandey N, Schild L, Netea M, Bistoni F, Hube B, Vecchiarelli A.** 2010. The Inflammatory Response Induced by Aspartic Proteases of Candida albicans Is Independent of Proteolytic Activity. Infection and immunity **78:**4754-4762.

42. **Mech F, Wilson D, Lehnert T, Hube B, Thilo Figge M.** 2013. Epithelial invasion outcompetes hypha development during *Candida albicans* infection as revealed by an image-based systems biology approach. Cytometry. Part A : the journal of the International Society for Analytical Cytology.

43. **Veses V, Richards A, Gow NA.** 2009. Vacuole inheritance regulates cell size and branching frequency of *Candida albicans* hyphae. Molecular microbiology **71:**505-519.

44. **Palanisamy SK, Ramirez MA, Lorenz M, Lee SA.** 2010. *Candida albicans PEP12* is required for biofilm integrity and in vivo virulence. Eukaryotic cell **9:**266-277.

45. **Hausauer DL, Gerami-Nejad M, Kistler-Anderson C, Gale CA.** 2005. Hyphal guidance and invasive growth in *Candida albicans* require the Ras-like GTPase Rsr1p and its GTPase-activating protein Bud2p. Eukaryotic cell **4:**1273-1286.

46. **Brand A, Vacharaksa A, Bendel C, Norton J, Haynes P, Henry-Stanley M, Wells C, Ross K, Gow NA, Gale CA.** 2008. An internal polarity landmark is important for externally induced hyphal behaviors in *Candida albicans*. Eukaryotic cell **7:**712-720.

47. **Nobile CJ, Andes DR, Nett JE, Smith FJ, Yue F, Phan QT, Edwards JE, Filler SG, Mitchell AP.** 2006. Critical role of Bcr1-dependent adhesins in *C. albicans biofilm* formation *in vitro* and *in vivo*. PLoS pathogens **2:**e63.

48. **Uhl MA, Biery M, Craig N, Johnson AD.** 2003. Haploinsufficiency-based large-scale forward genetic analysis of filamentous growth in the diploid human fungal pathogen *C.albicans*. The EMBO journal **22:**2668-2678.

49. **Xu D, Jiang B, Ketela T, Lemieux S, Veillette K, Martel N, Davison J, Sillaots S, Trosok S, Bachewich C, Bussey H, Youngman P, Roemer T.** 2007. Genome-wide fitness test and mechanism-of-action studies of inhibitory compounds in *Candida albicans*. PLoS pathogens **3:**e92.

50. **Zavrel M, Majer O, Kuchler K, Rupp S.** 2012. Transcription factor Efg1 shows a haploinsufficiency phenotype in modulating the cell wall architecture and immunogenicity of *Candida albicans*. Eukaryotic cell **11:**129-140.

51. **Lay J, Henry LK, Clifford J, Koltin Y, Bulawa CE, Becker JM.** 1998. Altered expression of selectable marker *URA3* in gene-disrupted *Candida albicans* strains complicates interpretation of virulence studies. Infection and immunity **66:**5301-5306.

52. **Bain JM, Stubberfield C, Gow NA.** 2001. Ura-status-dependent adhesion of *Candida albicans* mutants. FEMS microbiology letters **204:**323-328.

53. **Staab JF, Sundstrom P.** 2003. *URA3* as a selectable marker for disruption and virulence assessment of *Candida albicans* genes. Trends in microbiology **11:**69-73.

54. **Sharkey LL, Liao WL, Ghosh AK, Fonzi WA.** 2005. Flanking direct repeats of hisG alter *URA3* marker expression at the *HWP1* locus of *Candida albicans*. Microbiology **151:**1061-1071.

Figure 1.

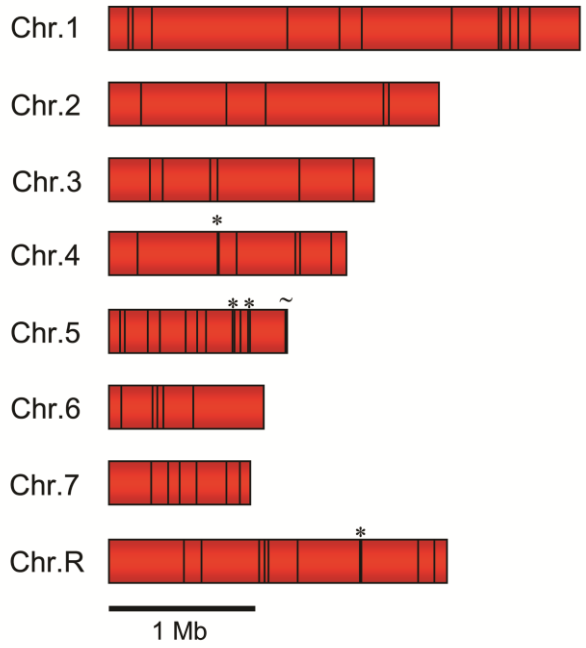


Figure 2.

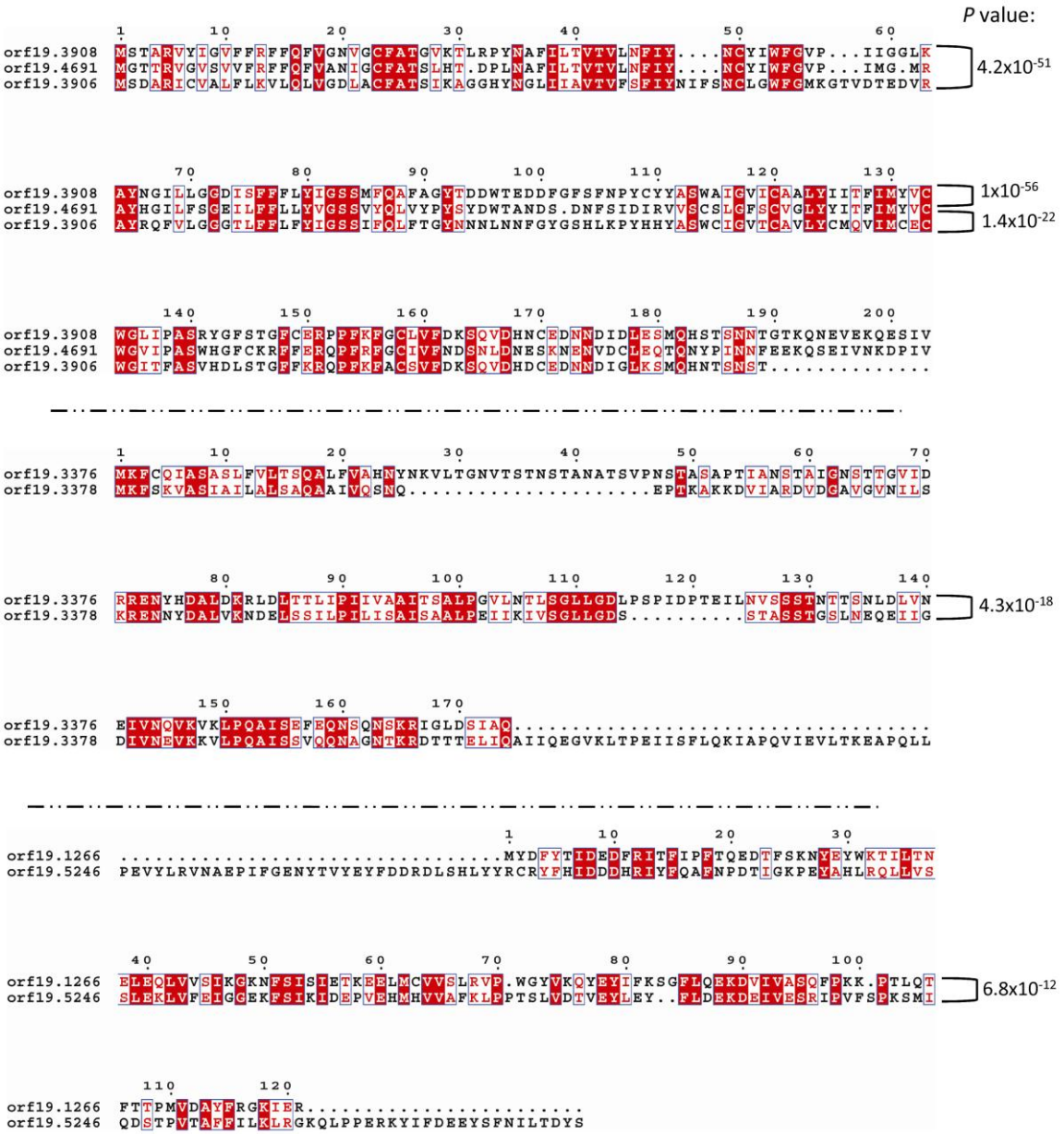
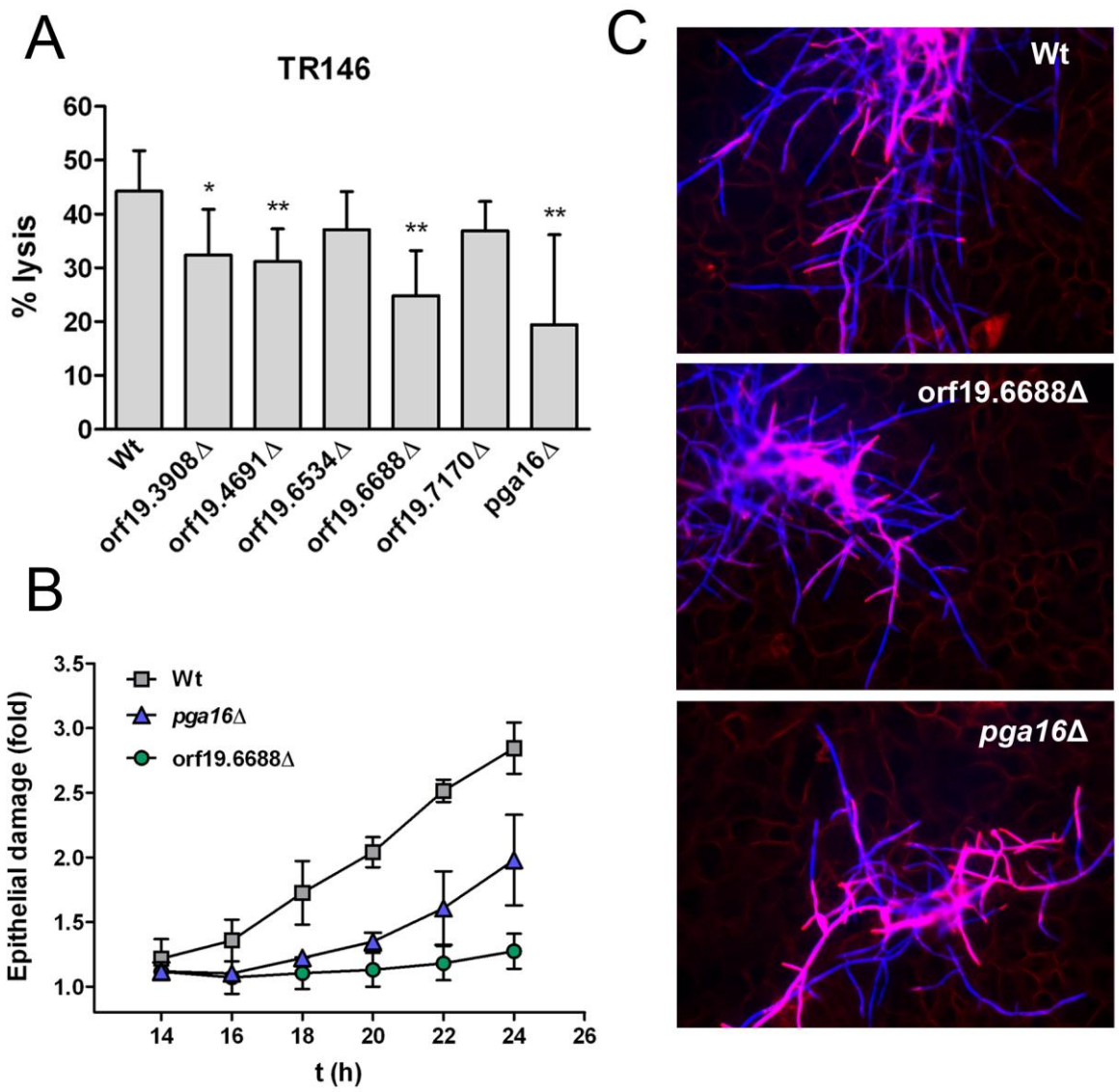

P value:
orf19.3908
orf19.4691
orf19.3906
4.2x10-51
1x10-56
1.4x10-22
orf19.3376
orf19.3378
4.3x10-18
orf19.1266
orf19.5246
6.8x10-12

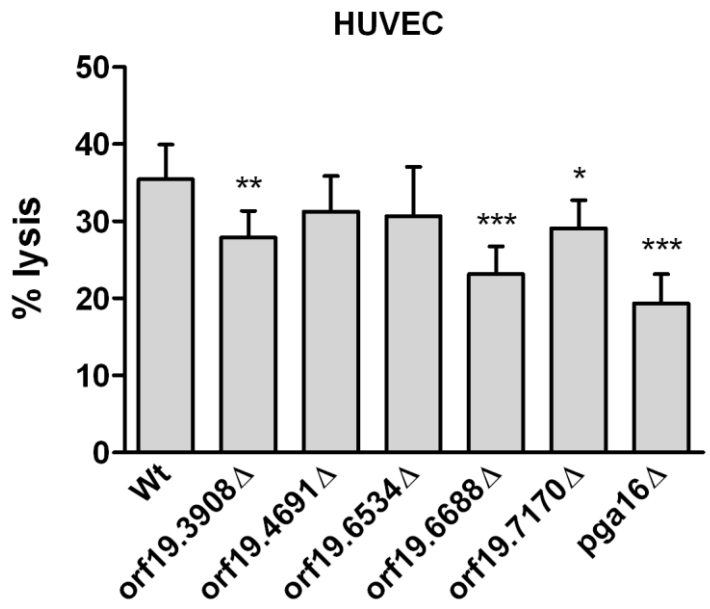
Figure 3.


A
TR146
% lysis
60
50
40
30
20
10
0
*
**
**
**
Wt
orf19.3908Δ
orf19.4691Δ
orf19.6534Δ
orf19.6688Δ
orf19.7170Δ
pga16Δ
B
Epithelial damage (fold)
3.5
3.0
2.5
2.0
1.5
1.0
Wt
pga16Δ
orf19.6688Δ
14
16
18
20
22
24
26
t (h)
C
Wt
orf19.6688Δ
pga16Δ

878

Figure 4.

879

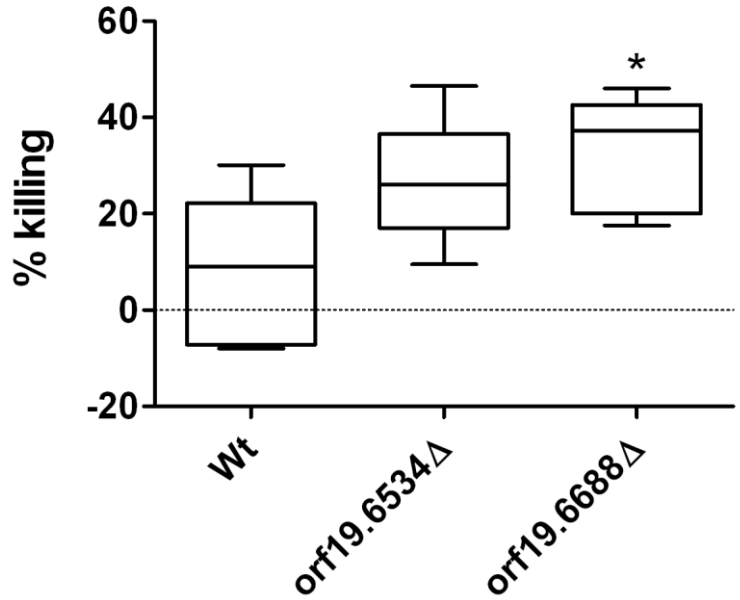


880

881

Figure 5.

882

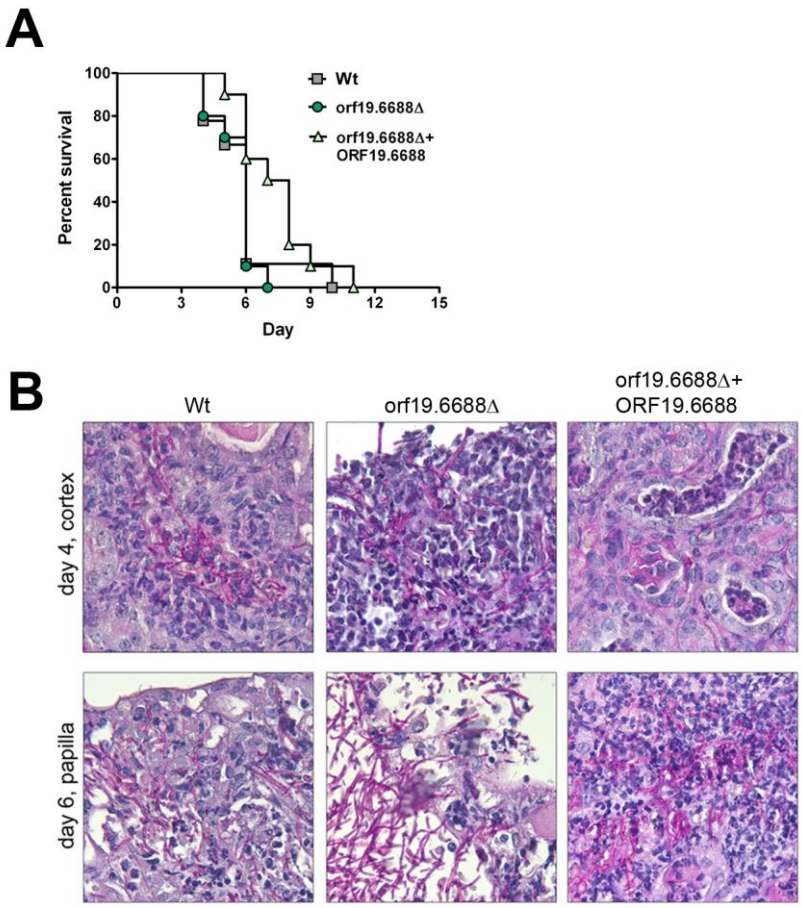


883

884

Figure 6.

885



886

887

888

889

890

891

892

Figure 7.

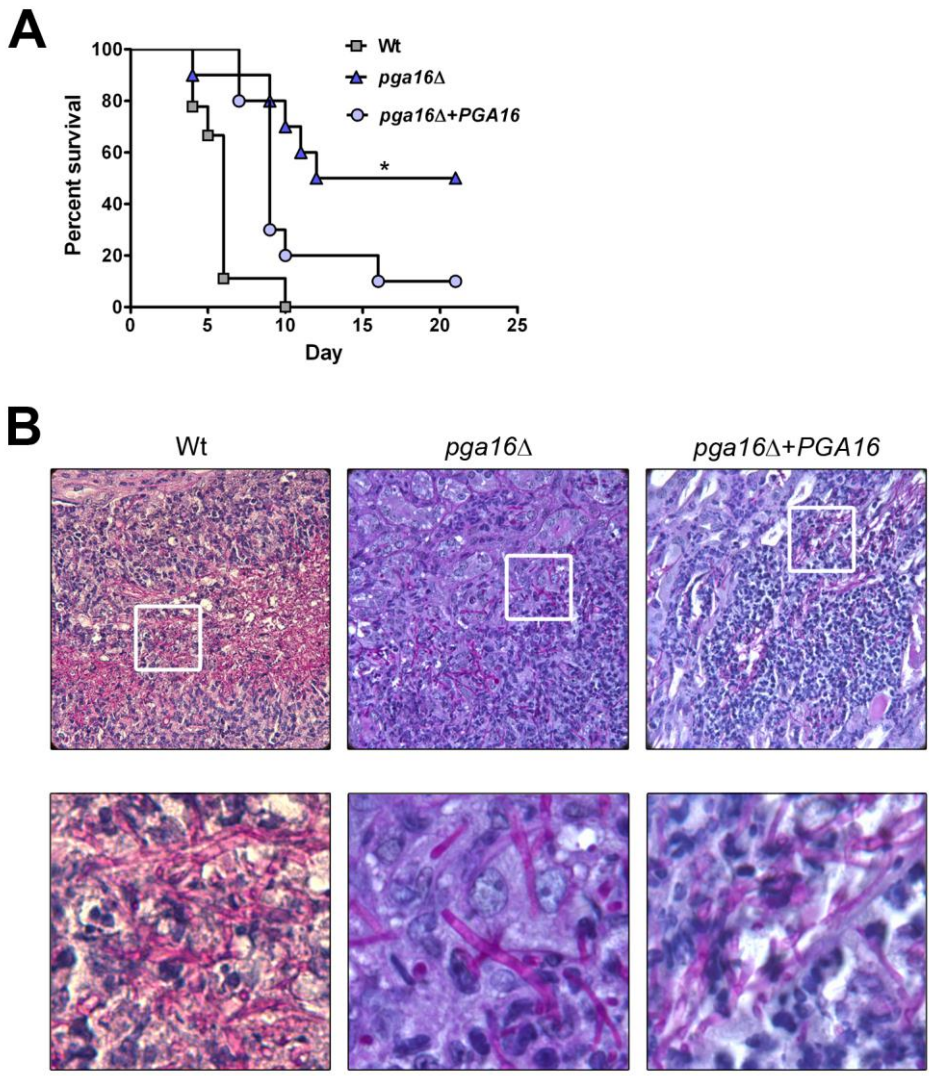
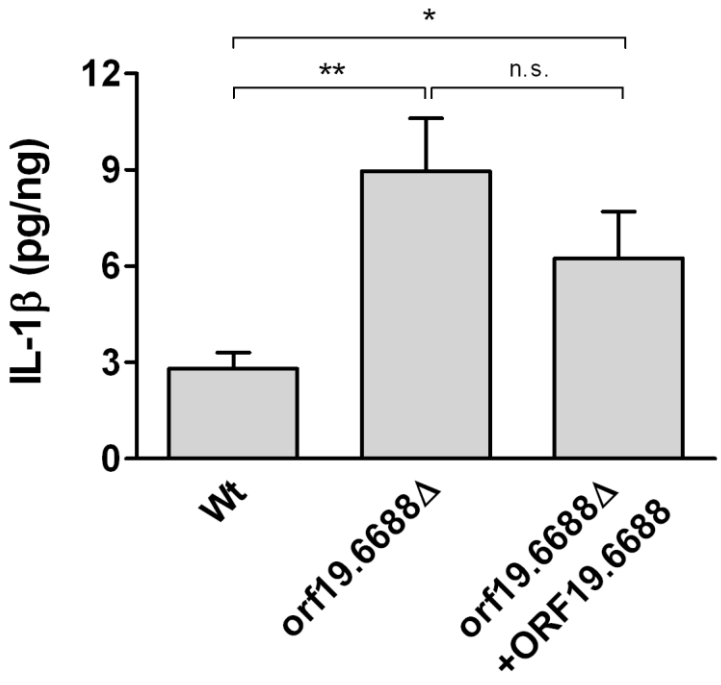

A
Wt
pga16Δ
pga16Δ+PGA16
Percent survival
Day
*
B
Wt
pga16Δ
pga16Δ+PGA16

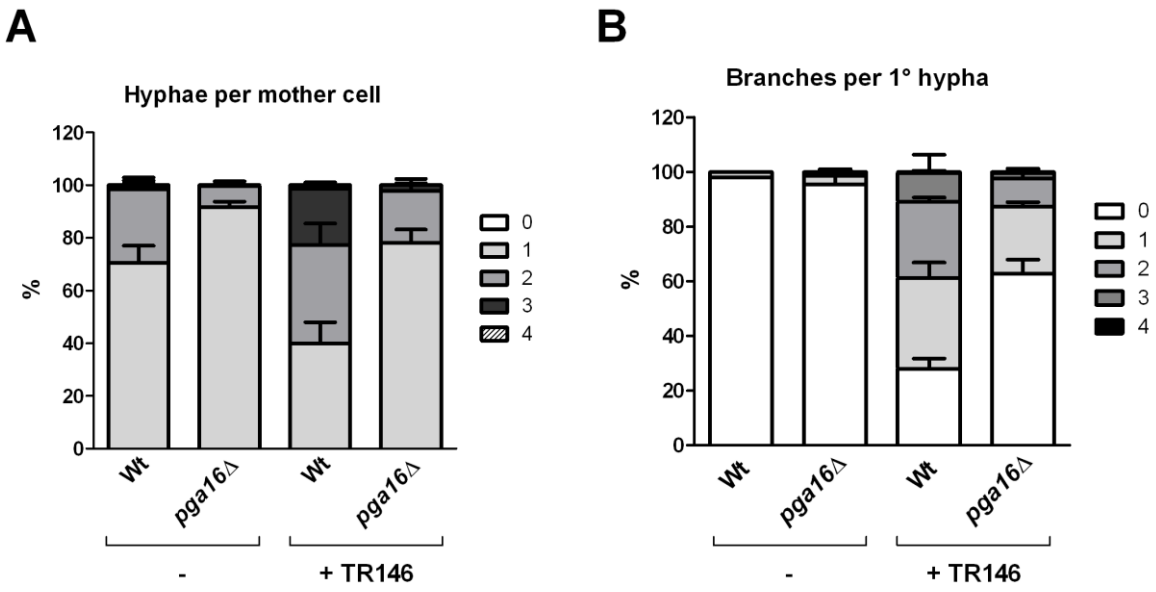
Figure 8.



910

Figure 9.

911



912

Figure 10.

913