**Candida albicans AGE3**, the Ortholog of the *S. cerevisiae* ARF-GAP-Encoding Gene GCS1, Is Required for Hyphal Growth and Drug Resistance

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**Abstract**

*Background:* Hyphal growth and multidrug resistance of *C. albicans* are important features for virulence and antifungal therapy of this pathogenic fungus.

*Methodology/Principal Findings:* Here we show by phenotypic complementation analysis that the *C. albicans* gene AGE3 is the functional ortholog of the yeast ARF-GAP-encoding gene GCS1. The finding that the gene is required for efficient endocytosis points to an important functional role of Age3p in endosomal compartments. Most *C. albicans* age3Δ mutant cells which grew as cell clusters under yeast growth conditions showed defects in filamentation under different hyphal growth conditions and were almost completely disabled for invasive filamentous growth. Under hyphal growth conditions only a fraction of age3Δ cells shows a wild-type-like polarization pattern of the actin cytoskeleton and lipid rafts. Moreover, age3Δ cells were highly susceptible to several unrelated toxic compounds including antifungal azole drugs. Irrespective of the *AGE3* genotype, C-terminal fusions of GFP to the drug efflux pumps Cdr1p and Mdr1p were predominantly localized in the plasma membrane. Moreover, the plasma membranes of wild-type and age3Δ mutant cells contained similar amounts of Cdr1p, Cdr2p and Mdr1p.

*Conclusions/Significance:* The results indicate that the defect in sustaining filament elongation is probably caused by the failure of age3Δ cells to polarize the actin cytoskeleton and possibly of inefficient endocytosis. The high susceptibility of age3Δ cells to azoles is not caused by inefficient transport of efflux pumps to the cell membrane. A possible role of a vacuolar defect of age3Δ cells in drug susceptibility is proposed and discussed. In conclusion, our study shows that the ARF-GAP Age3p is required for hyphal growth which is an important virulence factor of *C. albicans* and essential for detoxification of azole drugs which are routinely used for antifungal therapy. Thus, it represents a promising antifungal drug target.

**Introduction**

*Candida albicans* is one of the most prevalent human fungal pathogens. Depending on environmental conditions it is able to grow in several distinct cell forms, such as yeast cells, different pseudohyphal forms and true hyphae [1,2]. Apart from other properties of *C. albicans* [3] hyphal development strongly contributes to its success as a pathogen [4].

Hyphal growth of *C. albicans* can be induced *in vitro* by varying growth conditions [5] and is controlled by a complex network of transcriptional activators and repressors [6,7]. Recently, the group of David Kadotsh and we independently identified a new central activator of hyphal development, *UME6*, that is required under all conditions of hyphal induction [8,9]. Hyphae formation starts from a yeast-form cell (blastospore) by forming a germ tube which elongates until the first cell division. Before branching of the filament, further cell divisions in the growing filament take place only in the apical cell. Germ tube formation and hyphal elongation are the result of polarized growth [10]. The latter depends on the polarization of the actin cytoskeleton [11–14]. Several other hyphae-specific structures or properties depend on the polarized actin cytoskeleton. Among these, at the hyphal tip the Spitzenko¨rper (tip body), a hyphae-specific organelle closely beneath the polarisome, is present [15] and lipid rafts (sterol- and sphingolipid-enriched membrane domains) are highly concentrat-
ed at the tip [16].

Mucosal and systemic infections caused by *C. albicans* and other *Candida* species are treated by drugs belonging to several different chemical classes, e.g. azoles, polyenes and echinocandins [17]. However, antifungal therapy is often not successful and has become a serious problem due to the emergence of multidrug-resistant strains that result from extended use of antifungal drugs over the last decades [18]. Many *Candida* species including *C. albicans* have a high natural tolerance for antifungal drugs.
Several highly potent drug efflux pumps that reside in the cytoplasmic membrane have different but overlapping substrate spectra to transport toxic compounds out of the cell [19]. There are two families of drug transporters. The ABC (ATP-binding cassette)-transporter family, which includes Cdr1p and Cdr2p, use the energy of ATP hydrolysis to extrude their substrates. The MFS (major facilitator superfamily) proteins (e.g. Mdr1p) use a drug/proton antiport system. Among other mechanisms, multi-drug resistance of clinical Candida strains is often caused by higher expression of genes encoding drug efflux pumps [19–21]. Taken together, there is a high demand for the development of new antifungal drugs and the identification of potential drug targets.

The *Saccharomyces cerevisiae* gene *GCS1* [22,23] encodes an ARF-GAP (ADP-ribosylation factor (ARF) GTPase-activating protein (GAP)) [24]. Several recent reviews discuss the various functions and properties of ARF proteins and ARF-GAPs in detail [25–27]. The GTP-bound form of ARF proteins is required for vesicle coat formation. Uncoating and formation of the naked transport vesicle is triggered by the GTPase activity of ARFs, which strictly depends on activation by ARF-GAPs. Gcs1p activates the intrinsic activity of Arf1p, Arf2p [24] and Arl1p (ARF-like protein 1) [28]. In *S. cerevisiae* for the products of four genes an ARF-GAP activity has been demonstrated [29]: *GCS1*, *GLO3*, *AGE1* and *AEG2*. The functions of these proteins in distinct intracellular vesicle routes partially overlap. The *C. albicans* genome carries homologs for each of these genes. *GCS1* appears to be the most important ARF-GAP in yeast because it shows synthetic lethality with other ARF-GAPs [29], is involved in several routes of intracellular vesicle traffic and has functions in both exocytosis and endocytosis [28,30–32]. Co-localization studies revealed that Gcs1p is predominantly present in Golgi and endosomal compartments [33].

Apart from its ARF-GAP activity Gcs1p is also required for other processes in vesicle traffic, such as vesicle coat formation and vesicle docking with the target membrane by interaction with v-SNAREs [33,34]. Furthermore, Gcs1p is required for maintenance of mitochondrial morphology [35], for formation of the prospore membrane in sporulation [36] and, like ARF-GAPs of other organisms, for the proper actin cytoskeletal organization by stimulating actin polymerization [27,37].

In a systematic phenotypic study of *C. albicans* homologs of genes essentially required for ascospore formation in *S. cerevisiae*, we deleted the *C. albicans* homolog (orf19.3683) of SgGCS1. In consensus with Epp et al. [38], who have shown very recently that cells lacking this ORF are nearly avirulent in the murine model of disseminated infection and are killed by azole drugs instead of being growth inhibited, we call this *C. albicans* homolog “AGE3”, because the gene name “GCS1” has been used already for the *C. albicans* ortholog of the *S. cerevisiae* gene *GSH1* [39].

Here we show that AGE3 complements several defects of the *S. cerevisiae* gcs1Δ strain. Thus, *AGE3* is indeed the functional ortholog of yeast GCS1. A clear delay of endocytosis in ageΔ cells is also shown. Mutant ageΔ cells have a severe defect in filament formation under several different hyphal growth conditions. We found that this is probably caused by defects of polarization of the actin cytoskeleton and lipid rafts of ageΔ cells. We also show that ageΔ cells are highly susceptible to several unrelated toxic compounds including azoles which are in use in antifungal therapy. Results of three experiments strongly indicate that the drug susceptibility of ageΔ cells is not the consequence of inefficient transport to the cell membrane or low activity of the drug efflux pumps Cdr1p, Cdr2p and Mdr1p.

### Materials and Methods

#### Strains, media and growth conditions

The strains used in this study are listed in Table 1. For growing *S. cerevisiae* and *C. albicans* strains in the yeast-form, the cells were cultured at 30°C either in YPD (1% yeast extract, 2% bactopeptone and 2% dextrose) or in SD medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, 2% dextrose) supplemented with the appropriate amino acids for auxotrophic strains. For sporulation of diploid *S. cerevisiae* strains, cells were removed from YPD agar after fresh growth in patches of 1×1 cm for

### Table 1. *C. albicans* and *S. cerevisiae* strains used in this study.

| Strain | Parent | Relevant genotype (otherwise see parent genotype) | Source |
|--------|--------|--------------------------------------------------|--------|
| **C. albicans strains** | | | |
| SC5314 | Wild-type clinical isolate | | [80] |
| SNB7 | SC5314 | leu2Δ·leu2A his1Δ·his1A URA3·ura3Δ JOR1·lor1Δ | [47] |
| UZ22 | SNB7 | age3Δ·CmiEU2·AGE3 his1Δ·his1A | This study |
| UZ45 | UZ22 | age3Δ·CdHS5·age3Δ·CmiEU2 | This study |
| UZ55 | UZ45 | age3Δ·age3Δ·AGE3 (PACT-CaSAT1)* | This study |
| TL19 | SC5314 | AGE3·AGE3 CDR1·CDR1 RPS1·Rps1·Pact-CDR1·GFP (PACT-HygB)* | This study |
| TL20 | UZ45 | age3Δ·age3Δ·CDR1·CDR1 RPS1·Rps1·Pact-CDR1·GFP (PACT-HygB)* | This study |
| TL21 | SC5314 | AGE3·AGE3 MDR1·MDR1-GFP (PmdR1·MDR1 PACT-HygB)* | This study |
| TL22 | UZ45 | age3Δ·age3Δ·MDR1·MDR1-GFP (PmdR1·MDR1 PACT-HygB)* | This study |
| **S. cerevisiae strains** | | | |
| BY4743 | S288C | Mat a/a·his3A1·his3A1 leu2ΔΔ·leu2ΔΔ | [57] |
| ydl226c+/- | BY4743 | gcs1Δ·kanMX4·CDS1 | [81] |
| ydl226c/- | BY4743 | gcs1Δ·kanMX4·gcs1Δ·kanMX4 | [78] |
| UZ177 | ydl226c/- | gcs1Δ·kanMX4·gcs1Δ·kanMX4 (centromeric plasmid pCaAge3-2: URA3 PACT-CaAGE3) | This study |

*Gene fusions in parentheses denote integration of a complete plasmid carrying these fusions into the locus indicated.*

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16 hours at 30°C, resuspended in 1% potassium acetate at a concentration of 1 x 10^5 cells per ml and 100 µl of the suspension were spotted onto 1% potassium acetate agar containing 50 µg/ml doxycline (and for control, without doxycline). After five days of spore formation at 28°C, the cells were removed from the plates, resuspended and the sporulation efficiency determined after microscopic quantification.

For filamentous growth of C. albicans in liquid media the cells were incubated at 37°C in either of the following media: YPD + 10% bovine serum with or without buffering with 20 mM potassium phosphate to pH 7.5, 2.5 mM N-acetylgalactosamine (GlcNAc) in 0.335% yeast nitrogen base with ammonium sulphate, RPMI-1640 (GIBCO-BRL) or Spider medium [40]. Agar plates (2% agar) for filamentous growth were prepared using Spider medium. For filamentous growth under embedded conditions at 37°C YPS agar (1% yeast extract, 2% bactopeptone, 2% sucrose, 1% agar) was prepared and the cells embedded as described [41].

Plasmid constructions

Oligonucleotide primers used for PCR amplification and all plasmids used and constructed are listed in Tables S1 and S2, respectively.

Construction of pCaAge3-Sat2 used for reintegration of the AGE3 gene into the homozygous age3Δ strain was done as follows. First, a gene cassette which is composed of the SAT1 gene (conferring nourseothricin resistance) located between the ACT1 promoter and the ADH1 terminator sequence was PCR-amplified from plasmid pSD54 (Bito A., unpublished). The latter carries modified fragments from pSFS1A [42] and the cassette was amplified using the oligonucleotides CaAct1-Spe-Nar und CaAdh1-Aat followed by digestion with NarI and AelII. This cassette was cloned into YCplac33 [43], resulting in plasmid pCaAct-Sat1. The AGE3 gene including 100 bp upstream of the ORF was PCR-amplified with oligonucleotides CaAge3-9 and CaA0423-2. The PCR product was digested with Sall and SmaI and inserted into pCaAct-Sat1 resulting in plasmid pCaAge3-Sat2. The nucleotide sequence of the AGE3 gene was verified.

The plasmid pCaAge3-2 used for complementation of the yeast goA strain by CaAGE3 was constructed by amplification of the pCm252-AGE3 promoter from C. albicans genomic DNA using the primers CaAge3-7 and CaAge3-8, digestion of the PCR product by BamHI and insertion into plasmid pCM252-2. The latter was derived from pCM252 [44] by replacement of the resident SacII fragment by SacII and inserted into pCaAge3-Sat2. The gene deletion cassette was constructed by amplification of the SAT1 gene (defined by oligonucleotides CaAge3-7 and CaAge3-8, digestion of the PCR product by NgoI and CA0423-2. The PCR product was digested with NgoI and CmLEU2 and inserted into the genome of the heterozygous age3Δ strain. The heterozygous and homozygous age3 deletion strains, UZ22 and UZ45, were constructed as described by Noble and Johnson [47]. To this end, the regions (about 300 bp) immediately upstream and downstream of the AGE3 open reading frame, and the marker gene cassettes Gdh51 and CmLEU2 were amplified either from the Candida genome or from the plasmids pSN52 and pSN40 [47], respectively. The PCR primers used were CaAge3-d1 and CaAge3-d3, CaAge3-d4 and CaAge3-d6, and CaUniv-2 and CaUniv-3 for the region immediately upstream and downstream of the AGE3 ORF and the auxotrophic marker genes, respectively (Table S1). The gene deletion cassette was generated by fusion PCR and integrated into the genome of the heterozygous age3Δ strain, thereby reconstituting one wild-type AGE3 gene copy. After transformation, recombinant clones were selected on YPD agar containing 200 mg/L nourseothricin. For both the homozygous age3 deletion strains and the reconstituted strains the expected structural genomic alterations were confirmed by both PCR (not shown) and Southern blots (Fig. S1).

To construct strains that encode C-terminal fusions of GFP [48] to Cdr1p and Mdr1p, respectively, the plasmid pCdr1-GFP was linearized with the restriction enzyme AgeI and inserted into one allele of the C. albicans RPS1 gene, whereas pMdr1-GFP was linearized with KpnI and inserted into the MDRI ORF. After transformation of the wild-type strain SC5314 and the age3Δ strain with pCdr1-GFP or pMdr1-GFP, the transformants were selected on agar plates containing 200 µg/ml (for the age3Δ strain) or 1000 µg/ml hygromycin B (wild-type strain). Plasmid integration at the expected loci was confirmed by PCR. As the consequence of the different integration sites the expression of the CDR1-GFP cassette is controlled by the Tet promoter [49] whereas the MDR1-GFP cassette is controlled by the MDR1 promoter. Thus, chimeric gene expression of cells was induced by addition of 50 µg/ml doxycline (Cdr1p-GFP) or 25 µg/ml benomyl (Mdr1p-GFP) for four hours.

RNA isolation, Northern blotting and quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA was isolated from cells grown to logarithmic phase in YPD or induced for filamentous growth in YPD+scram using the RNeasy® Mini Kit from Qiagen. Northern blot analysis was done as described elsewhere [9]. For synthesis of the AGE3-specific probe, a PCR product was amplified by using the oligonucleotide primers CaAge3-9 and CaAge3-3 (Table S1). The antisense strand was labelled by incorporation of [32P]-dATP in a single-stranded PCR reaction. After hybridization, transcripts were made visible by phosphoimaging. As a control for RNA quantity and transfer efficiency, rRNA was stained on the blot with methylene blue. Quantitative AGE3 gene expression was determined by qRT-PCR analysis. After degrading residual DNA from the RNA samples by DNaseI, cDNA synthesis was performed using the GoScript™ Reverse Transcription System (Promega Corp.). Gene-specific fragments of ACT1 (control for constitutive expression) and AGE3 were amplified by real-time PCR [primer pairs CaAct1-10/CaAct1-11 and CaAge3-9A/CaAge3-10, respectively] in a Corbett Rotor-Gene RG-3000 machine using the GoTaq® qPCR Master Mix (Promega Corp.). The relative AGE3 expression levels of different strains were calculated by the ΔCt method (normalization to ACT1 expression). For each cDNA sample real-time PCR was done in duplicate and the experiments were repeated once on a different day for reproducibility.
Plasma membrane isolation and Western blotting

The plasma membranes of Candida yeast cells grown in YPD to exponential phase (OD₆₀₀ = 1.0) were purified as described by Panaretou and Piper [50]. For induction of CDR1 and CDR2 expression 10 μg/ml β-estradiol [51], and for MDR1 induction 25 μg/ml benomyl had been added to the medium for one hour before harvesting the cells. After separation of membrane proteins (10 μg) by SDS-PAGE (10% polyacrylamide gels) and blotting onto nitrocellulose membranes, the blots were incubated with polyclonal antibodies raised against Cdr1p, Cdr2p or Mdr1p (kindly provided by D. Sanglard) [52]. After incubation with secondary antibodies (goat anti rabbit) conjugated with alkaline phosphatase (AP) the drug transporters were visualized by staining the blots with the AP substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium (NBT).

Staining procedures, fluorescence microscopy and rhodamine 6G influx/efflux

Endocytosis under yeast growth conditions was investigated by staining of endocytotic vesicles with FM4-64 [53] as follows. Cells were grown in YPD to an OD₆₀₀ of 0.5, harvested by centrifugation and resuspended in 300 μl YPD containing 2.5 μM FM4-64 stock solution (stock 1.64 mM). The samples were incubated at 0°C for 40 minutes and then washed in 1 ml YPD. Then the cells were resuspended in 200 μl YPD to start endocytosis and incubated for different time periods at 30°C. Samples were taken and washed in 1x PBS before microscopic analysis.

Hyphal structures were stained according to procedures formerly published. Lipid rafts were stained with filipin [16] using a concentration of 16.6 μg/ml. F-actin was stained as described [54] with the modification that instead of rhodamine-conjugated phalloidin Alexafluor-488-conjugated phalloidin was used.

The structures were inspected with a Leica TCS SP5 confocal microscope using the appropriate excitation and emission wavelength.

The steady-state level of intracellular rhodamine 6G was determined using the protocol of Maesaki et al. [55]. The influx and efflux of rhodamine 6G was measured as described [56].

Testing susceptibility to metabolic inhibitors and zymolysis

To test strains for sensitivity to metabolic inhibitors, strains were initially grown for 16 hours in YPD medium. Then the cell suspensions were diluted in YPD to an OD₆₀₀ of 0.05 and the cells grown at 30°C for six hours. The cell number per ml was determined with a hemacytometer and serially diluted in cold H₂O. Each five μl suspension containing about 20, 200, 2000 or 20,000 cells were spotted onto YPD agar containing certain toxic compounds. For each compound three concentrations were initially tested for optimal results. After two or three days of growth at 30°C the agar plates were photographed. Growth susceptibility of S. cerevisiae strains to 80 mM NaF in YPD was tested by inoculating in medium with and without 50 μg/ml doxycycline and incubation for 46 hours at 30°C. The OD₆₀₀ was measured at certain time points.

Sensitivity to zymolysate™ (Seikagaku Biobusiness Corp., Japan) was tested as follows. The strains (wild type, age3Δ and reintegrant) were grown in YPD at 30°C to exponential phase (OD₆₀₀ about 0.5). After washing with water, the cells were resuspended in SCE+DTT (1 M Sorbitol, 10 mM Na-Citrat [pH 7.5], 20 mM EDTA, 20 mM DTT) for 30 minutes at 23°C. The cells were washed in SCE and an amount of 2.5×10⁸ cells per ml SCE including zymolysate at a concentration of 5, 10 or 20 μg/ml was incubated at 37°C for 30 minutes. One volume of 2% SDS was added and the OD₆₀₀ measured. The percentage of viable cells was determined according to the following equation: % viable cells = 100× (OD₆₀₀ with zymolysate/OD₆₀₀ of a control sample without zymolysate).

Results

Deletion of AGE3 from the C. albicans genome and gene expression

To study the biological role of AGE3 in C. albicans we made heterozygous and homozygous strains, UZ22 and UZA5 (Table 1), respectively, that lack one or both alleles of AGE3. In addition, as a control for phenotypic analyses an AGE3-reconstituted (“reintegrate”) strain, UZ55, was generated. In this strain, one copy of the wild-type AGE3 gene had been reintegrated into the age3Δ strain at the native genomic locus.

Both in YPD and SC medium age3Δ yeast cells formed aggregates (Fig. 1A) which disintegrate when the coverslip and the cells in suspension beneath are gently pressed to the slide. This indicates that age3 mutant cells possibly adhere to each other or have some defects in cell separation after division.

Gene expression analysis by Northern blotting (Fig. 1B) and qRT-PCR analysis (Table 2) revealed that the gene is expressed constitutively at similar high levels under yeast and hyphal growth conditions. Under these conditions both the heterozygous and the reintegrant strains showed a lower gene expression compared to the wild type. This is probably the reason for the intermediate phenotype (between wild-type and age3 mutant) shown by the heterozygote and the reintegrant strains in most of the experiments described in this study. The different AGE3 expression levels of the reintegrant strain and the heterozygous strain could be caused possibly by expression of the gene from the promoters of different alleles (was not investigated), because the promoter regions of the two AGE3 alleles differ from each other by several nucleotide insertions and deletions.

AGE3 is the functional ortholog of S. cerevisiae GCS1

The open reading frames (ORFs) of C. albicans AGE3 and S. cerevisiae GCS1 encode for proteins of 379 and 352 amino acids length, respectively. The protein sequences are 46.7% identical and 54.3% similar (E-value: 1.2e-89). The N-terminal ARF-GAP domains show the highest homology (83.6% identity, 91.0% similarity). To confirm that AGE3 is indeed the functional ortholog of GCS1 we investigated whether AGE3 is able to complement some known defects of the yeast gcs1Δ strain. To this end, the AGE3 ORF was cloned behind the doxycycline-dependent Tet promoter [44] on the centromeric plasmid pCM252-U. The resulting plasmid pCaAge3-2 was transformed into the homozygous diploid yeast gcs1Δ strain ydl226cΔ−/− from the EUROSCARF collection [57]. The resulting strain was called UZ177. We then compared the ability of UZ177, the gcs1Δ strain and the wild-type strain for gentamycin susceptibility and sporulation efficiency, which depend on Golgi and vacuole functions [58] and proper formation of the prosopere membrane [36]. Furthermore, the growth in the presence of sodium fluoride which inhibits glycolysis by binding to enolase [59] was compared. In each case the wild-type phenotype was nearly fully restored in the mutant strain when the C. albicans AGE3 ORF was expressed (Fig. 2). This suggests that C. albicans AGE3 is indeed the functional ortholog of S. cerevisiae GCS1 and probably functions as an ARF-GAP. In parallel we found that doxycycline did not have any influence on growth in liquid media on any S. cerevisiae strain used in this study (not shown).

age3 mutant cells show a strong delay in endocytosis

To confirm that age3Δ cells have defects in intracellular vesicle traffic, we studied their endocytotic efficiency after staining the
cytoplasmic membrane with the lipophilic fluorescent dye FM4-64. Although FM4-64 was internalized efficiently, many age3Δ cells showed bright fluorescent spots in the cytoplasm or irregular structures (even at later time points) which was observed less often in cells of the wild-type and reintegrant strains (Fig. 3A and 3B). Moreover, the age3 mutant cells had a clear delay of endocytosis compared to wild-type cells. In contrast to mutant cells the vacuolar membranes of many wild-type and reintegrant cells were stained weakly even ten minutes after release of endocytosis (temperature shift from 0 to 30°C after removing excess FM4-64) (Fig. 3A). Compared to wild-type cells it took about 20 minutes longer (this corresponds to about 1/3 of the doubling time) until 50% of the mutant cells had taken up FM4-64 into the vacuolar membrane (Fig. 3C). Although all mutant cells showed staining of the vacuolar membrane after one hour, there were still several bright spots observed in the cytoplasm and close to the cell membrane of many mutant cells (Fig. 3B). This and the generally higher background staining of cytoplasm indicates that mutant cells also endocytose quantitatively less efficiently than wild-type cells.

The age3Δ strain has severe defects in hyphal and invasive growth under several distinct conditions

We then studied filamentation of age3Δ cells under several distinct growth conditions, both in liquid media, on agar surfaces and when embedded in agar. Depending on the growth conditions, the mutant strain showed more or less severe defects in formation of long filaments in liquid media. True hyphae formation of the age3Δ strain was most efficient in YPD containing 10% bovine serum and germ tube formation occurred as efficiently as in wild-type background (albeit with a short delay and a reduced germ tube length compared to wild-type cells). However, during prolonged growth only 54.3% (standard deviation (SD) = 4.6; about 400 cells of three cultures each were determined) of the mutant cells further developed into filaments of normal true hyphae appearance, whereas 91.3% (SD = 1.4) of wild-type cells and 75.6% (SD = 3.9) of the heterozygous and 79.6 (SD = 2.4) of the reintegrated cells showed normal appearance. The rest of the mutant cells were single yeast cells, cells of aberrant shape and also single cells with bent shape of the germ tube were observed (Fig. 4A). Many filaments were shorter, sometimes of undulating or helical shape and some appeared swollen at the end of the apical cell. Filaments and cells grown in serum-containing medium form huge aggregates. Fixation in ethanol and subsequent separation of the filaments by digesting proteins with pepsin resulted in osmotic shrinkage of filaments and made it very difficult to distinguish filaments of different diameter and shape. Therefore we were not able to determine the fractions of different aberrant cell and filament forms reliably. Filaments of “normal” appear-

**Table 2.** AGE3 transcript levels in heterozygous and homozygous age3 mutant cells.

| Strain | AGE3 genotype | Growth condition1 | Relative AGE3 transcript level2 |
|--------|---------------|------------------|-------------------------------|
| SN87   | +/-           | Yeast            | 1.00                          |
|        |               | Hyphae 2 hours   | 1.01                          |
| UZ22   | +/-           | Yeast            | 0.93                          |
|        |               | Hyphae 6 hours   | 0.45                          |
| UZ45   | +/-           | Yeast            | 0.44                          |
|        |               | Hyphae 2 hours   | 0.44                          |
| UZ55   | +/- (+)       | Yeast            | 0.69                          |
|        |               | Hyphae 2 hours   | 0.63                          |

1The cells were grown either in YPD at 30°C to exponential phase (yeast) or induced for hyphal growth (hyphae) in YPD + bovine serum at 37°C for two or six hours.

2The transcript levels were determined by qRT-PCR and are given as mean levels of two parallel reactions compared to the AGE3 expression level of wild-type cells grown under yeast growth conditions.

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ance (without fixation) were composed of hyphal cells with a mean length of about 73% of wild-type cells (Fig. 4B). The mean lengths were 27.4 μm (±6.34) for wild-type hyphal cells and 20.1 μm (±1.69) for mutant cells. However, the first hyphal cell of mutant filaments was often as long as in wild-type filaments. A larger difference of hyphae formation between age3Δ and wild-type cells was observed in medium containing N-acetylglucosamine (GlcNAc) (Fig. 4C), which strongly induces hyphal growth of wild-type cells. Whereas around 80% of the wild-type cells germinated and formed true hyphae of up to three cells three hours after hyphal induction, only a fraction of about 8% of the age3Δ cells germinated and the few filaments formed were composed of no more than two cells. Many cells had an aberrant shape. After five hours nearly 100% of the wild-type blastospores germinated or the filaments had been elongated further, whereas most of the mutant “filaments” were not able to elongate or divide further (not shown). Also in Spider medium the age3Δ cells formed very few short filaments (not shown). Most mutant cells were of aberrant and often bent shape. The heterozygous and the reintegrant strains showed an intermediate phenotype in all media.

In conclusion, age3Δ cells are strongly compromised in maintaining polarized filamentous growth after germ tube formation in liquid media (the severity of these defects depending on the medium).

We also investigated filamentous growth of the age3Δ mutant when grown on Spider agar and when embedded in YPS agar (Fig. 5). Compared with liquid media, the mutant strain showed an even stronger filamentation defect on/in the agar media. Although being able to form long filaments in the center of the colonies grown on Spider agar, the colonies were easily washed from the agar surface (Fig. 5A). Moreover, the age3Δ colonies were very flat and larger compared to colonies formed by other strains. Under embedded conditions the age3Δ strain formed very short filaments instead of long and branched filaments observed with the wild-type strain (Fig. 5B). From these results we conclude that Age3 is essential for invasive filamentous growth in C. albicans.

Filamentation deficiency of age3Δ cells correlates with improper organization of the actin cytoskeleton and the absence of lipid raft polarization at the hyphal tip

We next investigated whether age3Δ cells induced for hyphal growth show polarization of the actin cytoskeleton and lipid rafts. As in other forms of polarized growth of fungi the development of true hyphae depends on the polarization of actin cables, which is established by regulatory and structural cytoskeletal proteins and by cortical patches.
Figure 3. Cells lacking AGE3 show a clear delay in endocytosis. (A) Cells of the strains SN87 (AGE3+/+), UZ45 (age3Δ/Δ) and UZ55 (age3Δ/Δ::AGE3) were grown in YPD to exponential phase. After staining the cytoplasmic membrane with the lipophilic fluorescent dye FM4-64 at 0 °C for 40 minutes, the excess dye was removed by washing. Then the cells were released for growth in YPD at 30 °C to allow endocytosis to occur. Samples were taken at the indicated time points. Staining of endocytic vesicles and the vacuole was visualized by confocal fluorescence microscopy. DIC images are shown for comparison. (B) One and two cell pairs of the wild-type and the age3Δ/Δ mutant strains, respectively, which were harvested 40 minutes after release of endocytosis are shown in higher magnification. The mutant cells show stronger background staining of the cytoplasm and more bright spots compared to the wild-type cells. The vacuolar membranes of the left mutant cells still have not taken up FM4-64. (C) Quantification of FM4-64 uptake into vacuolar membranes in cells of the strains mentioned above. Images of a similar experiment as described under (A) were analysed and the percentage of cells (about 100–150 in total for each strain and time point) with clearly stained vacuolar membranes determined. A similar experiment performed on another day with slightly different time points showed a very similar FM4-64 uptake kinetics.

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of actin [11–14,54]. Actin was stained with Alexafluor-488 phalloidin. The high enrichment of lipid rafts at the hyphal tip [16] were stained with the polyene filipin, which binds to ergosterol [60].

To study these structures, we induced mutant and wild-type cells for hyphal growth in GlcNAc-containing medium, because under these conditions we observed the mutant’s strongest defect in hyphal growth. The wild-type strain showed the expected concentration of actin patches at the tips of germ tubes and hyphae (Fig. 6A). However, for most of the germ tubes (or germ tube-like structures) and short filaments formed by age3Δ blastospores, actin patches were scattered along the cell bodies and a polarization pattern was rarely observed. A similar

Figure 4. Growth forms of the age3Δ/Δ strain after hyphal induction in YPD +10% bovine serum (A and B) or in GlcNAc medium (C). (A) The strains SN87 (AGE3+/+), UZ22 (AGE3+/Δ), UZ45 (age3Δ/Δ) and UZ55 (age3Δ/Δ:AGE3) were induced for hyphal growth in YPD +10% bovine serum at 37°C. After five hours of growth, samples were inspected under the microscope (most pictures in phase contrast and some in Nomarsky optics). Note that the homozygous age3 mutant strain forms several distinct forms of filaments. Some of them are not true hyphal filaments. Most strikingly, a fraction of germ tubes and filaments show an untypically curved or spiral shape (images e, g, h and i). Some other filaments show constrictions between cells which are typical for pseudohyphae (i). There are also cell clusters seen which are composed of yeast-form cells (c) and others which show only poor polarized growth (d, f and b arrow head). (B) Each one true hyphal filament of the wild-type and the age3 mutant strains grown under the same conditions as in (A) were stained with Calcofluor white and DAPI to visualize septa (arrow heads) and nuclei, respectively. Compared to wild-type filaments, age3 mutant filaments are composed of shorter hyphal cells (except for the first cell following the blastospore in many cases). (C) The strains SN87 (AGE3+/+), UZ45 (age3Δ/Δ) and UZ55 (age3Δ/Δ:AGE3) were induced for hyphal growth in GlcNAc-containing medium. After three hours of growth at 37°C for each clone about 200 cells and filaments were classified as wild-type-like germ tubes and hyphae, yeast cells and aberrant forms of single cells, germ tubes or filaments (curved shape, short pseudohyphal elements, cells with thick germ tubes; as shown in (A)). The experiment was done in triplicate. The mean percentage of these morphologies and standard deviation are shown. doi:10.1371/journal.pone.0011993.g004
Figure 5. Defective filamentous and invasive growth of the \textit{age3\Delta} strain on/in solid media. Note: All images of each row are shown in the same magnification. (A) The strains SN87 (\textit{AGE3}+/+), UZ22 (\textit{AGE3}+\Delta), UZ45 (\textit{age3}\Delta/\Delta) and UZ55 (\textit{age3}\Delta/\Delta::\textit{AGE3}) were induced for filamentous growth on Spider agar at 37°C for three and six days (two sets of agar plates). The colonies were photographed and the colony edges inspected under the microscope. To try to remove the colonies after three days of growth one set of agar plates was washed with water. Only the homozygous \textit{age3} mutant colonies could be removed easily. At the edges of the mutant colonies after wash single yeast cells and very short filaments are still attached to the agar. However, weak invasive growth into the agar by \textit{age3} mutant filaments is only visible in the colony center. After six days, filamentous growth is seen macroscopically for the wild-type, the heterozygous and the reintegrant strain. The mutant strain forms flat colonies.
difference was observed for the distribution of lipid rafts (Fig. 6B). Whereas wild-type hyphae showed the typical strong staining of hyphal tips and septa with filipin, ergosterol and lipid rafts were uniformly distributed in the cytoplasmic membrane of many age3 mutant filaments and germ tubes. As mentioned, age3Δ cells and filaments show a certain variability in shape. Filaments which are morphologically similar to the wild-type hyphae showed nearly normal staining patterns of actin patches and lipid rafts (not shown). The reintegrant strain was observed in parallel and showed both wild-type and mutant staining patterns (not shown).

Cells lacking AGE3 are highly susceptible to several unrelated toxic compounds

To identify other cellular functions of the Age3 protein in C. albicans we determined the growth behaviour of the mutant strain in the presence of several distinct metabolic inhibitors. As shown in Fig. 7, the age3Δ strain showed high susceptibility to hygromycin B, theazole drugs miconazole and itraconazole, SDS, Cd2+ ions and brefeldin A. In the presence of 5-fluorocytosine and amphotericin B, which are both used in antifungal therapy in combination with or without azoles [19], we did not observe any influence on growth in the absence of AGE3. The sensitivity of mutant cells to brefeldin A, which is an inhibitor of ARF-GEFs (ARF guanine nucleotide exchange factors; these are required for restoring the potentially active ARF-GTP complex) [61], confirms a role of the Age3 protein in the process of vesicle uncoating. The toxic compounds identified have different chemical structures and are involved in inhibition of distinct cellular processes and structures, such as protein glycosylation, cell wall and protein synthesis (hygromycin B), ergosterol biosynthesis and lipid composition of the plasma membrane (azoles), as well as other membrane or cell wall defects (SDS), and heavy metal toxicity (cadmium ions). Since yeast Gcs1p, and probably C. albicans Age3p as well, function as ARF-GAPs and are required for efficient vesicle traffic, the pleiotropic susceptibility spectrum may be caused mainly by improper or defective intracellular vesicle and protein transport to different organelles and membranes of most age3Δ cells. In particular, the high sensitivity of age3Δ cells to azoles, hygromycin B and cadmium may be caused by inefficient transport of drug efflux pumps to the plasma membrane. As a consequence age3 mutant cells could have a much lower capacity to remove these compounds from the cell.

The drug efflux pumps Cdr1p, Cdr2p and Mdr1p are properly localized in age3Δ cells

From the hypothesis described above, we expected that, as a consequence of inefficient transport of drug efflux pumps to the membrane, a high amount of these transporters may be present in the cytoplasm instead of the plasma membrane in age3Δ cells. To test this hypothesis, we constructed C. albicans strains expressing chimeric proteins composed of drug efflux pumps C-terminally fused with GFP. The efflux pumps chosen were Cdr1p [62] and Mdr1p [63,64], two well studied drug transporters belonging to two distinct classes of efflux pumps. Both proteins are able to efflux azoles [62,65]. However, both the GFP fusion protein of Cdr1p and that of Mdr1p are predominantly localized in the plasma membrane in both the age3Δ and Age3 wild-type background (Fig. 8A). That translocation of drug transporters to the plasma membrane does not significantly depend on Age3p function was further confirmed by Western blotting of plasma membrane proteins isolated from yeast-form cells of the wild-type, the age3Δ, and reintegrant strains and immunostaining with

Figure 6. Absence of polarization of lipid rafts and actin patches in age3Δ cells. The strains SN87 (AGE3+/+) and UZ45 (age3ΔΔ) were induced for hyphal growth in GlcNAc medium at 37°C. After three hours samples were taken and the cell structures shown were stained and visualized under the confocal microscope. For control pictures made with phase contrast or DIC optics are shown below the fluorescence picture. (A) At the tips of germ tubes of wild-type cells the actin patches, which were stained with Alexafluor 488-conjugated phalloidin, are highly concentrated. Most mutant cells do not show this polarization of the actin cytoskeleton. (B) Lipid rafts were stained with filipin. As expected, lipid rafts are concentrated at the hyphal tips and at septa in the wild type. Most mutant germ tubes (note that some have constrictions at the neck) and filaments show a uniform staining of the cytoplasmic membrane. Note that the figure shows only typical structures found in cultures of wild-type and mutant cells. However, a low fraction of mutant cells and filaments (see Fig. 4C) showed a wild type-like staining patterns. Also for the reintegrant strain (not shown) both wild-type and mutant staining patterns were observed.

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antibodies raised against Cdr1p, Cdr2p (which is also able to efflux azoles) and Mdr1p [kindly provided by D. Sanglard] [52]. The results revealed that the plasma membrane of mutant cells contained similar amounts of Cdr1p and Cdr2p and possibly a somewhat lower Mdr1p amount compared to the membranes isolated from wild-type and reintegrant cells (Fig. 8B). Moreover, the intracellular steady-state level of rhodamine 6G (R6G), a substrate of Cdr1p and Cdr2p, but not of Mdr1p [53], is only slightly increased (<3%) in age3Δ cells compared to wild-type cells after growth for one hour at 22, 30 or 37°C (Fig. 8C). Furthermore, de-energized (depleted for ATP) exponentially growing age3 mutant cells showed only a slight increase of R6G influx compared to wild-type cells (data not shown). The R6G efflux rate after glucose-addition (activation of the ATP-dependent efflux pumps) was similar (not shown). These results indicate that the higher drug susceptibility of age3Δ cells is not the result of poor transport to the cell membrane and lower activity of the Cdr1 and Cdr2 proteins.

The age3Δ cells are less susceptible to the cell wall-lytic enzyme zymolyase

Since hygromycin B is not a substrate of Cdr1p and Mdr1p [66] and susceptibility of age3Δ cells to hygromycin B and SDS points to defects in the cell wall, we investigated the sensitivity of age3Δ cells to the cell-wall lytic enzyme zymolyase. Unexpectedly, instead of being more sensitive, the mutant cells were more resistant to zymolyase compared to wild-type cells. This was tested by using two different approaches, cell viability determination (not shown) and measuring the decrease of optical density after spheroplast formation by zymolyase treatment and subsequent cell lysis with SDS (Fig. 9).

Discussion

In this paper we describe the phenotypic and physiological consequences of deleting the AGE3 gene from the C. albicans genome. Since it is able to complement defects of S. cerevisiae gcs1Δ cells, AGE3 is probably the functional ortholog of the S. cerevisiae GCS1 gene. The age3Δ strain showed a clear delay in endocytosis, which is consistent with an important function of Age3p in endosomal compartments as it has been also shown for its yeast ortholog [29,33].

Hyphal and invasive growth defects of the age3Δ mutant strain

As we have shown the poor ability of the cells to form true hyphae and the formation of aberrant cell and filament shapes under a number of hyphal growth conditions is possibly caused by the defects in actin polarization. Moreover, the morphology of age3Δ cells induced for hyphal growth closely resembles that of other mutants with defective polarization of the actin cytoskeleton [12,14]. This suggests that, similar to Gcs1p [37], Age3p can stimulate actin polymerization.

Proper formation of the actin cytoskeleton and cortical actin patch localization depends on both exo- and endocytosis [19]. Moreover, cell polarization depends on the localization of the Cdc42 GTPase, the master regulator of polarized growth, to the polar cap. Its essential role in hyphal growth of C. albicans is well studied [67–69]. It has been shown in yeast that Cdc42p shuttles in a dynamic flux between the cytoplasmic membrane at the polar cap and the cytoplasm [70]. Apart from GDP/GTP recycling on Cdc42p, robust maintenance of cell polarity depends on an actin-dependent efficient exocytic transport of internalized Cdc42p-containing vesicles and subsequent fusion with the cytoplasmic membrane [70]. As discussed above, age3Δ cells have a defect in the endocytic pathway. Thus the defect of age3Δ cells to maintain the balance of polarized versus isotropic growth, which is required to sustain true hyphal growth after germ tube formation, is probably a combination of disturbed polarization of the actin cytoskeleton and inefficient vesicle trafficking.

As observed for a C. albicans strain that lacks the gene encoding for the polarisome component Spa2p [14], the defect of the age3Δ strain in
hyphal growth was more pronounced on solid than in liquid media. Furthermore, we have shown that AGE3 is essential for invasive filamentous growth in vitro. It is commonly acknowledged that, apart from filamentous growth per se, the ability of C. albicans to invade host tissues strongly contributes to virulence of this and other pathogenic fungi [4,69,71]. Consistent with this, Epp et al. [38] found that the age3D strain is much less virulent compared to the wild-type strain. AGE3 is required for tolerance of several unrelated toxic compounds including azole drugs

The high susceptibility of age3 mutant cells to several structurally unrelated toxic compounds, which affect distinct functions of the cell, could be caused simply by the pleiotropic consequences of disturbed vesicle traffic of several routes to different compartments of age3Δ cells. Since the drug transporters Cdr1p, Cdr2p and Mdr1p were efficiently transported to the cell membrane in age3Δ cells and the steady-state level of rhodamine 6G was in a similar range as in wild-type cells, the high azole sensitivity of mutant cells is probably not the consequence of poor drug efflux activity. In addition, age3Δ cells did not show a higher susceptibility to amphotericin B which directly binds to ergosterol [19]. This strongly suggests that age3Δ cells have no defect in ergosterol biosynthesis, a process that is targeted by azoles. Taken together, other cellular functions that are involved in tolerance to azoles and

![Figure 8. Plasma membrane localization of Cdr1p, Cdr2p and Mdr1p and intracellular rhodamine 6G steady-state level in age3 mutant cells.](image)

![Figure 9. Increased resistance of age3Δ cells to zymolyase.](image)
possibly also to the other compounds affecting growth of \textit{age3A} cells must be compromised in \textit{age3A} cells.

Few studies revealed an important role of the vacuole inazole detoxification. \textit{C. albicans} mutant cells lacking the genes of the vacuolar protein sorting factors \textit{Vps1p}, \textit{Vps28p} or \textit{Vps32p} (\textit{Snf7p}) are more susceptible to fluconazole compared to wild-type cells [72,73]. Epp et al. [38] found that fluconazole exerts a very strong fungistatic activity on \textit{C. albicans} mutant cells lacking the Vma10 subunit of the vacuolar membrane H⁺-ATPase, which they also found for \textit{S. cerevisiae} mutants lacking other V-ATPase subunits.

Moreover, several fluconazole-resistant strains isolated from patients after fluconazole administration for several weeks highly accumulate the drug (or some modified form or degradation product) in vesicular vacuoles [74]. Altogether these studies strongly indicate that the vacuole has some important, but yet undefined function in detoxification of azoles.

Interestingly, the \textit{vps1}, \textit{vps28}, \textit{vps32} deletion strains [72,73] and the fluconazole-resistant strains described by Maebashi et al. [74] do not show an increased susceptibility to amphotericin B and 5-fluorocytosine compared to the wild type. This susceptibility pattern agrees with the one we found for \textit{age3A} cells. Moreover, we found a higher susceptibility of \textit{age3A} cells to hygromycin B, SDS and cadmium ions. In \textit{C. albicans} or \textit{S. cerevisiae} an involvement of the vacuole in susceptibility and/or detoxification of hygromycin B [75], SDS [73] and cadmium [76] has been found. From this compound susceptibility pattern and the endocytic defect of \textit{age3A} cells we conclude that the drug susceptibility of \textit{age3A} cells may be caused by some yet undefined defect in vacuolar function. A study to confirm this hypothesis is under way. Whether the glutathione S-conjugate pump \textit{Ycf1p} [76] or another glutathione-dependent (or -independent) ABC transporter in the vacuolar membrane, like \textit{Mlt1p} [77], is able to sequester azoles into the vacuolar lumen is unknown yet. Interestingly, it has been shown that glutathione S-transferases, which catalyse the conjugation of glutathione to xenobiotics and other substrates, are involved in fluconazole detoxification in \textit{Schizoacharyomycetes pombe} [78].

An involvement in vesicle and protein traffic from the Golgi apparatus to the vacuole via the endosomal route has been shown for yeast \textit{Gcs1p} [31,32,36,79]. The strong delay of \textit{C. albicans} \textit{age3A} cells in the endocytic route to the vacuole could result in a much lower amount of drug transporters in the vacuolar membrane and toxic compound-degrading enzymes in the vacuolar lumen. This would have a strong impact on susceptibility of the cell to toxic compounds.

The high sensitivity to the cell-wall perturbing compounds hygromycin B and SDS and the reduced sensitivity to cell wall degradation by Zymolyase™ (which includes several enzymes that are able to hydrolyse the major components of the fungal cell wall) compared to the wild type indicate that the cell wall composition of \textit{age3A} and wild-type cells differ.

In summary, we conclude that the mutant phenotypes of \textit{age3A} cells result from a defect in proper polarization of the actin cytoskeleton, inefficient endocytosis, and possibly inefficient vacuolar functions. The phenotypic consequences of the absence of \textit{Age3p} described here probably have strong impacts on virulence of \textit{C. albicans} and antifungal therapy which both are affected by deletion of \textit{AGE3} [38]. Therefore, the protein is an ideal candidate target for the development of new antifungal compounds.

Supporting Information

**Figure S1** Southern blots confirming the \textit{AGE3} genomic regions of the homozygous \textit{age3} mutant (A) and \textit{AGE3}-reconstituted strains (B). Genomic DNA isolated from the strains indicated was digested with restriction enzymes and the fragments separated by agarose gel electrophoresis. After blotting onto a nylon membrane and hybridization with a [32P]-labelled probe, \textit{AGE3} promoter-specific fragments were visualized by phosphoimaging. DNA was digested either with \textit{SmaI} and \textit{KpnI} (A) or with EcoRV and \textit{XbaI} (B). For both the homozygous \textit{age3} mutant and the \textit{AGE3}-reconstituted strains the expected fragment patterns were observed. (*) For the reconstituted strain two \textit{AGE3} promoter-specific fragments (ca. 1620 and 5000 bp) were expected.

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**Table S1** Oligonucleotides used in this study.

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**Table S2** Plasmids used in this study.

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**Author Contributions**

Conceived and designed the experiments: TL UZ AB. Performs the experiments: TL UZ MH AB. Analyzed the data: TL UZ AB. Contributed reagents/materials/analysis tools: MG MH MB. Wrote the paper: AB.
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