Multiple functions of *DOA1* in *Candida albicans*

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While searching for regulators of virulence attributes of the human-pathogenic fungus *Candida albicans*, a gene was identified similar to the genes encoding the mammalian phospholipase A2-activating protein (PLAP) and the *Saccharomyces cerevisiae* protein Doa1, which is known to play a key role during ubiquitin (Ub)-dependent protein degradation. All three proteins contain WD-repeats. Both PLAP and CaDoa1 contain a mellitin-like sequence with a central 'KVL'. This mellitin-like sequence was shown to be necessary for full function of CaDoa1. *CaDOA1* was expressed under all conditions investigated. Gene disruption of *CaDOA1* caused phenotypes including modified colony morphologies, temperature sensitivity, reduced secretion of hydrolytic enzymes and hypersensitivity to various compounds such as propranolol, butanol, caffeine, chelators, azoles, nocodazole and cadmium. Strikingly, mutants lacking *DOA1* were filamentous and grew as pseudohyphae and true hyphae under conditions that normally support yeast growth. Transcriptional profiling of Δ *doa1* indicated that several genes associated with Ub-mediated proteolysis, including *CDC48* and *UBI4*, are upregulated. These data suggest that *DOA1* of *C. albicans*, like its orthologue in *S. cerevisiae*, is associated with Ub-mediated proteolysis and has multiple functions. However, some functions of CaDoa1 seem to be unique for *C. albicans*. These results support the hypothesis that Ub-mediated proteolysis plays an important role in the regulation of morphology in *C. albicans*.

Received 22 September 2006

Revised 21 November 2006

Accepted 30 November 2006

INTRODUCTION

The human-pathogenic yeast *Candida albicans* has become one of the model microbes to investigate the principles of fungal pathogenicity and host–fungus interactions. Since the genome of *C. albicans* has been sequenced and sequence data made available to the scientific community (<http://www-sequence.stanford.edu/group/candida/>; <http://genolist.pasteur.fr/CandidaDB>), significant progress has been made in understanding the biology of this fungus on the basis of comparative and functional genomics (Jones *et al.*, 2004). This includes knowledge about genes and factors associated with virulence attributes of *C. albicans* such as adhesion factors, the ability to alter cell morphology between yeast, pseudohyphal and hyphal forms, and the secretion of hydrolases (Calderone & Fonzi, 2001). For the latter, gene

families have been discovered which encode secreted proteinases (Naglik *et al.*, 2003), lipases (Hube *et al.*, 2000) and phospholipases (Ghannoum, 2000). Genes encoding phospholipases B, C and D have been described and their impact on pathogenesis of *C. albicans* infections studied (Ghannoum, 2000; Hube *et al.*, 2001; Knechtle *et al.*, 2005; Kunze *et al.*, 2005). However, although earlier reports demonstrated extracellular phospholipase A activity (Goyal & Khuller, 1992; Takahashi *et al.*, 1991), no gene encoding a putative secretory phospholipase A has so far been identified.

With the increasing information gained about single virulence attributes and fitness factors, research has moved on to those factors which regulate the molecular and cellular processes associated with pathogenicity. One of the best examples of regulatory networks linked to processes essential for virulence of *C. albicans* is the regulation of morphogenesis (Kumamoto & Vines, 2005a). Here, two major pathways, a MAP-kinase and a cAMP pathway, are of central importance for the initiation of hypha formation, but several other factors have also been identified to be crucial for hyphal morphogenesis. In particular, the key

Abbreviations: DAG, diacylglycerol; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PLAP, phospholipase A2-activating protein; Ub, ubiquitin.

The GEO accession number for the data reported in this paper is GSE 6905.

transcriptional factor of the cAMP pathway, Efg1, was shown to be essential not only for hypha formation under most conditions (Lo *et al.*, 1997; Stoldt *et al.*, 1997), but also for the expression of virulence attributes associated with dimorphism, such as the adhesion molecule Hwp1 or the secreted aspartic proteinases Sap4–6 (Felk *et al.*, 2002; Kumamoto & Vences, 2005b; Sundstrom, 2002).

Regulation of cellular processes in fungi has been studied extensively in the yeast *Saccharomyces cerevisiae*. In principle, regulation of eukaryotic cells occurs on a number of different levels including genomic, transcriptional and post-transcriptional (Castrillo & Oliver, 2006). One of the central post-translational mechanisms of regulation in eukaryotic cells is the controlled proteolysis of proteins such as transcription factors via ubiquitination. Protein ubiquitination is catalysed by ubiquitin (Ub) ligases in concert with Ub-conjugating enzymes, which facilitate the formation of an isopeptide bond between the C-terminus of Ub and a lysine side chain of a target protein (Sung *et al.*, 1988). The specific biological signal mediated by a polyubiquitin chain is determined, in part, by chain topology, which is differentiated by the Ub lysine residue used for chain extension (Hofmann & Pickart, 2001; Pickart, 1997). Escort factors are involved in targeting ubiquitinated substrates to the proteasome (Rumpf & Jentsch, 2006). Furthermore, protein ubiquitination is reversible as Ub can be removed by deubiquitinating enzymes (Amerik *et al.*, 2000). These regulatory processes require a network of factors to ensure a timely, precise and specific degradation of proteins with distinct cellular functions. In *S. cerevisiae*, it has been shown that one of the proteins associated with the escort factor Cdc48 is Doa1, which acts as an adaptor that possesses a novel Ub binding domain (Mullally *et al.*, 2006).

While searching for regulators which may be involved in phospholipase A activation in *C. albicans*, we identified a gene homologous to the mammalian PLAP (phospholipase A2-activating protein) (Clark *et al.*, 1991) and the *S. cerevisiae* protein Doa1 (Ghislain *et al.*, 1996; Hochstrasser & Varshavsky, 1990; Johnson *et al.*, 1995; Mullally *et al.*, 2006). The orthologue of Doa1 is involved in multiple cellular processes in *C. albicans*, including filamentous growth, diacylglycerol production, cell wall integrity, mitotic spindle formation, heavy metal tolerance, growth on non-fermentable carbon sources and haemolytic activity.

METHODS

Strains and growth conditions. In this study we used *C. albicans* strains SC5314 and CAF2-1 as wild-type controls and CAI-4 to produce mutants either lacking or overexpressing *CaDOA1* (Fonzi & Irwin, 1993). Cells were grown in YPD [2% (w/v) glucose, 1% (w/v) yeast extract, 2% (w/v) bacto peptone] or SD [2% (w/v) glucose, 0.5% (w/v) (NH₄)₂SO₄, 0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulphate; Difco]. For phenotype screening and expression studies the following media were used: M199 (Sigma), CAA medium (Leuker *et al.*, 1997; Stoldt *et al.*, 1997), Sabouraud glucose medium [2% (w/v) glucose, 1% (w/v) bacto peptone], YCB-BSA (Hube *et al.*, 1994), blood agar {1 l bouillon

[0.3% (w/v) NaCl, 0.3% (w/v) Na₂HPO₄·2H₂O, 1% (w/v) bacto peptone, 1.4% (w/v) Lab-Lemco powder (Oxoid)], 15 g agar, 50 ml mutton blood}, Lee's medium pH 4.5 and 6.5 (Buffo *et al.*, 1984), egg yolk agar (Fu *et al.*, 1997), SLAD medium [6.7 g YNB l⁻¹ (Difco) without amino acids and ammonium sulphate plus 0.05 mM (NH₄)₂SO₄], Spider agar (Liu *et al.*, 1994), YPD plus 5% (v/v) fetal calf serum (FCS) (YSer) or SD containing cadmium sulphate, propranolol, butanol, caffeine, EDTA, LiCl, MnCl₂, ZnCl₂, CuCl₂, NaCl, H₂O₂, hygromycin B, cycloheximide, tetracycline, benomyl, Congo Red, SDS, amphotericin B, amorolfine, cyclosporin A, calcofluor, ciclopirox, isoflurane, nocodazole or itraconazole at appropriate concentrations.

For phenotypic screening of mutant strains a serial drop dilution test was used. Five microlitre suspensions of tenfold dilutions of cells were dropped onto the corresponding solid media.

For expression analysis, CAF2-1 was grown overnight at 30 °C in SD medium, diluted into fresh SD medium to give a concentration of 10⁶ cells ml⁻¹ and incubated at 37 °C to an OD₆₀₀ of 0.6. The culture was then divided into 40 ml aliquots, centrifuged and the pellet resuspended in 40 ml of one of the media described above. After a given incubation period, cells were harvested for RNA extraction (see below). For induction of the *PCK1* promoter we used media without glucose: SGlyc [SD with 2% (v/v) glycerol instead of glucose] or SGlycAc [SD with 3% (v/v) glycerol and 2% (w/v) potassium acetate instead of glucose], CAA medium and succinate medium (B medium) (Leuker *et al.*, 1997; Stoldt *et al.*, 1997).

For cloning procedures, *E. coli* TOP10 cells (Invitrogen) were used as described by the manufacturer.

Microscopic investigations. For viability staining, we used the LIVE/DEAD Yeast Viability Kit (Molecular Probes), according to the manufacturer's instructions. Briefly, 50 µl cells from a culture in late exponential phase were mixed with 1 ml sterile medium. Cells were harvested and resuspended in fresh medium at a cell density of 10⁶–10⁷ cells ml⁻¹. The sample was mixed with the stain FUN1 (final concn 20 µM) and incubated for 30 min at 30 °C in the dark. Calcofluor white was used to stain fungal chitin and to discriminate between true hyphal cells and pseudohyphae. Stained cells were analysed with a fluorescence microscope.

Disruption of *DOA1*. To disrupt *DOA1* we produced the following plasmids. Primers DOA1-3 fwd, containing a *Sall* restriction site, and DOA1-4 rev with genomic DNA from strain SC5314 were used to amplify a 433 bp fragment from the 3' end of *DOA1* (all primers are summarized in Table 1). The PCR product was cloned into pCR2.1-TOPO to give pDK-9. The *DOA1* insert was released with *HindIII* and *Sall* and ligated into those sites of vector pMB7 to give pDK-10. Primers DOA1-1b fwd and DOA1-2a rev were used to amplify a 745 bp fragment of the 5' end of *DOA1* and the PCR product was cloned into pCR2.1-TOPO to give pDK-11. Next, a *HindIII* and *KpnI* fragment of pDK-10 containing the 3' end of *DOA1* and the *hisG-URA-hisG* cassette was ligated into pDK-11 digested with the same enzymes to give the final disruption cassette, pDK-12. The disruption cassette was used to transform *C. albicans* CAI-4 as described below. Integration of the disruption cassette at the correct locus, disruption of *DOA1* and the genotypes of all other mutants, revertants and overexpressing strains described below were confirmed by PCR and Southern blot analysis. All strains produced in this study are summarized in Table 2.

Construction of a conditional *DOA1* mutant. To produce a conditional *DOA1* mutant we used primers DOA1-UE1 and DOA1-UE2, containing *BglII* sites, to amplify the entire *DOA1* gene, including 200 bp of the 3' end, using the Long Expand Kit (Roche). The PCR fragment was subcloned into pGEM-T Easy (Promega) to give

Table 1. Primers used in this study

Primer	Gene	Sequence (5'–3')
DOA1-3 fwd	DOA1	GTC GAC CGA GCC ACC ATT GAA
DOA1-4 rev	DOA1	GGT GAT TAA TTC TAG CGC TTG C
DOA1-1b fwd	DOA1	CTG TTT GCA AAC TAG TTG TAT TC
DOA1-2a rev	DOA1	ATC GGC ACT ACA AGT TAA ATA GTG
DOA1-UE1 fwd	DOA1	AGA TCT ATG TCT TAC AAA CTT AGT
DOA1-UE2 rev	DOA1	AGA TCT ATT AGA GAT TCA TAA GAA
DOA1-k1a-fwd	DOA1	GGT ACC GGC ACT GAC AAT AAA TTA TA
DOA1-k1a-rev	DOA1	GGT ACC AAG TGC TGG ATT TTC CAC AT
ACT1 fwd	ACT1	ACC GAA GCT CCA ATG AAT CCA
ACT1 rev	ACT1	GGA TGG ACC AGA TTC GTC GTA
EFB1 fwd	EFB1	AGT CAT TGA ACG AAT TCT TGG CTG
EFB1 rev	EFB1	TTC TTC AAC AGC AGC TTG TAA GTC

pDK-21. The fragment was released with *Bgl*II and ligated into pBI-1 (Stoldt *et al.*, 1997) fusing the 5' end to the *PCK* promoter (pDK-22). The plasmid was used to transform Δ *doa1* and CAI-4.

Construction of a *DOA1* mutant lacking the mellitin domain.

We amplified the *DOA1* gene lacking an internal 75 bp fragment (containing the KVL-site) by inverse PCR (Long Expand Kit; Roche) by use of plasmid pDK-21 as a template and primers DOA1-k1a fwd and rev, containing *Kpn*I sites. The linear fragment was digested with *Kpn*I and religated to give pDK-26. The insert of pDK-26 was released with *Bgl*II and ligated into pBI-1 at the *Bgl*II site to give pDK-27. The plasmid was used to transform Δ *doa1*.

Transformation of *C. albicans*. To transform *C. albicans*, two modified lithium acetate protocols (Sanglard *et al.*, 1997) and one electroporation protocol (De Backer *et al.*, 1999) were used. Transformants were screened by PCR and Southern blot analysis as described previously (Felk *et al.*, 2002).

RNA extraction and RT-PCR. Liquid cultures were grown as described for each experiment, and cells were harvested and RNA isolated as described previously (Felk *et al.*, 2002). For RT-PCR, 1.5 µg RNA was DNase-treated and cDNA was synthesized (Felk *et al.*, 2002). Controls included cDNA synthesis without addition of reverse transcriptase and amplification of an intron-containing fragment of the *EFB1* gene (Schaller *et al.*, 1999) (with primers EFB1 fwd and EFB1 rev in Table 1). To ensure that samples from the exponential phase of PCR amplification were examined, we used 20, 25, 30, 35 and 40 (or more) cycles. All RT-PCR experiments were done at least in duplicate with samples from two independent biological experiments. An additional internal standard control was the housekeeping gene *ACT1*, amplified with primers ACT1 fwd and ACT1 rev.

Microarray hybridization and analysis. For transcript profiling, we used *C. albicans* microarrays (Eurogentec) containing 6039 ORFs. Arrays were designed as described at www.galarfungail.org/

Table 2. Strains used in this study

Strain no.	Strain	Genotype	Source or reference
	SC5314	Wild-type strain	Clinical isolate, Gillum <i>et al.</i> (1984)
	CAF2-1	SC5314 derivative Δ <i>ura3::imm434/URA3</i>	Fonzi & Irwin (1993)
	CAI4	SC5314 derivative Δ <i>ura3::imm434/Δura3::imm434</i>	Fonzi & Irwin (1993)
DK1	Δ <i>doa1/DOA1</i>	CAI4 derivative, Δ <i>doa1::hisG-URA3-hisG/DOA1</i>	This study
DK1-1	Δ <i>doa1/DOA1</i>	CAI4 derivative, Δ <i>doa1::hisG/DOA1</i>	This study
DK2	Δ <i>doa1/Δdoa1</i>	CAI4 derivative, Δ <i>doa1::hisG/Δdoa1::hisG-URA3-hisG</i>	This study
DK2-1	Δ <i>doa1/Δdoa1</i>	CAI4 derivative, Δ <i>doa1::hisG/Δdoa1::hisG</i>	This study
DK3	Δ <i>doa1/Δdoa1</i> + pBI-1- <i>DOA1</i>	CAI4 derivative, Δ <i>doa1::hisG/Δdoa1::hisG</i> carrying pBI-1- <i>DOA1</i>	This study
DK4	CAI4 + pBI-1- <i>DOA1</i>	CAI4 carrying pBI-1- <i>DOA1</i>	This study
DK5	Δ <i>doa1/Δdoa1</i> + pBI-1	CAI4 derivative, Δ <i>doa1::hisG/Δdoa1::hisG</i> carrying empty pBI-1	This study
DK6	Δ <i>doa1/Δdoa1</i> + pBI-1- <i>DOA1</i> -KVL	CAI4 derivative, Δ <i>doa1::hisG/Δdoa1::hisG</i> carrying pBI-1- Δ <i>DOA1</i>	This study
DK7	Δ <i>doa1/Δdoa1</i> + <i>Clp10</i>	CAI4 derivative, Δ <i>doa1::hisG/Δdoa1::hisG rps1::Clp10/RPS1</i>	This study
DK8	Δ <i>doa1/Δdoa1</i> + <i>Clp10-DOA1</i>	CAI4 derivative, Δ <i>doa1::hisG/Δdoa1::hisG, rps1::Clp10-DOA1/RPS1</i>	This study

CaDOA1 is expressed in yeast and hyphal cells

To analyse under which conditions *CaDOA1* is expressed *in vitro*, we used semiquantitative RT-PCR. We analysed the expression of *DOA1* in media supporting the yeast growth form and media which induced hypha formation. Yeast cells from a preculture (Lee’s medium, pH 4.5, at 25 °C, or SD medium) were used to inoculate media supporting either yeast or hyphal growth with samples taken at several time points (Fig. 2). After 3 h, up to 99 % of all cells had produced germ tubes in the hypha-inducing media (Fig. 2). Using either *ACT1* or *EFB1* as internal controls and several rounds of PCR to ensure that samples from the exponential phase of PCR amplification were examined, we detected *DOA1* transcripts in all samples showing that this gene is constitutively expressed in yeast and hyphal cells under the conditions investigated. However, an increased expression of *DOA1* was observed at late stages of yeast growth (Fig. 2).

Gene disruption of DOA1

To analyse the function of *DOA1*, we produced mutants lacking this gene with the Ura-blaster protocol (Fonzi & Irwin, 1993). A disruption cassette was constructed which contained a 745 bp fragment corresponding to position

–274 to +471 bp relative to the ATG start codon of *DOA1* and a 433 bp fragment corresponding to position +1181 to +1614 bp relative to the ATG flanking the *hisG-URA3-hisG* cassette. The disruption cassette was used to disrupt both alleles of *DOA1*. Integration into both alleles was confirmed by Southern analysis and PCR. Several independent isogenic mutants were produced to verify that no further mutational events occurred during the transformation steps and the 5-fluoro-orotic acid treatments. For all experiments with the $\Delta doa1$ mutant (DK2; Ura⁺) we used the wild-type strains SC5314 and/or CAF2-1 (one copy of *URA3*) as reference strains.

Mutants lacking DOA1 are filamentous and show reduced growth under multiple conditions

Mutants lacking *DOA1* were analysed under a series of conditions. To analyse growth in liquid media, $\Delta doa1$ mutant (DK2) and wild-type cells (10⁶ cells ml^{–1}) from an SD preculture at 30 °C, which consisted predominantly of yeast cells, were inoculated into either SD or YPD medium at 37 °C. Microscopic investigation of cells grown under these conditions showed that wild-type cells grew in the yeast form, while $\Delta doa1$ mutant cells produced filaments (Fig. 3). By 4 h, 98 % of all wild-type cells grew as yeast forms in SD

Fig. 2. Expression of *DOA1* during yeast and hyphal growth using semiquantitative RT-PCR. SC5314 was grown in (a) SLAD, YPD plus serum, YPD or SG (Sabouraud glucose), or (b) Lee’s medium at either pH 4.5 or 6.5. RNA samples from different time points were used for RT-PCR analysis. Transcripts were amplified with 20–55 cycles using primer pairs that detect *CaDOA1*, *ACT1* or *EFB1*. *DOA1* is expressed under all conditions and only up-regulated at late time points in Lee’s medium supporting yeast growth.

Fig. 3. Filamentous growth of $\Delta doa1$ mutant and wild-type (CAF2-1) cells in liquid SD medium incubated at 37 °C for 2, 4 or 6 h.

medium (96 % in YPD), while 92 % of $\Delta doa1$ cells had formed filaments (YPD, 48 %). Detailed microscopy, including staining with calcofluor white, showed that the filaments produced by the $\Delta doa1$ mutant were a mixture of pseudohyphae and true hyphae (Fig. 3).

For analysis on solid media, we used a drop dilution test. For each medium we used two independently produced isogenic $\Delta doa1$ mutants, a heterozygote $\Delta doa1$ mutant (DK1, one copy of *URA3* and *DOA1*) and the wild-type strain CAF2-1 (one copy of *URA3*).

The most striking phenotype on solid media was filamentous growth as already shown under liquid conditions. Colonies of $\Delta doa1$ mutants grew mostly as filaments in media which normally do not induce hypha formation (Fig. 4). The hyperfilamentous phenotype was often associated with a crumpled colony surface. This includes colonies for which macroscopic production of filaments was not obvious. For example, wild-type growth on YPD at 30 or 37 °C appeared as smooth, creamy colonies and contained few hyphae, but $\Delta doa1$ colonies had a crumpled surface with filamentous cells which could be detached from the agar surface as a firm mass (Fig. 4c). As shown in Fig. 4(c), cells from CAF2-1 colonies consisted of yeast cells only, while cells from the mutant colonies were associated with each other as a network of mainly filaments and few yeast cells. Under conditions which induce hypha formation in wild-type cells (e.g. serum or spider medium), the mutant formed filaments more rapidly and mostly longer than in the wild-type. Growth at 25 and 40 °C under the same conditions did not alter these phenotypes.

In addition to the morphological differences, we observed a hypersensitivity of the $\Delta doa1$ mutants to several compounds (Fig. 4). For example, the addition of propranolol strongly reduced growth of the $\Delta doa1$ mutants. This compound is known to inhibit the lipid phosphate phosphohydrolases that convert phosphatidic acid (PA) into diacylglycerol (DAG) and hence hypha formation in *C. albicans* (Baker *et al.*, 2002). Furthermore, the metabolic inhibitor caffeine and the chelator EDTA caused severe growth inhibition of $\Delta doa1$ mutants. The inhibition caused by EDTA (more pronounced at 30 °C) was reversible by addition of either MnCl_2 (15 mM), ZnCl_2 (25, 50 μM) or CuCl_2 (50, 100 μM), with MnCl_2 being the most efficient and CuCl_2 the least efficient salt for reversing the effect (not shown). An increased susceptibility was also observed with antifungal agents such as itraconazole. Similar results were obtained with nocodazole, which causes disassembly of mitotic spindles. Since it had been shown that *S. cerevisiae* mutants lacking *DOA1* were sensitive to volatile anaesthetic agents such as isoflurane (Keil *et al.*, 1996), we also investigated the isoflurane sensitivity of the $\Delta doa1$ mutants from *C. albicans* and found they grew slower as compared to wild-type cells. The observed phenotypes varied with the temperature of incubation. For example, higher concentrations of CdSO_4 (120 μM) or butanol (4 %) were necessary at 30 °C compared with 30 μM CdSO_4 or 2 % butanol at 37 °C to cause growth inhibition of the $\Delta doa1$ mutants (not shown). Use of non-fermentable carbon sources such as glycerol or acetate instead of glucose led to reduced growth of the $\Delta doa1$ mutant at 37 °C, but not at 30 °C (not shown). To investigate the haemolytic activity of $\Delta doa1$ mutants, we also inoculated

Fig. 4. Colony and cellular morphology of $\Delta doa1$. (a) Wild-type (wt) (CAF2-1) and mutant cells were dropped at concentrations of 5×10^3 , 5×10^1 or 5 cells per $5 \mu\text{l}$ onto agar plates and incubated under the given conditions. (b) Morphology of wild-type and mutant colonies on protein agar (YCB/BSA) and blood agar. (c) Colony and cellular morphology of $\Delta doa1$ as compared to the wild-type. Cells were scratched from the colony surface, diluted in water and investigated under the microscope. Mutant cells are filamentous.

blood agar plates. At 37°C we observed strongly reduced growth and greyish and brown colonies with a clearing zone around colonies on this type of agar. In contrast, the $\Delta doa1$ mutants showed hyperfilamentous growth (as compared with the wild-type) with no or only reduced clear zones around the colonies at 30°C . On YCB-BSA agar, which can be used to test extracellular proteolytic activity, we observed an earlier and stronger production of filaments for the $\Delta doa1$ mutants at 30°C compared with 37°C and with the wild-type under the same temperatures. Phenotypes of $\Delta doa1$ mutants are summarized in Table 3.

Phospholipase activity of $\Delta doa1$

Since the human orthologue of CaDoa1, PLAP, is a phospholipase A2-activating factor, we investigated whether

disruption of *DOA1* has any influence on phospholipase activity in *C. albicans*. For these studies we tested LPC as substrate with culture supernatants and soluble intracellular protein samples after mechanical cell lysis as enzyme sources. These samples came from *C. albicans* cells grown under conditions which normally favour yeast growth. Since phospholipase activity may depend on the morphology of *C. albicans* cells and the phenotypic analysis of the $\Delta doa1$ mutant showed filamentation under conditions which normally favour yeast growth, we investigated the morphology of cells in each sample. Both wild-type (CAF2-1) and $\Delta doa1$ mutant (DK2) cells grew in the yeast form in Lee's medium at pH 4.5 at 25°C (not shown). However, growth in SD medium at 30 and 37°C caused at least moderate filamentation of the $\Delta doa1$ mutant (2–4%; not shown). Generally, the phospholipase activity was reduced below

that of wild-type in SD medium. Extracellular phospholipase activity was reduced by $68.4 \pm 3.5\%$ at pH 6.0 and $68.5 \pm 2.8\%$ at pH 7.5 at 30 °C. At 37 °C the activity was further decreased ($78.5 \pm 1.0\%$ at pH 6.0 and $74.9 \pm 5.2\%$ at pH 7.5). Intracellular phospholipase activity was reduced by $56.1 \pm 4.1\%$ at pH 6.0 and $49.4 \pm 1.9\%$ at pH 7.5 at 30 °C. At 37 °C the activity was less reduced ($25.4 \pm 7.3\%$ at pH 6.0 and $25.4 \pm 7.3\%$ at pH 7.5).

Reintroduction of *DOA1* into Δ *doa1* restored wild-type phenotypes

To fulfil Koch's postulates (Falkow, 1988), we reintroduced a native *DOA1* copy into the Δ *doa1* mutant (DK2) and investigated the phenotype of the retransformant (DK3) in comparison with the Δ *doa1* mutant on selected media. For this we fused the *DOA1* gene with the controllable *PCK1* promoter from *C. albicans* into the plasmid pBI-1 (Leuker *et al.*, 1997; Stoldt *et al.*, 1997). Genes controlled by the *PCK1* promoter are expressed under low concentrations of glucose. A PCR fragment containing the entire *DOA1* ORF and 200 bp of the untranslated 3' region was cloned into pBI-1 to produce the retransformation plasmid pDK-22. The

URA3-negative Δ *doa1* mutant was transformed with pDK-22. To produce a control strain, we also transformed the *URA3*-negative Δ *doa1* mutant with the empty plasmid pBI-1. Transformants with the *PCK1*-driven *DOA1* gene (DK3) and the mutant with the empty vector (DK5) were tested on media known to induce the *PCK1* promoter and containing compounds previously shown to inhibit growth of the Δ *doa1* mutants. These included CAA medium, succinate medium (B medium), SGlycAc medium and CAA medium with isoflurane or itraconazole ($1 \mu\text{g ml}^{-1}$) at 30 and 37 °C.

As shown in Fig. 5, extrachromosomal expression of *DOA1* reversed the altered growth phenotypes of *DOA1* disruption on all media. To show further that the observed phenotypes of the Δ *doa1* mutants were in fact due to the disruption of *DOA1*, we also cloned *DOA1* including its own promoter and 3' untranslated region into the vector CIP10 (Murad *et al.*, 2000) (to give pDK-24 and pDK-25) and inserted the gene into the *RPS1* locus of *C. albicans* (DK8). When compared to strains carrying the empty plasmid CIP10 (DK7), transformants carrying pDK-25 (DK8) reversed the phenotype observed for Δ *doa1* mutants into wild-type phenotypes (not shown).

Table 3. Phenotypes of Δ *doa1* mutants on solid media

The following abbreviations and symbols are used to describe the phenotype of the mutant: *hf*, hyperfilamentation; –, no differences; *in*, growth inhibition; *c*, crumpled colony surface; > *cz*, increased clearing zone; ND, not determined.

Growth condition and inhibitor	30 °C	37 °C
Standard media		
SD	–	–
YPD	<i>hf</i>	<i>c</i>
Sabouraud	–	–
YPD anaerobic	ND	<i>hf</i>
Hyphal induction		
Spider	<i>c</i>	<i>hf</i>
M199	<i>hf</i>	<i>hf</i>
FCS (5 %)	<i>hf</i>	<i>hf</i>
FCS (10 %)	–	<i>hf</i>
Extracellular hydrolytic activity		
Blood agar	<i>hf</i>	<i>in</i> , <i>c</i> , > <i>cz</i>
Egg yolk agar	> <i>cz</i>	–
YCB-BSA	<i>hf</i> , > <i>cz</i>	<i>hf</i>
Osmotic stress		
Sorbitol (6, 7 %)	–	–
Galactose (5 %)	<i>hf</i>	–
NaCl (0.5–1.25 M)	–	–
Oxidative stress		
Potassium acetate (2 %), glycerol (3 %) (SGlycAc)	–	<i>in</i>
Potassium acetate (2 %), glycerol (3 %) (SGlycAc) plus CuCl ₂ (10 mM)	<i>in</i>	ND
Potassium acetate (2 %), glycerol (3 %) (YGlycAc)	<i>c</i>	<i>c</i>
H ₂ O ₂ (0.44–0.88 mM)	<i>in</i>	ND
pH sensitivity		
pH 5.7	<i>hf</i>	–
pH 6.0	<i>hf</i>	<i>c</i>
pH 6.5	<i>hf</i>	<i>c</i>
pH 7.0	<i>hf</i>	<i>c</i>
pH 7.5	<i>hf</i>	<i>c</i>
pH 8.0	<i>hf</i>	–
Chelators		
EDTA (1 mM) (Mn ²⁺ , Ca ²⁺ , Mg ²⁺ , Ni ²⁺ , Zn ²⁺)	<i>in</i>	<i>in</i>
Ciclopirox (Fe ²⁺)	–	–
2,2'-Dipyridyl (Fe ²⁺)	–	<i>in</i>
Ions/signal transduction		
LiCl (0.24 M)	ND	–
CaCl ₂ (300 mM)	–	–
MnCl ₂ (30 mM)	–	<i>c</i>
Caffeine (10 mM)	–	<i>in</i>
Heavy metals		
Cadmium sulfate (15–100 µM)	–	<i>in</i>
Cadmium sulfate (120 µM)	<i>in</i>	ND
Protein synthesis inhibitors		
Hygromycine B (400 µg ml ^{–1})	–	<i>in</i>
Cycloheximide (1 µg ml ^{–1})	–	(<i>in</i>)

Table 3. cont.

Growth condition and inhibitor	30 °C	37 °C
Tetracycline (10 µg ml ^{–1})	–	–
Microtubule inhibitors		
Nocodazole (4 µg ml ^{–1})	–	<i>in</i>
Benomyl (10 µg ml ^{–1})	–	–
Cell wall inhibitors		
Calcofluor (800 µg ml ^{–1})	–	<i>in</i>
Congo Red (100 mg ml ^{–1})	–	ND
Cell surface integrity		
SDS (0.005 %)	<i>in</i>	<i>in</i>
Amphotericin B (0.2 µg ml ^{–1})	–	<i>in</i>
Amorolfine (3 µg ml ^{–1})	–	<i>in</i>
Itraconazole (1 µg ml ^{–1})	<i>in</i>	<i>in</i>
Casamino acids plus itraconazole	<i>in</i>	–
Succinate plus itraconazole	–	<i>in</i>
Non-glucose carbon sources		
Galactose 2 % (SGal)	–	–
Galactose 2 % (YGal)	–	–
Sucrose 2 % (SSucr)	<i>in</i>	ND
Casamino acids	<i>hf</i>	<i>c</i>
Succinate	<i>hf</i>	<i>c</i>
Calcineurine A inhibition		
Cyclosporin A (50–100 µg ml ^{–1})	–	–
PLD and PA phosphatase inhibitors		
Propranolol (50–500 µM)	–	<i>in</i>
Butanol (0.5–5 %)	<i>in</i>	<i>in</i>
Volatile anaesthetics		
Isoflurane (100–500 µl)	<i>in</i>	ND

The mellitin-like C-terminal region of Doa1 is essential for full function of Doa1

Clark *et al.* (1991) investigated the role of the mellitin-like region of the PLAP protein for the activation of phospholipase A2. To investigate the functional role of the mellitin-like region downstream of the WD repeats we deleted a small region containing the KVL motif in the *DOA1* gene sequence used to rescue the wild-type phenotype in the Δ *doa1* mutant. This was accomplished using plasmid pDK-21 as a template for an inverse PCR with primers DOA1-k1a fwd and rev. The resulting fragment lacking base pairs +1801 to +1875 after the ATG start codon of *DOA1* (representing the amino acids of CaDoa1 shown in Fig. 1b) was fused to the *PCK1* promoter in pBI-1 (pDK-27). Transformants (DK6) were analysed on *PCK1*-inducing media such as CAA, SGlycAc and blood agar at 30 and 37 °C in serial drop dilution tests. As shown in Fig. 6, the *PCK* promoter-driven *DOA1* gene lacking the 75 bp fragment of the mellitin-like region was not able to fully restore the phenotypes of the Δ *doa1* mutant on CAA (wild-type and retransformant have smooth surfaces, mutants have wrinkled surfaces) and blood agar (wild-type and retransformant produce smooth colony edges, mutants produce hyphae at the edge of the colony), suggesting that this region is essential for full function of CaDoa1.

Fig. 5. Reintroduction of *DOA1* into $\Delta doa1$ restored wild-type phenotypes. A native copy of *DOA1* was fused to the *PCK1* promoter in the plasmid pBI-1 and cultivated under conditions which promoted *PCK1* induction. Growth of the retransformant ($\Delta doa1/\Delta doa1+pBI-1-DOA1$) was compared with the $\Delta doa1$ mutant carrying the empty plasmid ($\Delta doa1/\Delta doa1+pBI-1$) and two isogenic mutants ($\Delta doa1/\Delta doa1$, carrying one copy of *URA3*). The wild-type phenotype (growth rate, colony morphology and cell morphology) was recovered in CAA medium, CAA containing itraconazole, B medium and medium containing glycerol and acetate as carbon source (SGlycAc).

Overexpression of *DOA1* has no influence on wild-type cells

Since disruption of *DOA1* generated altered phenotypes we questioned whether overexpression of *DOA1* may have an influence on cellular processes and growth. Therefore, we transformed the wild-type strain CAI-4 with plasmid pDK-22 containing the *PCK1* promoter-driven *DOA1* gene in pBI-1. Transformants (DK4) grown on SD, YPD, B medium, CAA medium, B medium or CAA medium containing 1 μg itraconazole ml^{-1} , SGlycAc medium and 5 or 10 % FCS agar at 30 or 37 °C did not show any growth differences when compared to strains carrying empty pBI-1.

DOA1 transcription under selected growth conditions

Phenotypic analysis of $\Delta doa1$ mutants showed that disruption of *DOA1* caused multiple growth effects under

distinct conditions. Since we reasoned that *DOA1* might be regulated under the conditions which caused growth defects for $\Delta doa1$ mutants, we investigated the transcriptional levels under selected conditions, such as SD medium containing 60 μM CdSO_4 or SGlyc medium at 37 °C, as compared with mRNA levels in SD medium at 37 °C in wild-type cells. Furthermore, we monitored the transcript level in SD medium at 42 °C. Only this heat-shock condition caused an increased transcriptional level of *DOA1* (Fig. 7).

Transcriptional profiling of $\Delta doa1$

To obtain further information about the functions of *DOA1*, we investigated the genome-wide transcription profile of the $\Delta doa1$ mutant (DK2) as compared to the wild-type strain CAF2-1 using microarrays. Cells from the $\Delta doa1$ mutant and the wild-type strain CAF2-1 were precultured in SD at 30 °C, and 10^6 cells ml^{-1} were inoculated in SD medium at

Fig. 6. Removal of a small mellitin-like region containing the conserved KVL motif caused loss of full function of Doa1. Strains containing a *DOA1* gene without this region ($\Delta doa1$ +pBI-1-*DOA1*-KVL) are not able to fully restore the wild-type phenotype in CAA (colony morphology) and blood agar (colony and cell morphology) as compared with the wild-type and the retransformant strain containing the native gene ($\Delta doa1$ +pBI-1-*DOA1*).

37 °C for 7 h before RNA was isolated. Transcriptional array data confirmed the expression data obtained with RT-PCR. Including the genes investigated by RT-PCR, 96 genes were significantly differentially expressed in both strains ($P<0.05$, t -test). Genes were 11.1- to 1.4-fold differentially expressed. Only two genes were identified as downregulated (both 2.1-fold) (Table 4). Fig. 8 shows the functional categories to which the 96 differentially expressed genes could be associated. One-third of all the genes (36 genes) encode proteins of unknown function (Fig. 8). Beside this group, the largest group of genes is associated with protein degradation, mostly linked to the proteasome, Ub or Ub-independent proteolysis. Other functional groups were transcription and replication, transport, fat metabolism and energy, amino acid metabolism, stress and oxidative stress. Despite the fact that the mutant produced mainly

pseudohypha and few true hypha, we identified the true hypha-associated gene *HWP1* as being upregulated. For selected genes (*HWP1*, *RBT6*, *ACB1*, *PRE10*, *PRE6*, *SMT3*, *IPF3262* and *IPF3262*) we again used RT-PCR to verify the microarray data (not shown).

Virulence of $\Delta doa1$

Since disruption of *DOA1* caused multiple phenotypes, including hyperfilamentation, we analysed the virulence of mutants lacking *DOA1* (DK2) by intravenous challenge in mice. Despite the filamentous phenotypic tendency of the $\Delta doa1$ mutant, no significant differences were observed in terms of mouse survival and organ burden compared with wild-type strain CAF2-1. Cell morphologies in the inoculum suspension comprised only yeast forms and short pseudo-

Fig. 7. Transcriptional level of *DOA1* as measured by semiquantitative RT-PCR in wild-type cells under selected conditions such as SD medium at 37 or 42 °C, SD medium containing CdSO₄ (CD) or SGlyc medium (SGlyc) as compared to the expression of the housekeeping gene *ACT1*. Only an increased temperature caused higher expression levels of *DOA1*.

Table 4. Genes differentially expressed in Δ *doa1*

Proteosome, Ub-dependent or -independent proteolysis (13)	Transcription/DNA/RNA (11)	Transporter (7)
<i>PUP2, UBI4, SMT3, CDC48, PRE6, PRE10, RPT1, RPT3, RPT6, IPF19983, IPF13356, IPF7227, IPF3262.3</i>	<i>HIR1</i> (down), <i>RNR21, DCP1, IFA3, URA5, MBF1, Zorro1a, IPF19974, IPF7666, IPF4814, IPF14248</i>	<i>CAN2, GAP6, GAP3, SSU1, CAF16, TOM22, GAP7.3eoc</i>
Energy/electron transfer/oxygen transport (5) <i>YHB1, EBP1, PCK1, GPM2, PGM2</i>	Sterol metabolism/lipid metabolism, -catabolism, -biosynthesis (5) <i>ACB1.exon2, SCS7, MUQ1, ARE2, DAK2.3eoc</i>	Oxidative stress (3) <i>TTR1, TRR1, IPF6231</i>
Stress/chaperones (2) <i>CPR6, RBL2</i>	Hypha specific (1) <i>HWP1</i>	Amino acid biosynthesis/metabolism (2) <i>MET3</i> (down), <i>IPF13176.3</i>
Unspecific (several) functions (11) <i>ERO1, UGP1, HEM14, MAK3, CRD1, IPF199, IPF6041, IPF7400, IPF11379, IPF3492, IPF1969</i>	Unknown functions (GO unknown) (36) <i>IPF14850, CDC123, IPF17510, IPF18234.3, ECM29.exon2, IPF6305.exon2, IPF3432, IPF19815, IPF4284, IPF3491, IPF18207, TCI1, IPF8762, IPF9851, IPF351, IPF6461, IPF4514, IPF11766, IPF17945, IPF4311, IPF8746, IPF18161, IPF2997, IPF14109, IPF1461, IPF15706, IPF2878, IPF3401, IFA1, IPF6156, IPF17975, IPF8725, IPF165, IPF13038, IPF16843, IPF1020</i>	

hyphae. The cells in the kidneys of moribund animals were filamentous, as were those in the kidneys of animals infected with wild-type *C. albicans*.

DISCUSSION

The aim of this study was the identification and characterization of putative regulators of virulence attributes, such as phospholipase A activity in *C. albicans*. One of the genes identified was similar to the human gene PLAP known to be associated with the regulation of phospholipase A activity (Lis & Romesberg, 2006) and Doa1 in *S. cerevisiae* (Tzermia *et al.*, 1994). Due to the high level of similarity to ScDOA1, we named this gene (*Ca*)DOA1. Although DOA1 was transcribed under all conditions investigated in yeast and pseudohyphal/filamentous forms of *C. albicans*, this gene is not essential for growth as mutants lacking DOA1 were viable. It is not clear whether DOA1 has a direct influence on phospholipase activity; however, Δ *doa1* mutants had reduced phospholipase activity.

The Doa1 amino acid sequence showed strong similarities to members of the WD-repeat protein family. These proteins have diverse cellular functions, such as regulation of signal transduction, transcription, pre-mRNA processing or cytoskeleton assembly (Smith *et al.*, 1999). Typically, WD-repeat proteins do not have catalytic functions, but are involved in multi-protein complexes in direct interaction with other proteins via the WD repeats (Neer *et al.*, 1994). However, some WD-repeat proteins are fused to a functional domain and have catalytic activities, for example

the mammalian protein PLAP (Neer *et al.*, 1994). In PLAP, the functional domain contains a mellitin-like sequence (Clark *et al.*, 1991) which is used to activate phospholipase A₂. A similar sequence exists in CaDoa1 as shown in this study. We showed that this putatively catalytic domain is essential for full function in *C. albicans*. Therefore, it may be possible that Doa1 in *C. albicans* has a catalytic function. The protein with the highest overall similarity to CaDoa1 is ScDoa1. Similar to CaDoa1, the second and seventh WD repeat of ScDoa1 (Keil *et al.*, 1996) differ in the consensus

Fig. 8. Functional categories of genes upregulated in the Δ *doa1* mutant in SD medium at 37 °C compared with wild-type cells as monitored by microarray analysis. Only two genes, *MET3* and *HIR1*, out of 96 differentially expressed genes were found to be downregulated.

sequence, confirming relatively high similarity between these two orthologues. However, the most similar regions between ScDoa1 and the mammalian PLAP are beyond the WD repeats in the C-terminal part, but exclude the catalytic mellitin-like sequence of PLAP. In contrast, CaDoa1 and PLAP are more similar within the mellitin-like sequence.

ScDoa1 has been shown to play a key role during Ub-dependent protein degradation, regulation of the cellular Ub concentration and DNA damage response (Johnson *et al.*, 1995; Lis & Romesberg, 2006). In two hybrid assays, ScDoa1 bound to Cdc48 and other factors via the C terminus (Decottignies *et al.*, 2004; Ghislain *et al.*, 1996; Johnson *et al.*, 1995; Keil *et al.*, 1996; Seigneurin-Berny *et al.*, 2001). The human orthologue of the chaperone Cdc48 is a central element in a major Ub-associated escort pathway (Rumpf & Jentsch, 2006). Recently, Mullally *et al.* (2006) showed that ScDoa1 is a Cdc48 adaptor that possesses a novel Ub binding domain. A number of phenotypes and the transcript profiles of mutants lacking CaDoa1 suggest that this orthologue in *C. albicans* has both distinct and similar functions and may also be involved in ubiquitination.

Deletion of *ScDOA1* caused resistance to isoflurane and other anaesthetics, and increased sensitivity to cadmium (Keil *et al.*, 1996; Wolfe *et al.*, 1998). The Δ *doa1* mutant of *C. albicans* is moderately more sensitive to both isoflurane and cadmium. The mode of action of isoflurane on fungal cells is unclear; however, an influence of isoflurane on membrane lipids and proteins, and on their role in membrane integrity has been proposed (Keil *et al.*, 1996). In contrast, Wolfe *et al.* (1998) postulated a possible direct or indirect correlation with Ub metabolism. Likewise, sensitivity to cadmium can be linked to Ub-mediated proteolysis. Cadmium reacts with thiol groups of proteins and can substitute Zn^{2+} ions. These modified proteins are likely to be substrates for proteolytic degradation via the proteasome. For example, mutants lacking Ub-conjugating proteins such as Ubc1, Ubc4 and Ubc7, or subunits of the proteasome such as Pre1 are hypersensitive to cadmium in *S. cerevisiae* (Jungmann *et al.*, 1993).

Microarray analyses of the Δ *doa1* mutant further hint at a role of Doa1 in the Ub degradation pathway. Several genes upregulated in the mutant are linked with Ub degradation. Of particular interest are *UBI4* and *CDC48*.

Overexpression of *ScUBI4* complements the defects of Δ *doa1* in *S. cerevisiae* (Johnson *et al.*, 1995) and the orthologue of *UBI4* from *C. albicans* can complement the defects of Δ *ubi4* in *S. cerevisiae* (Roig & Gozalbo, 2003). *C. albicans* wild-type cells upregulate *UBI4* during thermal stress and starvation, and mutants lacking Ubi4 have a moderately increased sensitivity to heat shock (Roig & Gozalbo, 2003). However, Δ *ubi4* mutants of *C. albicans* have a clear morphological dysfunction as increased formation of hyphal and pseudohyphal cells was observed (Roig & Gozalbo, 2003). The same expression pattern and phenotypes were shown for Δ *doa1* in this study.

As mentioned above, Mullally *et al.* (2006) suggest that ScDoa1 acts as an adaptor between Cdc48 and Ub. *CDC48* is upregulated in the Δ *doa1* mutant of *C. albicans* and, due to the high similarities between ScDoa1 and CaDoa1, it is likely that CaDoa1 has similar functions within the Ub degradation pathway. In this context, it is worth mentioning that the Ub binding domain in ScDoa1, the PFU domain (Mullally *et al.*, 2006), is also conserved in CaDoa1 (aa 354–448).

However, in addition to the similar features, there must be differences in function since the most obvious phenotype of the Δ *doa1* mutant of *C. albicans* was filamentous growth under conditions that normally support yeast morphology. These filaments were a mixture of true hyphae and pseudohyphae as indicated by the location of septa within the filaments (Sudbery, 2001). Surprisingly, we observed that the known hypha-specific gene *HWPI* was upregulated in the Δ *doa1* mutant compared to wild-type cells, even though the mutant cells grew mostly as pseudohyphae at the time of sampling.

Filamentous phenotypes have been observed in a number of mutants, such as strains lacking *SSN6* (Hwang *et al.*, 2003), *SPT3* (Laprade *et al.*, 2002), *UBI4* (Roig & Gozalbo, 2003), *RAD6* (Leng *et al.*, 2000), *CDC4* (Atir-Lande *et al.*, 2005) and *GRR1* (Butler *et al.*, 2006) in *C. albicans*, with the last four genes putatively linked to the Ub degradation pathway. Therefore, ubiquitination seems to be an important and previously underestimated regulator of morphology in *C. albicans*.

In addition to the filamentous morphology, we observed further phenotypes for the Δ *doa1* mutant of *C. albicans*, which were not described for the corresponding mutant in *S. cerevisiae*. For example, Ghislain *et al.* (1996) did not observe reduced growth for mutants lacking Doa1 (Ufd3) in *S. cerevisiae* when cells were exposed to caffeine (inhibitor of cAMP phosphodiesterase), while growth of the Δ *doa1* mutant of *C. albicans* was dramatically reduced when exposed to caffeine. Furthermore, propranolol, an inhibitor of phosphatidate phosphohydrolase, the enzyme which can convert PA to DAG, strongly reduced growth of Δ *doa1*. DAG, which can also be produced by phosphatidate phosphohydrolase-independent pathways, is an essential molecule for certain molecular processes (Kearns *et al.*, 1997). It was suggested to be a regulator of morphology in *C. albicans* as mutants lacking phospholipase D, which cannot produce PA from PC, have reduced abilities to produce hyphae (Hube *et al.*, 2001). Since propranolol had such a strong effect on growth, it must be concluded that other pathways of DAG production, such as PLC-mediated activity, are blocked or inhibited in the Δ *doa1* mutant.

Several observations, including increased susceptibility to SDS, amphotericin B, amorolfine or itraconazole, suggest a defect of the Δ *doa1* mutant in cell surface integrity. These defects could also be responsible for the reduced haemolytic activity observed on blood agar. Furthermore, cell membrane or cell wall defects may have caused reduced

protection against extracellular exposure to inhibitors, such as cycloheximide and hygromycin B, which cause increased growth inhibition in the Δ doa1 mutant. The view that disruption of DOA1 caused a defect in membrane integrity was supported by the observation that genes associated with sterol metabolism, such as SCS7 or ACB1 (Faergeman *et al.*, 2004; Gaigg *et al.*, 2001; Jensen-Pergakes *et al.*, 2001; Swain *et al.*, 2002; Yang *et al.*, 1996), or oxidative or nitrogen stress, such as TTR1, TRR1 (Fradin *et al.*, 2005) or YHB1 (Hromatka *et al.*, 2005), were upregulated in the mutant.

Although the Δ doa1 mutant had severe growth defects under a number of conditions and was filamentous, the virulence in a mouse model of systemic infections was not attenuated. Since both yeast and filamentous cell forms seem to be essential for full virulence in *C. albicans* (Kumamoto & Vines, 2005a, b) it must be concluded that other factors compensate the loss of function of Doa1 *in vivo*.

ACKNOWLEDGEMENTS

We thank members of the NG5 team of Antje Flieger, Robert Koch-Institut, for help with the phospholipase assay. This work was supported by the Robert Koch-Institut and the Deutsche Forschungsgemeinschaft (Hu528/7).

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Edited by: J. Pla