Autophagy in the pathogen *Candida albicans*

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Autophagy is a major cellular process that facilitates the bulk degradation of eukaryotic macromolecules and organelles, through degradation within the lysosomal/vacuole compartment. This has been demonstrated to influence a diverse array of eukaryotic cell functions including adaptation, differentiation and developmental programmes. For example, in *Saccharomyces cerevisiae* autophagy is required for sporulation and survival of nitrogen starvation. The opportunistic pathogen *Candida albicans* has the ability to colonize and cause disease within a diverse range of mammalian host sites. The ability to adapt and differentiate within the host is liable to be critical for host colonization and infection. Previous results indicated that the vacuole plays an important role in *C. albicans* adaptation to stress, differentiation, and survival within and injury of host cells. In this study the importance of vacuole-mediated degradation through the process of autophagy was investigated. This involved identification and deletion of *ATG9*, a *C. albicans* gene required for autophagy. The deletion strain was blocked in autophagy and the closely related cytoplasm to vacuole (cvt) trafficking pathway. This resulted in sensitivity to nitrogen starvation, but no defects in growth rate, vacuole morphology or resistance to other stresses. This indicates that the mutant has specific defects in autophagy/cvt trafficking. Given the importance of autophagy in the development and differentiation of other eukaryotes, it was surprising to find that the *atg9Δ* mutant was unaffected in either yeast–hypha or chlamydospore differentiation. Furthermore, the *atg9Δ* mutant survived within and killed a mouse macrophage-like cell line as efficiently as control strains. The data suggest that autophagy plays little or no role in *C. albicans* differentiation or during interaction with host cells.

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INTRODUCTION

Autophagy is a major pathway by which eukaryotes are able to degrade cellular material. This has been shown to influence a wide array of biological phenomena including resistance to stress and starvation, cellular differentiation, development and ageing, programmed cell death, antigen presentation, clearance of intracellular pathogens, tumorigenesis and neurodegenerative disease (Mizushima, 2005). In the model yeast *Saccharomyces cerevisiae*, autophagy is critical for survival during nitrogen starvation, and to complete the differentiation process of sporulation (Takeshige *et al.*, 1992; Tsukada & Ohsumi, 1993). The molecular mechanism behind autophagy has been intensively studied in yeast and mammalian cells, and many of the components that are required have been identified (Abeliovich & Klionsky, 2001; Klionsky, 2005). A portion of cytoplasmic material is bound in a double-membrane structure known as an autophagosome, which fuses with the vacuolar membrane to release a single-membrane-bound compartment within the vacuole lumen. Degradation of this

material by the hydrolytic enzymes within the vacuole recycles cellular building blocks such as amino acids, which can be used in the synthesis of new macromolecules. Thus autophagy is typically considered a non-specific means to degrade 'bulk' cytoplasmic material. However, recent studies have revealed that under some conditions certain cargo is preferentially targeted for sequestration within the autophagosome (Nair & Klionsky, 2005). For example, in *S. cerevisiae* under nutrient-replete conditions (i.e. when autophagy is repressed), the vacuolar hydrolases aminopeptidase I (API) and α -mannosidase (AMS) are delivered from cytoplasm to vacuole via the cvt (cytoplasm to vacuole trafficking) pathway. The cvt pathway closely resembles autophagy, and involves sequestration of the cargo proteins within a double-membrane vesicle, which subsequently fuses to the vacuole to release its contents within the lumen. The vesicles formed are smaller than autophagosomes and appear to specifically deliver the API and AMS hydrolases. Interestingly, the cvt and autophagy pathways rely on much of the same cellular machinery, and many mutants defective in autophagy are also blocked in the cvt pathway (Abeliovich & Klionsky, 2001; Thumm *et al.*, 1994; Tsukada & Ohsumi, 1993). Furthermore, under starvation conditions API and AMS are delivered to the vacuole in the

Abbreviations: AMS, α -mannosidase; API, aminopeptidase I; CPY, carboxypeptidase Y; DIC, differential interference contrast; SAP, secreted aspartyl protease.

autophagosome, underscoring the close relationship between these pathways.

Candida albicans is a commensal organism of some mammalian species including humans, where it commonly resides on mucosal surfaces. Under conditions of host immunosuppression *C. albicans* can invade host tissues to cause a diverse range of diseases. Infection of mucosal surfaces is common, with HIV patients being particularly susceptible to oral and oesophageal candidiasis (de Repentigny *et al.*, 2004), while neutropenic patients are at risk of disseminated disease, with a high mortality rate (Maertens *et al.*, 2001).

Given the importance of autophagy in a diverse array of eukaryotic cellular processes, we decided to establish the role of autophagy in *C. albicans* survival and differentiation within the host. To date the role the vacuole or indeed autophagy plays during *Candida*–host interaction has not been established. We hypothesized that autophagy is required for *C. albicans* adaptation and differentiation, two properties critical for survival within and infection of the mammalian host.

METHODS

Sequence analysis. *C. albicans* *ATG9* and *LAP4* homologues were identified through BLASTP searches on the *C. albicans* genome database (<http://www.candidagenome.org/>) using the *S. cerevisiae* protein sequences (<http://www.yeastgenome.org/>) as the query sequence. Protein sequences were aligned using the EMBOSS pairwise align algorithm (<http://www.ebi.ac.uk/emboss/>). Kyte–Doolittle hydropathy plots (Kyte & Doolittle, 1982) were performed within the DS gene program (Accelrys).

Strain construction. Strains used in this study are described in Table 1. Gene deletion strains were constructed by the PCR-based approach described by Wilson *et al.* (1999), using the *ura3 his1 arg4* strain BWP17, kindly provided by Dr A. Mitchell (Columbia University). *atg9Δ::ARG4* and *atg9Δ::HIS1* deletion cassettes were amplified by PCR using pARGΔSpe and pGEMHIS1 plasmids, respectively, as template with primers ATG9DISF and ATG9DISR (Table 2). BWP17 was first transformed with *atg9Δ::ARG4* to generate heterozygote strains BAA1 and BAA4. Each heterozygote strain was then transformed with the *atg9Δ::HIS1* cassette to generate the

double deletion strains BAA1H1 and BAA4H4. Correct integration of either cassette was confirmed at each step by PCR analysis using primer pairs ATG9DETF and ATG9DETR, ATG9AMPR and ARG4DET2, or ATG9AMPF and HIS1F1268 (Table 2). Southern blot analysis was also performed using an *ATG9*-specific probe to the 3'-UTR of *ATG9*, amplified using primers ATG9PBF and ATG9PBR (Table 2). Correct gene deletion resulted in the replacement of 2858 bp of the 2859 bp *ATG9* ORF, with either the 2822 nt *HIS1* or 2161 nt *ARG4* encoding cassettes. Finally a wild-type copy of *ATG9* including 5' and 3' flanking sequences was introduced to the deletion strains on pLA2, to produce a prototrophic 'reconstituted' strain. Prototrophic deletion strains were produced by transforming the deletion strains with plasmid vector alone (pLUX). Either plasmid was digested with *NheI* prior to transformation to target integration into (and reconstitution of) the *URA3* loci. The presence/absence of *ATG9* in the prototrophic deletion/reconstituted strains was confirmed by amplification using the ATG9DETF/R primer pair.

Strains harbouring API–GFP fusions were derived from BWP17 (*ATG9/ATG9*), BAA1 and BAA4 (*ATG9/atg9Δ*), and BAA1H1 and BAA4H4 (*atg9Δ/atg9Δ*) using the PCR-based tagging approach described by Gerami-Nejad *et al.* (2001). Primers LAP4GFPF and LAP4GFPFR (Table 2) were designed to amplify a GFP:: *URA3* cassette from plasmid pGFPURA3 (Gerami-Nejad *et al.*, 2001) with 78 bp of homology on either end to direct integration at the 3' end of the *C. albicans* *LAP41* gene to form an in-frame *LAP41*–GFP fusion. The amplified cassette was transformed into the above strains; transformant colonies were selected, and screened for correct integration by PCR. Genomic DNA was prepared from transformants and PCR-amplified using primers LAP4DETR and URA3-5 (Table 2); a product of 470 bp confirmed the expected integration event.

DNA manipulations. PCR was performed using standard reagents. Plasmids pGEMURA3, pGEMHIS1 and pRSARGASpe (Wilson *et al.*, 1999) were provided by Dr A. Mitchell (Columbia University). Plasmid pLUX (Ramon & Fonzi, 2003), was provided by Dr W. Fonzi (Georgetown University). Plasmids pLA2 and pLA9 were made by amplifying the *ATG9* ORF with 693 bp of 5'-UTR and 214 nt 3'-UTR from genomic DNA with ATG9AMPF and ATG9AMPR (Table 2) to incorporate *Bam*HI sites. The resulting product was cloned into the *Bam*HI site of pLUX.

Growth conditions. Strains were routinely grown on YPD (1% yeast extract, 2% Bacto Peptone, 2% glucose) at 30 °C, supplemented with uridine (25 µg ml⁻¹) when necessary (Guthrie & Fink, 1991). For growth curves, overnight cultures were subcultured to 20 ml fresh YPD medium to OD₆₀₀ 0.2 and incubated at 30 °C with

Table 1. Strains

Strain	Relevant genotype	Source
SC5314	<i>ATG9/ATG9</i>	
YJB6284	<i>ATG9/ATG9</i>	Bensen <i>et al.</i> (2002)
BWP17	<i>ATG9/ATG9 ura3Δ/ura3Δ his1Δ/his1Δ arg4Δ/arg4Δ</i>	Wilson <i>et al.</i> (1999)
BAA1/4	<i>ATG9/atg9Δ::ARG4 ura3Δ/ura3Δ his1Δ/his1Δ arg4Δ/arg4Δ</i>	This study
BAA1H1/BAA4H4	<i>atg9Δ::ARG4/atg9Δ::HIS1 ura3Δ/ura3Δ his1Δ/his1Δ arg4Δ/arg4Δ</i>	This study
AGD1 and AGD5	<i>atg9Δ::ARG4/atg9Δ::HIS1 ura3Δ/ura3Δ::URA3 his1Δ/his1Δ arg4Δ/arg4Δ</i>	This study
AGR1 and AGR5	<i>atg9Δ::ARG4/atg9Δ::HIS1 ura3Δ/ura3Δ::ATG9::URA3 his1Δ/his1Δ arg4Δ/arg4Δ</i>	This study
BL1/2	<i>ATG9/ATG9 LAP4/LAP4-GFP</i>	This study
LA1/2	<i>ATG9/atg9Δ::ARG4 LAP4/LAP4-GFP</i>	This study
DAL3/51	<i>atg9Δ/atg9Δ::ARG4 LAP4/LAP4-GFP</i>	This study

Table 2. Oligonucleotides

Primer	Sequence (5'→3')
ATG9AMPR*	TCATCAGGATCCAATAGAAAATAAAATCAACCC
ATG9AMPF*	TCATCAGGATCCACTTCTTTTGACGATCAACCC
ATG9DISF	TTCACTTATCCTACTTTTCTTCTTTTGAAGAATAACACAAGCTAACTTGTTCA- TGCTGGATGATAAGATTTGTGGAATTGTGAGCGGATA
ATG9DISR	CTCTCATTCCCTCCAAAAATAACATTATATAAACCAATGTATGTAAACTAAC- GTAGTAATACTAATTACCTTTCCCAGTCACGACGTT
ATG9PBF	CGTTAGTTTTACATACATTGG
ATG9PBR	AAGTATCACGATTAGTTCGAG
ATG9DETF	TCATATATAATCAACAGGGGC
ATG9DETR	AGTTATAGCCCAAATTGTCCC
LAP4GFPF	ATTAGGTATTAATTCCTCTATGGTTTCTTCAAGAATTGGAGAGATGTCTATG- ATAATTTTGTTGATTAGGTGGTGGTTCTAAAGGTGAAGAATTATT
LAP4GFPR	TAAACTAACAAATAACTAATACTTGCAAATCAACTTGCAAATCAACTTTTAATG- ATTCCTTTTCCTGTTGTTCAATGTTTCGATCTAGAAGGACCACCTTTGATTG
LAP4DETR	TTAATGTACAAGCCTTGCGGC
ARG4DET2	ATCAATTAACACAGAGATACC
HIS1F1268	CCGCTACTGTCTCTACTTTG
URA3-5	CCTATGAATCCACTATTGAACC

**Bam*HI sites shown in italic.

shaking. OD₆₀₀ was determined from samples taken hourly. Transformants were selected on minimal media [6.75 g l⁻¹ yeast nitrogen base plus ammonium sulfate and without amino acids, 2 % glucose, 2 % Bacto agar (YNB)] supplemented with the appropriate auxotrophic requirements, as described for *S. cerevisiae* (Burke *et al.*, 2000), except for uridine, which was added at 25 µg ml⁻¹.

Phenotypic assays. Resistance to temperature stress was determined on YPD agar at 37 and 42 °C, and osmotic stress was determined on YPD agar plus 2.5 M glycerol or 1.5 M NaCl. Secreted aspartyl protease (SAP) secretion was examined on BSA+YE agar (Crandall & Edwards, 1987). Carboxypeptidase Y (CPY) activity was measured using a colorimetric assay as previously reported (Palmer *et al.*, 2003). Resistance to nitrogen starvation was determined using an assay similar to that of Noda *et al.* (2000). Each strain was grown in YNB broth for 48 h at 30 °C. Cells were washed twice in SD–N and 10⁷ cells resuspended in 2 ml SD–N medium (0.17% yeast nitrogen base without ammonium sulfate or amino acids, 2% glucose). Samples taken at intervals were plated to YPD agar, and viability determined as c.f.u. after 2 days at 30 °C. Accumulation of autophagic bodies within the vacuole was assayed by transferring cells grown overnight in YPD to SD–N medium in the presence of 1 mM PMSF (Noda *et al.*, 2000). PMSF inhibits the breakdown of the delivered autophagic bodies. After 6–24 h at 30 °C, cells were examined by differential interference contrast (DIC) microscopy. Filamentation on M199 and 10% fetal calf serum (FCS) agar was performed as previously described (Palmer & Sturtevant, 2004). Cells from overnight cultures were also induced to filament in 10% FCS (in distilled H₂O) at 37 °C after inoculation at 10⁶ cells ml⁻¹. Chlamydospores were induced on cornstarch-Tween agar as previously described (Palmer *et al.*, 2004). Sensitivity to H₂O₂ and rapamycin was determined by measuring growth in YPD medium supplemented with the appropriate compound. Approximately 1000 cells from an overnight culture were inoculated to 200 µl growth medium within the wells of a 96-well plate. After 48 h incubation at 30 °C (250 r.p.m.), growth was measured at OD₆₀₀ using a plate reader.

Phagocytic assays. The murine macrophage cell line, J774A.1 (ATCC TIB-67) was grown according to ATTC instructions in DMEM, high glucose, 4 mM glutamine, 10 % fetal bovine serum at 37 °C under 5 % CO₂. The same culture conditions were used for incubations with *C. albicans*. *C. albicans* strains were incubated with J774A.1 cells as described by Lorenz *et al.* (2004). Briefly, J774A.1 cells were seeded overnight in 12-well plates (2 × 10⁵ per well) on 18 mm coverslips and incubated at 37 °C under 5 % CO₂. *C. albicans* strains were grown overnight at 30 °C, washed and incubated with J774A.1 cells at an m.o.i. of 2.0 (4 × 10⁵ per well) unless otherwise noted.

Macrophage survival. *C. albicans* and J774A.1 cells were incubated for 1, 5 and 24 h and then wells were washed and incubated with 0.2 μ M calcein AM (final concentration) (LIVE/DEAD Viability/Cytotoxicity Kit, Molecular Probes). *C. albicans* cells were simultaneously stained with calcofluor (0.225 μ M). Coverslips were removed from wells and observed under a fluorescence microscope. Macrophages that fluoresced green were viable. Macrophage survival was quantified by counting at least four fields for each well. Results are presented as the mean number of macrophages observed per field.

C. albicans survival. This was assessed using an end-point dilution assay as described by Rocha *et al.* (2001). Macrophages were seeded overnight in 96-well plates at 5×10^3 per well. *C. albicans* (50 μ l) were added to the first column of cells (150 μ l), then serially diluted 1:4 for six columns so that the resulting m.o.i. were between 2 and 1.9×10^{-3} . The plates were incubated for 48 h. Controls were wells with *C. albicans* but no macrophages. The lowest dilution of the control well where it was possible to discriminate distinct colonies was counted. The same dilution was counted for the *C. albicans* plus macrophage well. Results are presented as (number of colonies in the presence of macrophages/number of colonies in the absence of macrophages) $\times 100$. Each experiment was set up in quadruplicate and *P* values determined using the unpaired Student's *t*-test.

Vacuole morphology and API localization. Vacuole morphology was visualized using the fluorescent dye FM4-64, as reported (Palmer *et al.*, 2003; Vida & Emr, 1995). In order to localize the API-GFP fusion protein, cells were grown into the exponential phase in YPD medium, stained with FM4-64, washed twice in distilled water, and visualized using an Olympus BX51 fluorescence microscope. All cells were applied to polylysine-coated slides prior to viewing.

Western blot analysis. Western blot analysis was performed basically as described previously (Palmer & Sturtevant, 2004). Cell extracts of *C. albicans* were prepared by lysis with glass beads in the presence of a protease inhibitor cocktail (Sigma). Lysates were microfuged at 13 000 r.p.m. to yield supernatant and pellet fractions. The pellet was resuspended in 50 μ l SDS-PAGE sample buffer. Protein lysates (pellet and supernatant fractions) prepared from equivalent numbers of cells were loaded per lane in 12 % SDS-PAGE gels. Gels were transferred to PROTRAN (Schleicher and Schuell) and blotted with a rabbit polyclonal anti-GFP antibody (Anaspec). Equivalent loading of SDS-PAGE gels was confirmed by staining with Coomassie blue, and membranes were stained with Ponceau Red (Sigma) following the manufacturer's directions. A horseradish-peroxidase-conjugated goat anti-rabbit secondary antibody (Rockland) was used for detection with reagents supplied by Pierce.

RESULTS

Cloning and deletion of *C. albicans* ATG9

S. cerevisiae ATG9 (also known as CVT7 and APG9) encodes an integral membrane protein of 998 amino acids, which is required for autophagosome and cvt vesicle formation (Lang *et al.*, 2000; Noda *et al.*, 2000). Deletion of ATG9 results in complete abrogation of autophagy and cvt pathways, leading to a block in API and AMS delivery, sensitivity to nitrogen starvation and an inability to complete sporulation (Lang *et al.*, 2000; Noda *et al.*, 2000). BLAST searches to the *C. albicans* genome sequence database (www.candidagenome.org/) identified a single ATG9 orthologue, predicted to encode a protein of 952 amino acids, sharing 32 % identity and 47.6 % similarity with *S. cerevisiae* Atg9p. A Kyte–Doolittle plot of the predicted *C. albicans* Atg9p sequence suggests six to eight transmembrane domains, which is in good agreement with the five to eight predicted transmembrane domains of the *S. cerevisiae* protein (Lang *et al.*, 2000; Noda *et al.*, 2000). In order to investigate the importance of autophagy (and the related cvt pathway) in the pathogen *C. albicans*, we constructed a strain in which both ATG9 alleles were deleted. Correct genotype was checked by both PCR detection and Southern blot analysis. The deletion mutant was readily produced, indicating that this gene is not essential for viability. A wild-type copy of ATG9 including 5' and 3' flanking sequences was then reintroduced to the deletion strain to produce a 'reconstituted' strain.

The *atg9* Δ mutant had a growth rate comparable to wild-type and reconstituted strains in liquid YPD culture, and was not sensitive to osmotic, temperature or oxidative stresses (data not shown). Levels of vacuolar CPY activity were also determined to be comparable between mutant and

control strains, suggesting that trafficking from the Golgi apparatus to the vacuole was not affected in the *atg9* Δ mutant (data not shown), and the mutant strain was unaffected in SAP activity (data not shown). The mutant strain was not sensitive to the autophagy-inducing drug rapamycin, or the replication checkpoint inhibitor caffeine (data not shown). Vacuole morphology was analysed using the fluorescent dye FM4-64 (Vida & Emr, 1995). The *atg9* Δ mutant was observed to have an intact vacuole morphology, similar to that of ATG9⁺ control strains (data not shown).

C. albicans atg9 Δ is defective in autophagy

We examined resistance to nitrogen starvation by measuring viability (as c.f.u.) after transfer to medium lacking a nitrogen source (SD–N) (Fig. 1a). Wild-type and reconstituted strains underwent two to three further cell divisions after shifting to SD–N medium, and then maintained viability for the duration of the experiment (30 days). However, the *atg9* Δ strain did not continue to divide after the shift to SD–N and c.f.u. steadily declined to 0, clearly demonstrating that *atg9* Δ cells are sensitive to nitrogen

Fig. 1. *C. albicans atg9* Δ mutant is defective in autophagy. (a) Resistance to nitrogen starvation was determined by measuring viability following shift to nitrogen-free (SD–N) medium. Viability was determined as c.f.u. and represented as a percentage of the c.f.u. at time 0. Data are given as the mean $\pm$ SD of three experiments. (b) Autophagosome formation was induced in SD–N medium, supplemented with PMSF to inhibit the breakdown of the autophagic bodies following delivery to the vacuole. Cells were inspected by DIC microscopy after 16 h at 30 °C. Arrowheads indicate vacuole containing accumulated autophagic bodies (ATG9⁺ strains), and vacuole lacking autophagic bodies (*atg9* Δ /*atg9* Δ mutant).

starvation. We next induced the formation of autophagosomes in SD–N media containing 1 mM PMSF. PMSF is a serine protease inhibitor which blocks the action of vacuolar PrB, an enzyme required for degradation of autophagic bodies within the vacuole (Noda *et al.*, 2000). Under these conditions wild-type and *ATG9* reconstituted strains accumulate autophagic bodies within the vacuole, giving the vacuole a granular appearance (Fig. 1b). As expected, the *atg9Δ* strain did not accumulate autophagic bodies under these conditions, confirming that our *atg9Δ* mutant is defective at an early step of autophagy, possibly autophagosome formation.

C. albicans atg9Δ is defective in cvt trafficking

In order to establish if Atg9p functions in the cytoplasm to vacuole trafficking pathway, we tagged the cvt cargo protein API (encoded by the *LAP4* gene in *S. cerevisiae*). Using the *S. cerevisiae* Lap4p sequence we identified two *LAP4*-like ORFs, designated *LAP4* and *LAP41* by the *C. albicans* genome database. The predicted amino acid sequences had 57 % (Lap41p) and 34 % (Lap4p) identity to *S. cerevisiae* Lap4p. We therefore selected the *LAP41* ORF for GFP tagging using a PCR-based procedure (Gerami-Nejad *et al.*, 2001), to generate a C-terminal Lap41–GFP fusion, in *ATG9*⁺ and *atg9Δ* backgrounds. Tagged strains were grown under nutrient-replete conditions (YPD) to the exponential phase of growth, conditions where cvt trafficking occurs but not autophagy, and observed by fluorescent microscopy (Fig. 2a). *ATG9*⁺ cells had fluorescence in three distinct patterns: 1, single intense spot outside the vacuole; 2, single intense spot within vacuole lumen; 3, diffuse staining of the vacuole lumen. These patterns resemble API–GFP localization in *S. cerevisiae* (Suzuki *et al.*, 2002) and correspond nicely with the cvt trafficking events: 1, oligomerized API–GFP precursor within the cytoplasm, or the membrane-bound cvt vesicle prior to vacuolar delivery; 2, cvt body released into the vacuole; 3, mature API–GFP released into the vacuole lumen following degradation of cvt vesicle (Wang & Klionsky, 2003). The distribution of API–GFP in the *atg9Δ* background mutant was altered (Table 3). No diffuse staining of the vacuole lumen was detected, and an increased proportion of cells had a single spot distribution. Furthermore, the spots were of increased intensity in the *atg9Δ* mutant as compared to the *ATG9*⁺ control strains, suggesting an accumulation of API–GFP in the oligomeric cytoplasmic cvt complex.

In *S. cerevisiae*, API is synthesized as an inactive precursor, which is activated upon delivery to the vacuole by proteolytic cleavage of an N-terminal propeptide of 45 amino acids (Oda *et al.*, 1996). This can be followed as a shift in molecular mass on a Western blot (Klionsky *et al.*, 1992; Suzuki *et al.*, 2002). In order to further confirm the cvt trafficking defects, we immunodetected the API–GFP fusion protein in *ATG9*⁺ and *atg9Δ* backgrounds, using a polyclonal anti-GFP antibody. The predicted molecular mass of GFP is 26.9 kDa and that of *C. albicans* Lap41p 56.7 kDa (prior to proteolytic maturation). In *ATG9*⁺

Fig. 2. *C. albicans atg9Δ* mutant is defective in cvt trafficking. (a) The cvt cargo protein API (encoded by *LAP41*) was C-terminally GFP tagged in *ATG9*⁺ and *atg9Δ* genetic backgrounds. Cells were grown into the exponential phase and labelled with FM4-64 (red) to locate each cell's vacuole. Phase-contrast and GFP-FM4-64 combined images are shown for each strain. Arrows indicate faint diffuse GFP within the vacuole lumen (*ATG9*⁺ strains only). (b) API–GFP was detected using a polyclonal anti-GFP antibody. The antibody detected a non-specific band of around 25 kDa, and one of approximately 80 kDa (*atg9Δ* only), which corresponded with the expected molecular mass of Lap41–GFP. Two independently constructed GFP-tagged strains were analysed for each genotype, and both experiments repeated three times. Strain YJB6284 was used as the minus GFP control strain.

strains, delivery of API–GFP to the vacuole would expose the GFP tag to the proteolytically active vacuole lumen, which is liable to have resulted in its cleavage and/or degradation. This may account for the inability to detect the API–GFP fusion species by Western blotting (Fig. 2b), and the fairly low level of fluorescence detected within the vacuole lumen. Unfortunately the anti-GFP antibody recognized a peptide in the minus GFP negative control (lane 1, Fig. 2b), of

Table 3. Vacuolar trafficking of API–GFP is dependent upon *ATG9*

The distribution of API–GFP was scored in *ATG9*⁺ and *atg9Δ* genetic backgrounds. Cells were scored from three fields of view (100 × magnification) for each of two independently constructed strains of each genotype. The proportion of cells in each GFP distribution category was calculated as a percentage of the total number of cells within the field. Data for all six fields for a given genotype were used to calculate the mean and standard deviation for each genotype. Results from a single experiment are shown. The experiment was repeated twice with similar results.

Genotype	API–GFP distribution		
	Vacuolar*	Point†	None‡
<i>ATG9/ATG9</i>	71 ± 1.4	23.1 ± 4.7	5.9 ± 4.8
<i>ATG9/atg9Δ</i>	65.7 ± 6.2	24.1 ± 2.5	10.3 ± 3.7
<i>atg9Δ/atg9Δ</i>	0 ± 0§	73.8 ± 10.1§	26.2 ± 10.1§

*Vacuolar, diffuse GFP within vacuole lumen, with or without intense GFP spot in cytoplasm.
†Point, intense GFP spot in cytoplasm, with no detectable staining of vacuole lumen.
‡None, no detectable GFP.
§*P* < 0.05.

around 25 kDa. A similar non-specific band has been described in *S. cerevisiae* (Suzuki *et al.*, 2002) and may have obscured the detection of peptide species of a similar size, including any GFP that is cleaved upon vacuolar delivery. However, a species of 75–80 kDa, corresponding to the predicted API–GFP molecular mass was detected in the *atg9Δ* background, indicating accumulation of API–GFP in the *atg9Δ* mutant. This species was detected in the pelletable fraction of the cell extracts, but not the soluble fraction. This suggests that the API–GFP has accumulated as part of a large complex before vacuole delivery, perhaps either the highly oligomerized precursor API (cvt body), or even within a cvt vesicle (Yorimitsu & Klionsky, 2005). These data clearly demonstrated that, similar to the *S. cerevisiae atg9Δ* mutant, our *C. albicans atg9Δ* mutant has a defect in cvt trafficking to the vacuole.

Autophagy is not required for *C. albicans* differentiation

In the model eukaryote *S. cerevisiae*, mutants defective in autophagy are unable to complete sporulation (Takeshige *et al.*, 1992; Tsukada & Ohsumi, 1993). Moreover, autophagy has been intimately associated with a wide range of differentiation and development events in higher eukaryotes. We therefore assessed the consequences of a defect in autophagy on two major differentiation processes of *C. albicans*. Yeast–hypha differentiation was examined on solid and in liquid M199 and FCS media. In each case the *atg9Δ* mutant produced filaments that were indistinguishable from the *ATG9*⁺ control strains (data not shown),

indicating that under *in vitro* conditions autophagy is not essential for yeast–hypha differentiation. Similarly, when induced on cornstarch–Tween agar, the *atg9Δ* mutant produced chlamydospores with similar kinetics and abundance as the *ATG9*⁺ control strains (data not shown). Together these results suggest that autophagy is not required for two major differentiation events in *C. albicans*.

Autophagy is not required for *C. albicans* survival within or killing of a macrophage cell line

Previous studies using two *C. albicans* mutants with defects in vacuole biogenesis demonstrated that some vacuolar function(s) are required for survival within, and killing of a mouse macrophage-like cell line (J774A.1) (Palmer *et al.*, 2005). We considered that while not required for yeast–hypha differentiation or chlamydospore formation, autophagy may be important for normal interaction with host cells or survival within host tissues. To test this we analysed the outcome of *atg9Δ* interaction with the J774A.1 cell line, by determining macrophage and *C. albicans* survival rates. Following co-culture, the macrophages rapidly phagocytosed wild-type *C. albicans*. This was followed by filamentation of *C. albicans* within the macrophage, which resulted in stretching of the J774A.1 cells, followed by macrophage lysis. The *atg9Δ* mutant killed the J774A.1 cells as efficiently as *ATG9*⁺ strains (data not shown), indicating that autophagy is not required for killing of the macrophage cell line. *C. albicans* survival of the macrophage challenge was determined using an end-point dilution assay (Rocha *et al.*, 2001). We found that the *atg9Δ* cells exhibited similar susceptibility to the J774A.1 interaction as *ATG9*⁺ control strains (data not shown), indicating that this pathway does not aid *C. albicans* survival within the J774A.1 cells.

DISCUSSION

Autophagy is a well-conserved pathway in eukaryotes, and serves to facilitate a diverse range of cellular activities. In this study we investigated the importance of autophagy and the closely related cvt pathway during differentiation and pathogenesis of the fungal pathogen *C. albicans*. We hypothesized that autophagy may be an important mechanism for survival and/or differentiation within host tissues. We considered that during adaptation to new host environments *C. albicans* may need to recycle endogenous macromolecules to provide nutrients until host sources can be tapped, or that in order to facilitate rapid differentiation within the host an ‘elimination’ of inappropriately expressed proteins may occur via autophagic degradation. We addressed these questions by analysing a mutant strain defective in autophagy. As expected, the *atg9Δ* mutant strain constructed within this study was blocked in autophagy and the closely related cvt pathway, and was sensitive to nitrogen starvation. However, the *atg9Δ* mutant had a normal growth rate, vacuolar CPY activity, resistance to osmotic and temperature stress, and vacuole morphology. These findings

suggest that the *atg9Δ* mutant does not have a generalized defect in vacuolar function (Palmer *et al.*, 2005), but a specific defect in cvt/autophagy trafficking.

The *atg9Δ* mutant has no detectable defect in either chlamydospore or yeast–hypha differentiation, a result which was surprising given the importance of this pathway in a diverse array of eukaryotic biological processes, including *S. cerevisiae* sporulation. Microarray studies by Lorenz *et al.* (2004) demonstrated that upon phagocytosis by J774A.1 cells, *C. albicans* undergoes a starvation-like transcriptional response, suggesting that the phagolysosome is a nutritionally poor environment. Vacuolar proteases including API, CPY and PrB are upregulated following macrophage ingestion, suggesting that vacuole-mediated proteolysis may be important for survival following phagocytosis. However, our *atg9Δ* mutant was not deficient in its ability to survive within or kill a mouse macrophage-like cell line, suggesting that autophagy may not be required for *C. albicans*–macrophage interaction. This prompted us to conduct a small-scale pilot study to test the virulence of the *atg9Δ* mutant in the well-defined mouse model of haematogenously disseminated candidiasis. Only three mice were infected per *C. albicans* strain, but our results (mean survival time and fungal burden in the kidneys) clearly suggested that the *atg9Δ* mutant is fully virulent (data not shown). Taken together, these results indicate that autophagy plays little or no role in *C. albicans* differentiation or pathogenesis. It remains possible that autophagy is important for the long-term survival of *C. albicans* as a commensal organism, or to reside on a peripheral host site such as skin, or perhaps within the environment.

Previously we found a *C. albicans vps11Δ* mutant to be defective in vacuole biogenesis and to exhibit a range of pleiotropic phenotypes, including a reduced growth rate, sensitivity to a range of stresses and to nitrogen starvation, and reduced vacuolar and secreted protease activities (Palmer *et al.*, 2003, 2005). Moreover, this mutant is unable to undergo yeast–hypha differentiation and was unable to kill the J774A.1 macrophage cell line. Work with a partially functional *vps11* allele revealed that restoring a normal growth rate, stress resistance, and SAP and vacuolar hydrolase activities was not sufficient to restore normal filamentation or macrophage killing. Both *vps11* mutants have highly fragmented vacuole morphology and are sensitive to nitrogen starvation, suggesting either of these vacuolar functions to be important in yeast–hypha differentiation and macrophage killing. As outlined above, the major role of the vacuole during nitrogen starvation is through the process of autophagy. The results presented here clearly demonstrate that vacuolar functions relating to autophagy are of little consequence in either yeast–hypha differentiation or interaction with macrophages.

Gow & Gooday (1982, 1984) originally observed the vacuole to undergo a dynamic expansion during *C. albicans* germ-tube emergence from a parental yeast cell. Furthermore, during apical extension of the germ-tube, asymmetrical

division of the protoplasm yields subapical compartments composed almost entirely of vacuole, whereas the protoplasm migrates at the hyphal tip (Barelle *et al.*, 2003; Gow & Gooday, 1984). After a delay, the highly vacuolated compartments regenerate cytoplasm and the vacuoles recede. This pattern of vacuole inheritance also seems to influence cell cycle progression and branching frequency of the hyphae (Barelle *et al.*, 2003; Veses *et al.*, 2005). At present the mechanism by which vacuole expansion is mediated, and its importance during host interaction, are unknown. Our results further strengthen the hypothesis that the major role the vacuole plays in yeast–hypha differentiation relates to its morphology, and perhaps its physical expansion to generate ‘empty’ hyphal compartments. Moreover, our results eliminate the autophagic and cvt trafficking pathways as mediating germ-tube induced vacuole expansion.

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