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Autophagy promotes survival in aging submerged cultures of the filamentous fungus *Aspergillus niger*

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Abstract Autophagy is a well-conserved catabolic process constitutively active in eukaryotes that is involved in maintaining cellular homeostasis by the targeting of cytoplasmic content and organelles to vacuoles. Autophagy is strongly induced by the limitation of nutrients including carbon, nitrogen, and oxygen and is clearly associated with cell death. It has been demonstrated that the accumulation of empty hyphal compartments and cryptic growth in carbon-starved submerged cultures of the filamentous fungus *Aspergillus niger* is accompanied by a joint transcriptional induction of autophagy genes. This study examines the role of autophagy by deleting the *atg1*, *atg8*, and *atg17* orthologs in *A. niger* and phenotypically analyzing the deletion mutants in surface and submerged cultures. The results indicate that *atg1* and *atg8* are essential for efficient autophagy, whereas deletion of *atg17* has little to no effect on autophagy in *A. niger*. Depending on the kind of oxidative stress confronted with, autophagy deficiency renders *A. niger* either more resistant (menadione) or more sensitive (H_2O_2) to oxidative stress. Fluorescence microscopy showed that mitochondrial turnover upon carbon depletion in submerged cultures is severely blocked in autophagy-impaired *A. niger* mutants. Furthermore, automated image analysis demonstrated that autophagy promotes survival in maintained carbon-starved cultures of *A. niger*. Taken together, the results suggest that besides its function in nutrient recycling, autophagy plays important roles in physiological adaptation by organelle turnover and protection against cell death upon carbon depletion in submerged cultures.

Keywords Mitophagy · Carbon starvation · Cell death · Automated image analysis

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Introduction

The filamentous fungus *Aspergillus niger* is an economically important and versatile cell factory commonly exploited for the industrial-scale production of a wide range of enzymes and organic acids (Andersen et al. 2011; Archer 2000; Pel et al. 2007). Although numerous studies have been conducted aiming at improving our knowledge of catabolic cellular activities that determine product yields in *A. niger*, including secretion of proteases and the unfolded protein response (Braaksma et al. 2009; Carvalho et al. 2012; MacKenzie et al. 2005; Mattern et al. 1992; Peberdy 1994), the possible role of autophagy in relation to protein production has yet not been studied in *A. niger*.

Autophagy is an intracellular degradation process functioning in the delivery of cytoplasmic proteins and organelles to vacuoles for macromolecule turnover and recycling (Bartoszewska and Kiel 2011; Inoue and Klionsky 2010). During autophagy, cellular components are sequestered in

double-membrane vesicles, termed autophagosomes, which are targeted to lytic compartments. The outer membrane of the autophagosome fuses with the vacuolar membrane, whereupon a single-membrane vesicle is released into the lumen. Following lysis of the autophagic membrane and degradation of its contents by hydrolytic enzymes, the breakdown products are transported back into the cytoplasm for reuse by the cell.

This pathway is highly conserved from yeast to higher eukaryotes and is tightly regulated (Bartoszewska and Kiel 2011). To date, more than 30 autophagy-related (*atg*) genes have been identified for *Saccharomyces cerevisiae* and other fungi (Kanki et al. 2011; Xie and Klionsky 2007). One key player controlling the levels of autophagy is the autophagy-related protein Atg1, which is a serine/threonine protein kinase (Bartoszewska and Kiel 2011; Inoue and Klionsky 2010). Upon induction of autophagy, this kinase interacts with Atg17, Atg29, and Atg31 in an Atg13-dependent manner, forming the Atg1 kinase complex that initiates the formation of autophagosomes (Cheong et al. 2008; Kabeya et al. 2005). Deletion of the *atg1* ortholog in *Podospora anserina* abolished autophagy and caused several developmental defects (Pinan-Lucarré et al. 2005). Mutants displayed fewer aerial hyphae and did not form protoperithecia. Similar phenotypic traits were observed in *P. anserina* Δ*atg8* mutants (Pinan-Lucarré et al. 2005). Atg8 is coupled to the membrane lipid phosphatidylethanolamine, forming an essential component of autophagic vesicle membranes (Bartoszewska and Kiel 2011; Inoue and Klionsky 2010). The *atg8* gene has also been deleted in the filamentous fungus *Aspergillus oryzae*, resulting in mutants that were defective in autophagy and did not form aerial hyphae and conidia (Kikuma et al. 2006).

Autophagy plays an important role in cellular homeostasis by efficient removal of damaged organelles. For filamentous fungi, it has been shown that endogenous recycling of cellular products by autophagy facilitates foraging of hyphae and fuels conidiation under nutrient starvation (Richie et al. 2007; Shoji et al. 2006; Shoji and Craven 2011). The hyphae that are formed during this starvation-induced (cryptic) regrowth show fewer new branches and significantly decreased diameters (Pollack et al. 2008). In the older portions of the mycelium, vacuolation increases dramatically following starvation, resulting in fragmentation and, eventually, dying of the hyphae.

It has been shown that the morphological response to carbon starvation in submerged batch cultures of the filamentous fungus *A. niger*, including emergence of empty hyphal ghosts and (cryptic) outgrowth of thinner non-branching hyphae, is accompanied by a concerted induction of genes related to autophagy (Nitsche et al. 2012). To gain insights into the function of autophagy during submerged carbon starvation, *A. niger* autophagy mutants were generated by deletion of *atg1*, *atg8*, and *atg17* orthologs. The mutants were phenotypically characterized during growth in surface and submerged cultures applying nutrient limitation and oxidative stress. Cytological effects of autophagy deficiency were assessed by investigation of fluorescent reporter strains allowing the visualization of cytoplasm, vacuoles, and mitochondria. The results indicate that autophagy plays important roles in the metabolic adaptation to carbon starvation in submerged cultures and promotes the survival of hyphae formed before depletion of the carbon source.

Material and methods

Strains, media, and molecular techniques

The *Aspergillus* strains used (see Table 1) were grown at 30 °C on solidified (20 g l^{-1} agar) nitrate minimal medium (MM; Alic et al. 1991) or complete medium containing 0.5 % (*w*/*v*) yeast extract and 0.1 % (*w*/*v*) casamino acids in addition to MM. The pH of the synthetic medium for bioreactor cultivations was adjusted to 3 and contained, per liter: 4.5 g NH_4Cl, 1.5 g KH_2PO_4, 0.5 g KCl, and 0.5 g $MgSO_4$ $7H_2O$ use and 1 ml trace metal solution modified from Vishniac and Santer (1957). After autoclaving, the synthetic medium was supplemented with filter-sterilized 10 % (*w*/*v*) yeast extract and autoclaved 50 % (*w*/*v*) glucose solutions to give final concentrations of 0.003 % (*w*/*v*) and 0.8 % (*w*/*v*), respectively.

All cloning steps were performed according to the methods described by Sambrook and Russell (2001) using *Escherichia coli* strain DH5α. The transformation of *A. niger* was performed as previously described (Meyer et al. 2010). Hygromycin-resistant transformants were isolated from plates supplemented with 200 μg ml^{-1} hygromycin and 50 μg ml^{-1} caffeine and subsequently purified on plates with 100 μg ml^{-1} hygromycin. Phleomycin-resistant transformants were isolated and purified on plates supplemented with 100 μg ml^{-1} phleomycin. Southern blot analysis was performed as described by Sambrook and Russell (2001) using either [α-32P]dATP-labeled probes synthesized with the Rediprime II DNA labeling System (GE Healthcare, Fairfield, CT) or DIG-labeled probes generated by PCR with the PCR DIG Probe Synthesis Kit (Roche Applied Science, Penzberg, Germany). MM for sensitivity plate assays was solidified with 2 % (*w*/*v*) agar and supplemented with H_2O_2 or menadione, as indicated.

Construction of strains

The vector for the constitutive expression of mitochondrially targeted green fluorescent protein (GFP) was constructed as

Table 1 *Aspergillus* strains used in this study

Strain name[a]	Genotype	Source
A. niger		
N402 (ATCC 64974)	*cspA1*	Bos et al. (1988)
AB4.1 (ATCC 62590)	*cspA1*, *pyrG*$^-$	van Hartingsveldt et al. (1987)
BN30.2 (FGSC A1871)	N402, Δ*atg1*::*hyg*^{R}	This study
BN29.3 (FGSC A1872)	N402, Δ*atg8*::*hyg*^{R}	This study
BN32.2 (FGSC A1873)	N402, Δ*atg17*::*hyg*^{R}	This study
BN38.9 (FGSC A1874)	AB4.1, P*gpdA*::*NcitA*::*gfp*, *pyrG*$^+$	This study
BN39.2 (FGSC A1875)	BN38.9, Δ*atg1*::*hyg*^{R}	This study
BN40.8 (FGSC A1876)	BN38.9, Δ*atg8*::*hyg*^{R}	This study
AW20.10 (FGSC A1877)	BN38.9, Δ*atg17*::*hyg*^{R}	This study
BN56.2 (FGSC A1878)	BN30.2, P*gpdA*::*gfp*, *phl*^{R}	This study
BN57.1 (FGSC A1879)	BN29.3, P*gpdA*::*gfp*, *phl*^{R}	This study
BN58.1 (FGSC A1880)	BN32.2, P*gpdA*::*gfp*, *phl*^{R}	This study
AR19#1 (FGSC A1881)	AB4.1, P*gpdA*::*gfp*, *pyrG*$^+$	Vinck et al. (2005)
AW24.2 (FGSC A1882)	BN56.2, *atg1*, *amdS*$^+$	This study
AW25.1 (FGSC A1883)	BN57.1, *atg8*, *amdS*$^+$	This study
AW26.1 (FGSC A1884)	BN58.1, *atg17*, *amdS*$^+$	This study
A. nidulans		
SRS29	SRF200 (ATCC 200171 x FGSC 851), P*gpdA*::*NcitA*::*gfp*	Suelmann and Fischer (2000)

[a]Accession numbers for public strain repositories are indicated in parentheses

follows. A 1.1-kb *NcitA*::*gfp* fragment was PCR-amplified from genomic DNA of the *Aspergillus nidulans* strain SRS29 (Suelmann and Fischer 2000), blunt-end ligated into pJET1.2 (Fermentas, St. Leon-Rot, Germany), and sequenced. Subsequently, the fragment was excised using *Bgl*II and *Bam*HI restriction enzymes and ligated into the 3.5-kb *Bgl*II-*Bam*HI backbone of pAN52-1N (GenBank: Z32697.1). Next, a 2.2-kb *Bgl*II-*Nco*I P*gdpA* fragment isolated from pAN52-1N was inserted into the *Bgl*II-*Nco*I opened intermediate construct. Finally, a 3.9-kb *Xba*I *pyrG** fragment was isolated from pAB94 (van Gorcom and van den Hondel 1988) and inserted at the *Xba*I site to give the final construct, *PgpdA-NcitA*::*gfp-TtrpC-pyrG**, which was transformed to *A. niger* strain AB4.1 (van Hartingsveldt et al. 1987). Single-copy integration at the *pyrG* locus was confirmed by Southern blot analysis according to the method previously described (Meyer et al. 2010; see Fig. S1 in the Supplementary Material). The strain was named BN38.9.

Constructs for gene replacements with the hygromycin resistance cassette were generated as follows. Approximately 1-kb flanking regions of the *atg1* (An04g03950), *atg8* (An07g10020), and *atg17* (An02g04820) open reading frames were PCR amplified from genomic DNA of the N402 wild type strain using primer pairs according to Table S1 in the Supplementary Material, blunt-end ligated into pJET1.2 (Fermentas), and sequenced. Flanks were isolated from the pJET1.2 vectors using enzymes cutting at the outermost restriction sites (as indicated in Table S1 in the Supplementary Material) and three-way ligated into a *Not*I-*Kpn*I-opened pBluescript II SK(+) vector (Fermentas). For finalizing the *atg1* and *atg17* deletion constructs, the hygromycin resistance cassette was isolated as the *Nhe*I-*Xba*I fragment from pAN7.1 (Punt et al. 1987) and ligated between the flanking regions within the corresponding intermediate pBluescript constructs. For insertion of the hygromycin resistance cassette between the *atg8* flanks, *Xho*I and *Xba*I were used accordingly. Linearized *atg1*, *atg8*, and *atg17* deletion cassettes were transformed to *A. niger* strain N402 and homologous integration was confirmed by Southern blot analysis (see Fig. S2 in the Supplementary Material), giving the strains BN30.2, BN29.3, and BN32.2, respectively (see Table 1).

For constitutive expression of cytosolically targeted GFP in the autophagy deletion mutants, the vector pGPDGFP (Lagopodi et al. 2002) was co-transformed with pAN8.1 (Mattern et al. 1988) to *A. niger* strains BN30.2, BN29.3, and BN32.2 (see Table 1). Positive transformants were isolated by screening for cytosolic fluorescence and named BN56.2, BN57.1, and BN58.1, respectively (see Table 1). For complementation of the autophagy deletion strains BN56.2, BN57.1, and BN58.1, the corresponding open reading frames and approximately 1-kb flanking regions were PCR amplified using genomic DNA of the N402 wild type strain as the template and primer pairs, as

indicated in Table S1 in the Supplementary Material. The individual fragments were blunt-end ligated into pJET1.2 and sequenced. Subsequently, the *amdS* cassette was PCR amplified from the *mnsA* deletion construct described by Carvalho and Arentshorst (2011) using the primers indicated in Table S1 in the Supplementary Material, blunt-end ligated into pJet1.2, and sequenced. Subsequently, the *amdS* cassette was isolated as an *Xho*I-*Hin*dIII fragment and ligated into pBluescript II SK(+). The amplified autophagy gene loci were isolated from the intermediate pJET1.2 vectors via *Not*I digestion and ligated into pBluescript II SK(+) harboring the *amdS* cassette. The resulting plasmids were transformed to *A. niger* strains BN56.2, BN57.1, and BN58.1, respectively. Random integration of the constructs was confirmed by Southern blot analysis (Fig. S3 in the Supplementary Material) and the resulting strains were named AW24.2, AW25.1, and AW26.1 (Table 1).

Deletion of *atg1*, *atg8*, and *atg17* in the strain expressing the mitochondrially targeted GFP was performed by transforming linearized *atg1*, *atg8*, and *atg17* deletion constructs to *A. niger* strain BN38.9. Homologous integration of the constructs at their respective loci was confirmed by Southern blot analysis (see Fig. S4 in the Supplementary Material) and the resulting strains were named BN39.2, BN40.8, and AW20.10, respectively (Table 1).

Bioreactor cultivation and sampling

Bioreactor cultivations were performed as previously described by Jørgensen et al. (2010). Briefly, autoclaved 6.6-l bioreactor vessels (BioFlo3000, New Brunswick Scientific, NJ) holding 5 l sterile synthetic medium were inoculated with 5×10^9 conidia. During cultivation, the temperature was set to 30 °C and pH 3 was maintained by the addition of titrants (2 M NaOH, 1 M HCl). The supply of sterile air was set to 1 l min^{-1}. To avoid loss of hydrophobic spores through the exhaust gas, the stirrer speed was set to 250 rpm and air was supplied via the head space during the first 6 h of cultivation. After this initial germination phase, the stirrer speed was increased to 750 rpm, air was supplied via the sparger, and 0.01 % polypropylene glycol P2000 was added to prevent foaming. O_2 and CO_2 partial pressures of the exhaust gas were analyzed with a Xentra 4100C analyzer (Servomex BV, Zoetermeer, The Netherlands). Dissolved oxygen tension and pH were measured electrochemically with autoclavable sensors (Mettler Toledo, Columbus, OH). At regular intervals, samples were taken from the cultures. Aliquots for microscopic analysis were either directly analyzed (fluorescence microscopy) or quickly frozen in liquid nitrogen (automated image analysis); the remainder of the samples was vacuum-filtrated using glass microfiber filters (GE Healthcare). The retained biomass and filtrates were directly frozen in liquid nitrogen and stored at −80 °C. Biomass concentrations were gravimetrically determined from freeze-dried mycelium of a known mass of culture broth.

Microscopic and image analysis

For the analysis of hyphal diameters, microscopic samples were slowly defrosted on ice and subsequently fixed and stained in a single step by mixing them at a 1:1 ratio with Lactophenol blue. Per sample, a minimum of 40 micrographs were taken using a ×40 objective and an ICC50 camera (Leica, Wetzlar, Germany). The microscope and camera settings were optimized to obtain micrographs with strong contrast. To measure hyphal diameters in an automated manner, a previously developed macro (Nitsche et al. 2012) for the open source program ImageJ (Abràmoff et al. 2004) was used. Differential interference contrast (DIC) and fluorescence images were taken with a Zeiss (Jena, Germany) Axioplan 2 imaging microscope equipped with DIC optics. For the GFP settings, an epifluorescence filter cube XF 100-2 with excitation of 450–500 nm and emission of 510–560 nm was used. Confocal images were obtained using a Zeiss Observer microscope equipped with a LSM 5 exciter. Excitation in the GFP settings was achieved with a 488 argon laser line with emission of 505–550 nm.

Results

Autophagy-related genes *atg1* and *atg8*, but not *atg17*, are essential for efficient autophagy in *A. niger*

To study the phenotypes of autophagy-deficient *A. niger* mutants in surface and submerged cultures, the orthologs of three genes known to encode essential components of the autophagic machinery in *S. cerevisae* (Cheong et al. 2005; Kabeya et al. 2005; Matsuura et al. 1997; Tsukada and Ohsumi 1993) were identified and deleted in *A. niger*. Two of the target genes encode proteins that are part of the regulatory Atg1 kinase complex, namely, the kinase Atg1 itself and the scaffold protein Atg17. The third target gene encodes the ubiquitin-like protein Atg8, which is a structural component required for the formation of autophagosomal membranes. The following ortholog pairs were identified by reciprocal best BlastP hit analysis *Scatg1*/An04g03950 ($E=4e^{-151}$), *Scatg8*/An07g10020 ($E=4e^{-67}$), and *Scatg17*/An02g04820 ($E=4e^{-12}$), which correspond to those suggested at the *Aspergillus* Genome Database (Arnaud et al. 2010, 2012). The identified target genes were deleted in the *A. niger* laboratory wild type strain N402 by

replacement with a hygromycin resistance cassette. Gene deletions were confirmed by Southern blot analysis (see Fig. S2 in the Supplementary Material) and the selected strains were named BN30.2 (Δ*atg1*), BN29.3 (Δ*atg8*), and BN32.2 (Δ*atg17*) and listed in Table 1.

Studies in *A. oryzae* (Kikuma et al. 2006) and *Penicillium chrysogenum* (Bartoszewska et al. 2011) have demonstrated the localization of cytosolic fluorescent proteins to vacuoles under starvation conditions in wild type strains, whereas mutants impaired in autophagy did not show vacuolar localization of cytosolic GFP. In order to assess whether deletion of the selected target genes impairs autophagy in *A. niger*, the Δ*atg1*, Δ*atg8*, and Δ*atg17* strains were transformed with a P*gpdA*-GFP construct to obtain the corresponding mutant strains BN56.2, BN57.1, and BN58.1 (see Table 1) with constitutive expression of cytosolic GFP. Vacuolar localization of cytosolic GFP was observed for the wild type strain during nutrient limitation, while both Δ*atg1* and Δ*atg8* mutants did not show GFP fluorescence inside vacuoles, indicating deficient autophagy. Interestingly, deletion of *atg17* did not affect the vacuolar localization of cytosolic GFP (see Fig. 1). Complementation studies showed that vacuolar localization of cytosolic GFP during starvation could be restored in the *atg1* and *atg8* complemented mutants (strains AW24.2 and AW25.1, respectively), demonstrating that the described phenotypes of strains BN56.2 and BN57.1 were caused only by the deletion of the *atg1* and *atg8* genes, respectively (see Fig. 1). Together, these results suggest that both *atg1* and *atg8*, but not *atg17*, are essential for efficient autophagy in *A. niger*.

To monitor autophagy-mediated turnover of the mitochondria, also referred to as mitophagy (Kanki et al. 2011), induced by carbon starvation, wild type and *atg* deletion strains with constitutive expression of mitochondrially targeted GFP (see Table 1) were generated. The approach described by Suelmann and Fischer (2000), who showed that N-terminal fusion of the mitochondrial targeting sequence from the citrate synthase A to a fluorescent protein efficiently labeled mitochondria in *A. nidulans*, was followed. Similar to *A. nidulans*, wild type (strain BN38.9) and *atg* mutant reporter strains (strains BN39.2, BN40.8, and AW20.10) showed fluorescent tubular structures inside hyphae under nutrient-rich conditions (as examplarily shown for Δ*atg1* and Δ*atg8* in Fig. 7a). During carbon starvation, however, microscopic analysis indicated considerable differences in mitochondrial morphology (see Fig. 2). The phenotypes correspond to those observed for the localization of cytosolically targeted GFP (see Fig. 1). Localization of GFP remained mitochondrial in both Δ*atg1* and Δ*atg8* strains (strains BN39.2 and BN40.8, respectively) upon starvation, whereas wild type and Δ*atg17* strains (strains BN38.9 and AW20.10, respectively) showed vacuolar localization of mitochondrially expressed GFP. These results show that mitochondrial turnover induced by carbon starvation is mediated by autophagy, which is severely impaired in *A. niger* upon deletion of either *atg1* or *atg8*, but not *atg17*.

Phenotypes of Δ*atg1*, Δ*atg8*, and Δ*atg17* strains in surface cultures

Depending on which species of filamentous fungi is studied and which *atg* gene is under investigation, defective autophagy results in complete or severe impairment of conidiation during surface growth (Bartoszewska et al. 2011; Kikuma et al. 2006; Richie et al. 2007). To test whether conidiation is affected as well in the Δ*atg1*, Δ*atg8*, and Δ*atg17* mutants (strains BN30.2, BN29.3, and BN32.2, respectively), conidia from colonies grown for 7 days on solid MM were harvested and counted. Although colonies of the Δ*atg1* and Δ*atg8* strains developed conidiophores and turned dark, the colonies showed slightly attenuated pigmentation (see Fig. 3a), indicating reduced spore densities and/or differences in the melanization of spores. Indeed, the amount of spores recovered from the colonies was significantly reduced (see Fig. 3c). Conidiation was most affected in the Δ*atg8* strain, with a decrease of 70 %. Interestingly, although deletion of *atg17* did not repress vacuolar localization of either cytosolic GFP (see Fig. 1) or mitochondrial GFP (see Fig. 2) under carbon starvation, and its colony appearance was indistinguishable from that of the wild type, the amount of recovered conidia was reduced by more than 20 %. This thus suggests an intermediate phenotype for the deletion of *atg17* in *A. niger*. In agreement with studies in *A. oryzae* (Kikuma et al. 2006), colonies of the Δ*atg8* strain showed slower radial growth on MM (see Fig. 3b). Even after correcting for this difference in colony size, conidiation was most reduced for the Δ*atg8* strain, as shown by the spore densities (see Fig. 3c). However, compared to the *A. oryzae* Δ*atg8* mutant, which was reported not to develop aerial hyphae and conidia (Kikuma et al. 2006), the conidiation phenotype in *A. niger* is much less pronounced.

In filamentous fungi, autophagy has been suggested to contribute to nutrient recycling along the mycelial network, promoting the foraging of individual substrate exploring hyphae and conidial development (Richie et al. 2007; Shoji et al. 2006; Shoji and Craven 2011). To elucidate the role of autophagy in nutrient recycling in *A. niger*, the phenotypes of Δ*atg1*, Δ*atg8*, and Δ*atg17* strains (strains BN30.2, BN29.3, and BN32.2, respectively) were

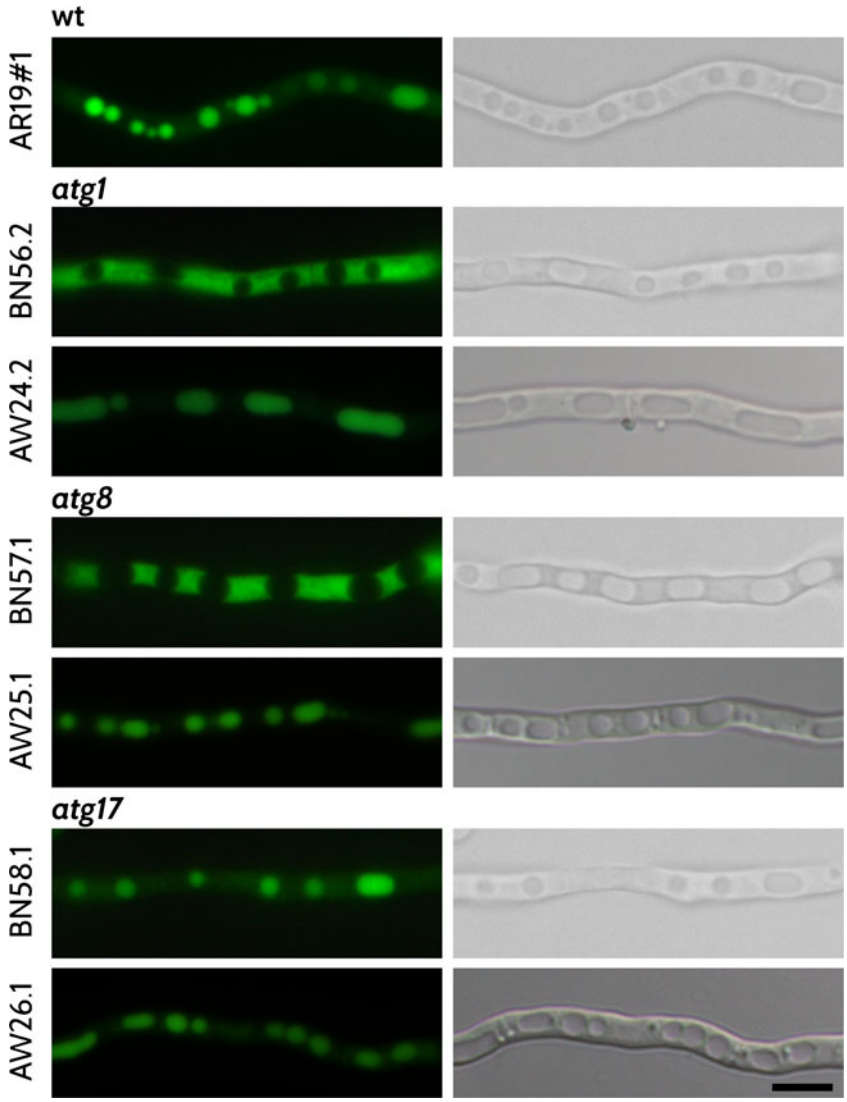


Fig. 1 Localization of cytosolically expressed GFP during carbon starvation. The strains were pre-grown for 8 h at 30 °C on coverslips in Petri dishes with liquid MM. Subsequently, coverslips with adherent hyphae were washed and transferred to MM without carbon source. Micrographs were taken 40 h after transfer. Wild type (strain AR19#1) and the Δ*atg17* mutant (strain BN58.1) showed vacuolar localization of GFP, whereas both Δ*atg1* and Δ*atg8* mutants (strains BN56.2 and BN57.1, respectively) showed cytosolic localization. Complementation with the corresponding wild-type loci restored vacuolar localization of GFP for the Δ*atg1* and Δ*atg8* mutants (strains AW24.2 and AW25.1, respectively). The phenotype of the complemented Δ*atg17* mutant (strain AW26.1) corresponds to that of the non-complemented strain BN58.1. *Scale bar*, 5 μm

investigated in comparison to the wild type under nitrogen and carbon limitation on solid MM (see Fig. 4a). The Δ*atg1* and Δ*atg8* strains were clearly more affected by nutrient limitation than the wild type, as shown by their strong conidiation phenotypes. The Δ*atg8* strain was more sensitive to nitrogen limitation than the Δ*atg1* strain, whereas both deletion strains were comparably affected by carbon limitation. Figure S5 in the Supplementary Material shows that these phenotypes induced by carbon and nitrogen limitation could be restored through complementation with the corresponding wild type *atg* loci. In accordance with the results shown in Figs. 1, 2, and 3a, deletion of *atg17* results in a phenotype that is indistinguishable from that of the wild type strain N402.

In addition to its role in nutrient recycling, numerous reports have shown that autophagy is closely associated with programmed cell death (PCD; Codogno and Meijer 2005; Pinan-Lucarré et al. 2005; Veneault-Fourrey et al. 2006). Among the major triggers of PCD are reactive oxygen species (ROS) and the damage they can cause to lipids, carbohydrates, DNA, and proteins. Oxidative stress-related phenotypes of the Δ*atg1*, Δ*atg8*, and Δ*atg17* mutants (strains BN30.2, BN29.3, and BN32.2, respectively) in comparison to the wild type strain N402 were thus investigated. Sensitivity assays with H_2O_2 and the superoxide anion generator menadione, which have been shown to cause distinct oxidative stress responses in yeast and filamentous fungi (Jamieson 1992; Pócsi et al. 2005; Thorpe et al. 2004; Tucker and Fields 2004), are shown in Fig. 4b, c. In comparison to the wild type, all mutants displayed differential phenotypes in response to treatment with the two compounds. Interestingly, H_2O_2 and menadione had opposing effects. The autophagy mutants were more sensitive to H_2O_2, while their resistance to menadione was increased. The Δ*atg17* strain displayed an intermediate phenotype, which was more comparable to that of the wild type.

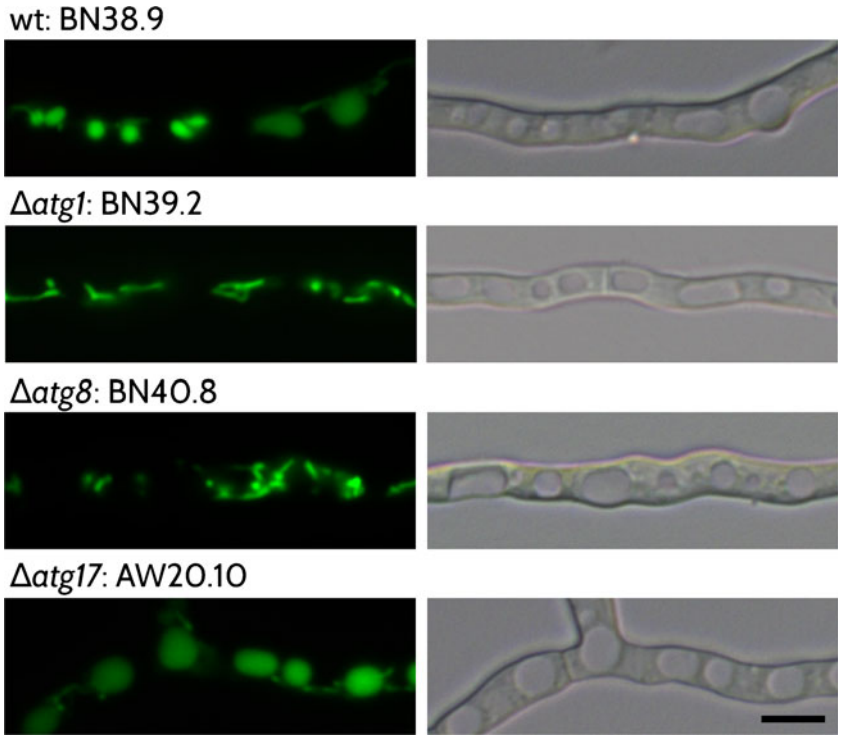


Fig. 2 Localization of mitochondrially expressed GFP during carbon starvation. The strains were grown as described in Fig. 1. Wild type (strain BN38.9) and Δ*atg17* mutant (strain AW20.10) showed vacuolar localization of mitochondrially targeted GFP upon carbon starvation, whereas both Δ*atg1* and Δ*atg8* mutants (strains BN39.2 and BN40.8, respectively) showed cytosolic localization. Under nutrient-rich conditions, all strains showed fluorescent signals resembling tubular mitochondrial networks as described by Suelmann and Fischer (2000), as exemplarily shown for the Δ*atg1* and Δ*atg8* strains in Fig. 7. *Scale bar*, 5 μm

Complementation studies showed that all described oxidative stress-related phenotypes could be partly restored upon expression of the corresponding wild type gene (Fig. S5 in the Supplementary Material).

Taken together, the phenotypic characterizations suggest that autophagy is severely impaired in *A. niger* upon deletion of either *atg1* or *atg8*. Contrary to this, deletion of *atg17* showed little to no phenotypic effect when compared to the wild type. For the subsequent analysis of autophagy impairment during submerged cultivation, the analysis was therefore restricted to the investigation of Δ*atg1* and Δ*atg8* mutants.

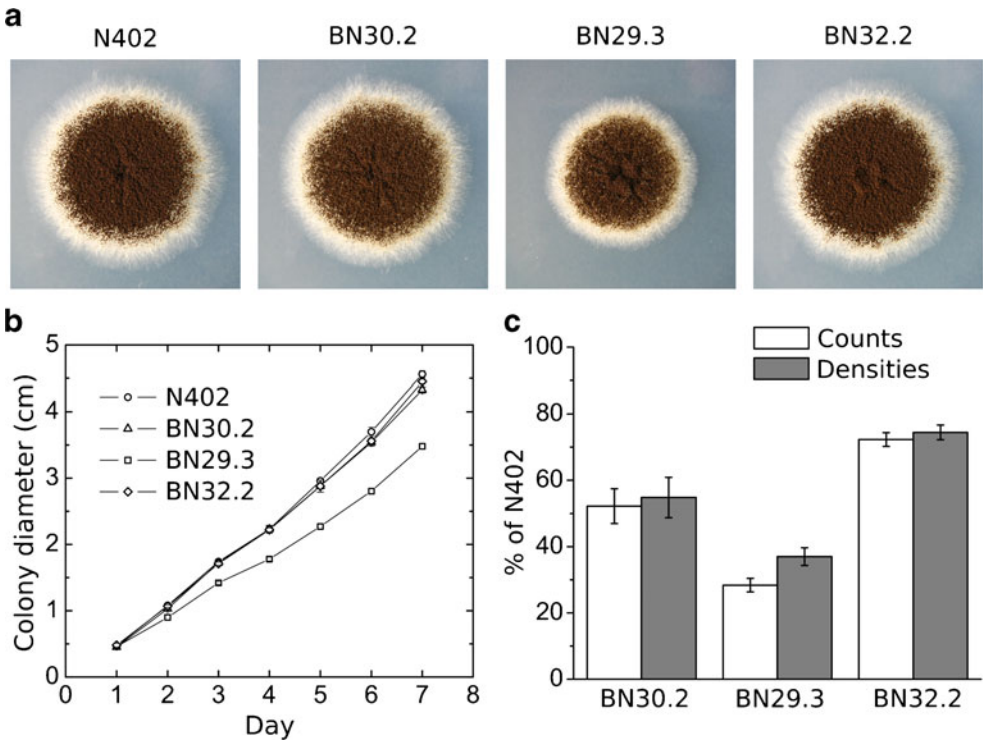


Fig. 3 Conidiation and colony expansion. Wild type (strain N402) and the Δ*atg1*, Δ*atg8*, and Δ*atg17* mutants (strains BN30.2, BN29.3, and BN32.2, respectively) were grown for 7 days on solid MM at 30 °C. **a** Colony appearance. **b** Colony diameters (n=3). **c** Recovered conidia (n=3) including spore densities corresponding to spore counts corrected for the colony area

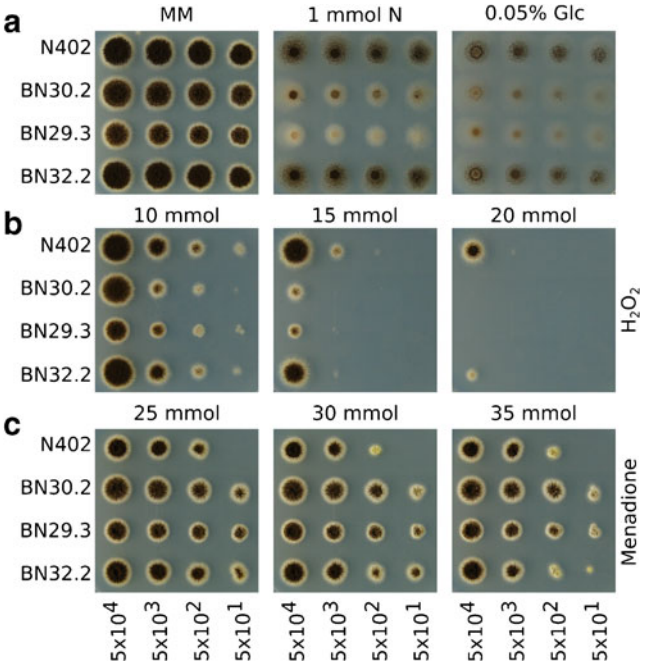


Fig. 4 Sensitivity assay of Δ*atg1*, Δ*atg8*, Δ*atg17*, and wild type strains (strains BN30.2, BN29.3, BN32.2, and N402, respectively). Tenfold dilutions (5×10^4–50 conidia) were spotted on plates with MM and MM with N (nitrate) or C (glucose) limitation (**a**) as well as MM supplemented with H_2O_2 (**b**) or menadione (**c**). Plates were incubated for 4 days at 30 °C

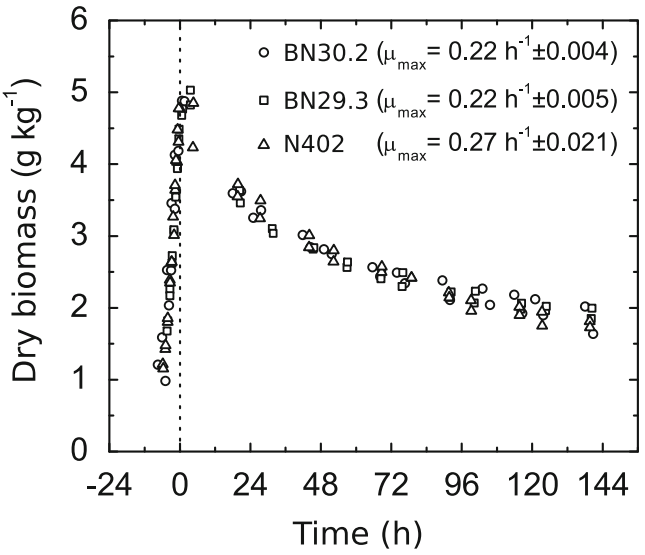


Fig. 5 Carbon starvation in submerged batch cultures. Duplicate biomass profiles of the wild type (strain N402) and the Δ*atg1* and Δ*atg8* mutants (strains BN30.2 and BN29.3, respectively) are shown. The time points of carbon depletion were set to 0 h and used for synchronization of (replicate) cultures

Phenotypes of Δ*atg1* and Δ*atg8* strains during submerged growth

It has been demonstrated that the majority of genes related to autophagy show joint transcriptional induction during the post-exponential phase in carbon-limited submerged batch cultures of *A. niger* (Nitsche et al. 2012). Concomitantly, old hyphae grown during the exponential phase undergo cell death, resulting in an increased fraction of empty hyphal compartments and secondary (cryptic) growth of thin non-branching hyphae. Although it has been shown for filamentous fungi that autophagy plays an important role in nutrient recycling during surface growth (Richie et al. 2007; Shoji et al. 2006; Shoji and Craven 2011), its function in submerged cultures remains obscure. To gain insights into the role of autophagy during submerged carbon starvation and to investigate how autophagy is related to the phenomena of cell death and secondary growth, Δ*atg1* and Δ*atg8* mutants (strains BN30.2 and BN29.3, respectively) were grown in bioreactors and maintained starving up to 6 days after carbon depletion (see Fig. 5).

The application of bioreactor technology allowed highly reproducible culture conditions. Monitoring of the physiological parameters (data not shown), including dissolved oxygen, O_2, and CO_2 partial pressures in the off-gas and titrant (NaOH and HCl) addition, allowed synchronization of (replicate) cultures. The described cultivation conditions prevented the formation of mycelial aggregates (pellets) and guaranteed a dispersed macromorphology during all cultivations. Interestingly, in contrast to the colony expansion rates on solid media (see Fig. 3a, b), the maximum specific growth rates for both mutants during exponential growth were affected to the same extent. The mutants grew slower (μ_{max}=0.22±0.005 h^{-1}) than the wild type (μ_{max}=0.27± 0.021 h^{-1}). The biomass profiles did not show any considerable differences during the post-exponential phase (see Fig. 5).

Flow chamber experiments with *A. oryzae* (Pollack et al. 2008) have shown that carbon depletion induces the outgrowth of hyphae with strongly reduced diameters. A similar morphological response has been observed during carbon starvation in submerged cultures of *A. niger* (Nitsche et al. 2012). An automated image analysis approach allowed for monitoring hyphal population dynamics of the cytoplasm-filled mycelial fraction and demonstrated a gradual transition from thick (old) to thin (young) hyphae during the post-exponential phase. This transition reflects cell death, resulting in the emergence of empty thick compartments fueling secondary (cryptic) regrowth in the form of non-branching thin hyphae. To examine whether autophagy affects this transition dynamics, microscopic pictures of the wild-type and the Δ*atg1* and Δ*atg8* strains from carbon-limited bioreactor batch cultures were analyzed accordingly (see Fig. 6). During exponential growth (day 0), all three strains displayed single populations of thick

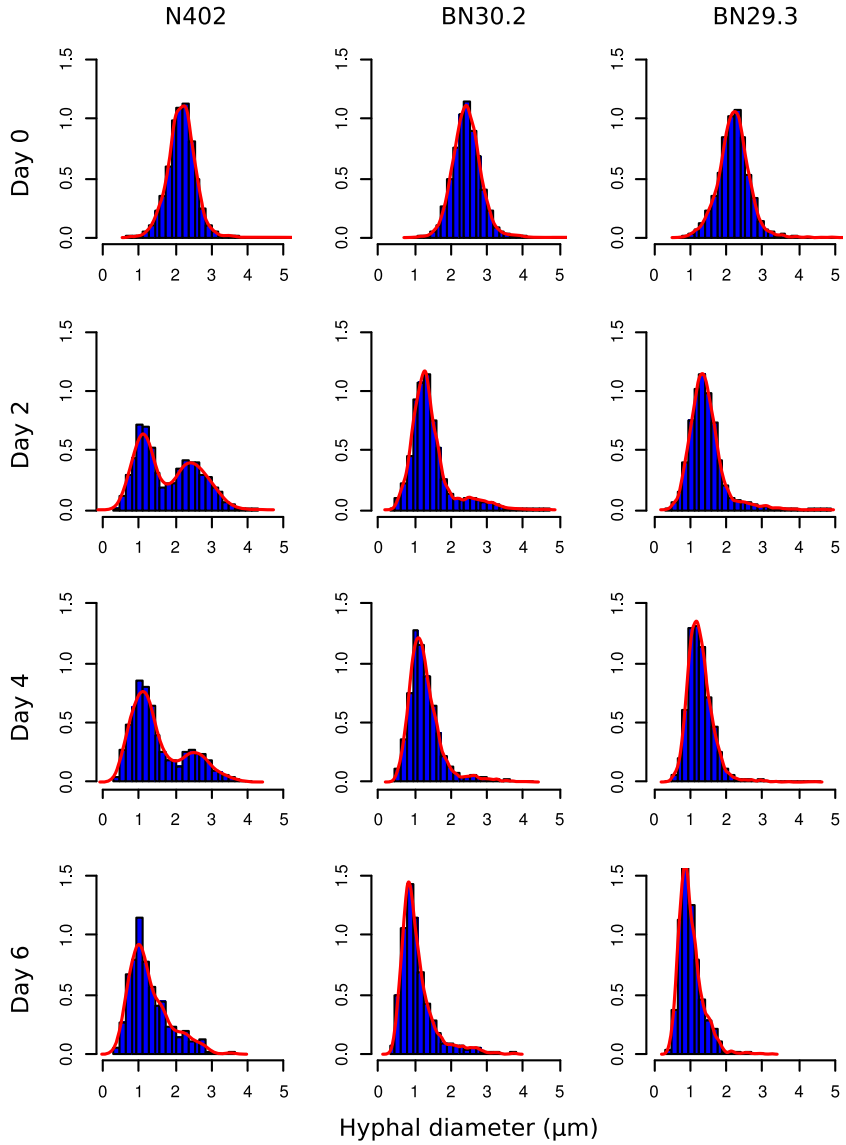


Fig. 6 Hyphal diameter populations. Population dynamics of hyphal diameters for the wild type (strains N402) and the Δ*atg1* and Δ*atg8* mutants (strains BN30.2 and BN29.3, respectively) are shown. Micrographs (≥40) of dispersed hyphae were analyzed by automated image analysis per strain and time point. *Day 0* refers to the exponential growth phase, whereas the subsequent three time points refer to 2, 4, and 6 days of carbon starvation

hyphae with mean diameters of approximately 2.2 µm. After carbon depletion, populations of thin hyphae with mean diameters of around 1 µm emerged. This transition was gradual for the wild type, while it was clearly accelerated for the Δ*atg1* and Δ*atg8* mutants, suggesting enhanced cell death rates for the older mycelium (thick hyphae) upon impairment of autophagy.

It has been shown that autophagy is important for mitochondrial maintenance and degradation of excess mitochondria during the stationary phase of *S. cerevisiae* cultures, which is of outermost importance because mitochondria play a key role in metabolism and cell death signaling (Zhang et al. 2007). In order to monitor whether the degradation of mitochondria in *A. niger* is similarly affected during carbon starvation in submerged cultures, the reporter strains BN38.9 (wild type), BN39.2 (Δ*atg1*), and BN40.8 (Δ*atg8*) with constitutive expression of mitochondrially targeted GFP (see Fig. 2) were cultured in carbon-limited bioreactor batch cultures and monitored using fluorescent microscopy. Hyphae from the exponential growth phase showed fluorescent tubular structures resembling those described by Suelmann and Fischer (2000), and no difference in mitochondrial morphology was observed between the three strains (see Fig. 7a). However, clear differences became apparent upon depletion of the carbon source. The mitochondrially targeted GFP was located inside the vacuoles of the wild type reporter (strain BN38.9), whereas no vacuolar GFP signal was detected for both Δ*atg1* and Δ*atg8* mutants (strains BN39.2 and BN40.8, respectively), as shown in Fig. 7b, c. The density of mitochondrial structures decreased in the wild type hyphae, but accumulated in the intervacuolar space for both mutants. Furthermore, there

were considerable differences in the mitochondrial morphology. The remaining mitochondrial structures were largely tubular in the wild type, while they appeared as fragmented and punctuated structures in the mutants.

Fig. 7 Localization of mitochondrially targeted GFP during submerged carbon starvation. Constitutive expression of mitochondrially targeted GFP in the wild type (strain BN38.9) and the Δ*atg1* and Δ*atg8* mutants (strains BN39.2 and BN40.8, respectively) during carbon-limited batch cultures. Differential interference contrast and fluorescence microscopy of hyphae from the exponential growth phase (**a**) and 7 h post-carbon depletion (**b**). **c** Confocal laser scanning microscopy of mycelial biomass at 14 h post-carbon depletion. *Scale bars*, 5 μm

Discussion

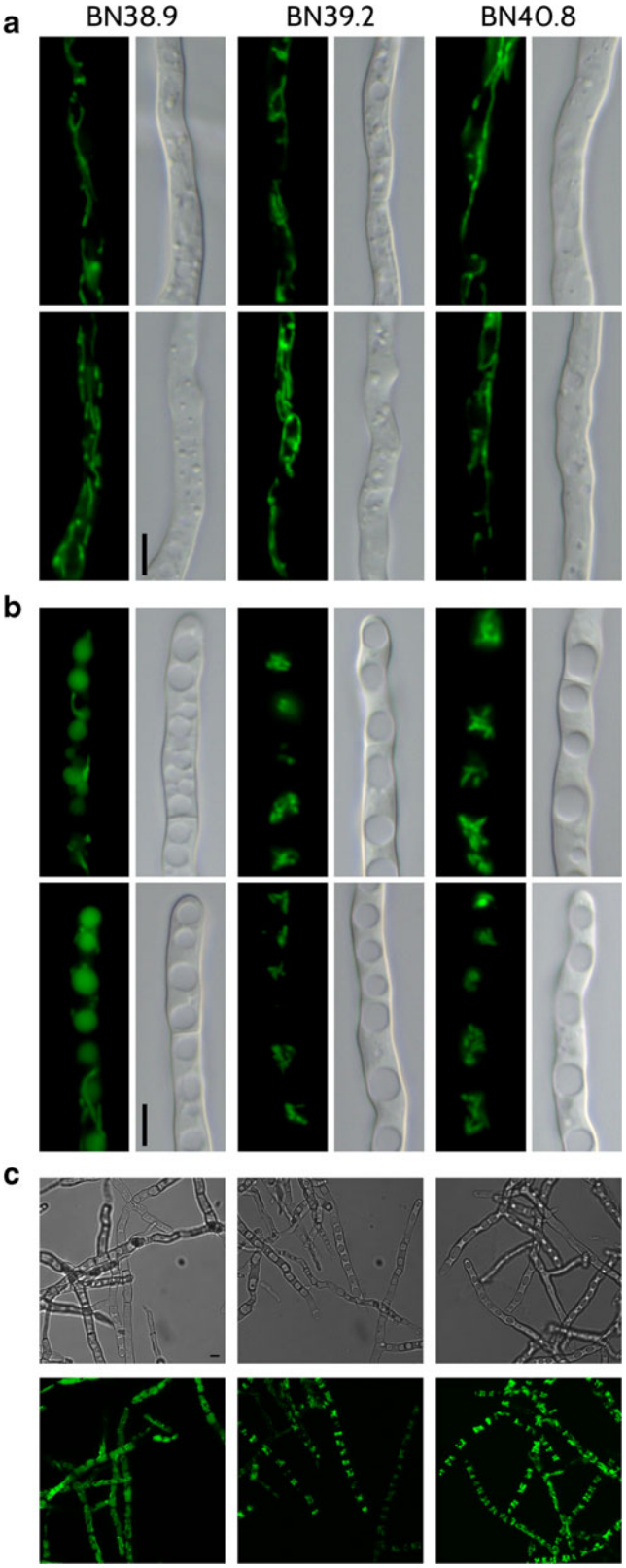
To our knowledge, this is the first published study investigating autophagy in the industrially important filamentous fungus *A. niger*. Improving our understanding of this catabolic pathway and its role during submerged cultivation is of great interest because autophagy has been shown to be involved in endogenous recycling and the regulation of cell death, both of which can have a direct impact on the yield of bioprocesses (Bartoszewska and Kiel 2011; Zustiak et al. 2008). For different filamentous fungi, several studies have described the phenomena of carbon-starved submerged cultures (Emri et al. 2004, 2005, 2006; White et al. 2002). A generic term that has emerged frequently in this context is autolysis. This term has been generally used to describe hallmarks of aging cultures including declining biomass, increasing extracellular ammonia concentration, hyphal fragmentation, and increasing extracellular hydrolase activities (White et al. 2002). Considerable effort has been made to analyze extracellular hydrolase activities (Emri et al. 2005; McIntyre et al. 2000; McNeil et al. 1998) as well as developmental mutants differentially affected in aging carbon-starved cultures (Emri et al. 2005). However, the role of autophagy in those cultures has not attained much attention yet. In a recent system-level analysis of the *A. niger* transcriptome during submerged carbon starvation, autophagy has been identified as a predominantly induced key process (Nitsche et al. 2012). This present study thus aimed at elucidating whether autophagy protects from or promotes loss of hyphal integrity, which was mainly observed by the formation of empty hyphal ghosts.

Two genes encoding components of the regulatory Atg1 kinase complex, namely the genes homologous to the kinase Atg1 itself and the scaffold protein Atg17, were deleted in *A. niger*. In addition, a homolog of the *atg8* gene encoding a membrane protein required for autophagosome formation and extension was deleted in *A. niger*. In agreement with studies in yeast and other filamentous fungi (Bartoszewska and Kiel 2011), the results demonstrate that deletion of either *atg1* or *atg8* is sufficient to severely impair autophagy in *A. niger*.

However, conflicting with results obtained in *S. cerevisiae* (Cheong et al. 2005; Kabeya et al. 2005), where the absence of Atg17 severely reduces the level of autophagy, deletion of *atg17* in *A. niger* did not show clear phenotypes. Except for a slightly attenuated spore formation (see Fig. 3) and intermediate phenotypes in response to oxidative stress (see Fig. 4), deletion of *atg17* was indistinguishable from the wild type. Similarly, the deletion of *atg13* in *A. oryzae* encoding another subunit of the Atg1 kinase complex was reported to only gently affect autophagy, whereas deletion of its counterpart in *S. cerevisiae* clearly impaired it (Kikuma and Kitamoto 2011). The authors suggested that Atg13 acts as an amplifier, resulting in higher autophagic activities in *A. oryzae*. Probably, Atg17 has a similar enhancing role during autophagy induction in *A. niger*, leading to the intermediate phenotypes described in this study. However, although the selected *atg17* ortholog (An02g04820) is highly conserved among filamentous fungi, its homology to *Scatg17* is considerably less ($E = 4e^{-12}$). Further functional analyses remain to be done to elucidate whether An02g04820 is indeed a functional ortholog of *Scatg17*.

Endogenous recycling of nutrients by autophagy has been supposed to be an important mechanism for nutrient trafficking along the mycelial network, promoting foraging of substrate exploring filaments and the formation of aerial hyphae-bearing conidiophores (Richie et al. 2007; Shoji et al. 2006; Shoji and Craven 2011). Similar to studies with other filamentous fungi (Bartoszewska et al. 2011; Kikuma et al. 2006; Richie et al. 2007), impairment of autophagy in *A. niger* reduced conidiation considerably (see Fig. 3), a phenotype which was much enhanced by carbon and nitrogen starvation (see Fig. 4a). However, in comparison to other filamentous fungi, this phenotype is much less pronounced, potentially indicating an important difference between *A. niger* and other filamentous fungi including *Aspergillus fumigatus* and *A. oryzae*. Alternatively, the explanation could possibly lie in the fact that the *A. niger* wild type strains used in this study have short conidiophores (*cspA1* mutant background; Bos et al. 1988), in which conidiophore development might be less affected because nutrients have to traffic along a shorter distance when conidiophore stalks are short.

In addition to its role in nutrient recycling, autophagy has been shown to be associated with PCD, which is classically categorized into three types, namely apoptotic (type I), autophagic (type II), and necrotic (type III) cell death. Although



autophagy is also referred to as type II PCD, it is not explicitly causative to cell death. Depending on the organism, cell type, and stressor, autophagy has been shown to promote both cell death and survival. In filamentous fungi, it has, for example, been demonstrated to protect against cell death during the heterokaryon incompatibility reaction in *P. anserina* (Pinan-Lucarré et al. 2005) or during carbon starvation in *Ustilago maydis* (Nadal and Gold 2010). Contrary to this, autophagy-induced cell death is required for rice plant infection by *Magnaporthe grisea* (Veneault-Fourrey et al. 2006). Loss of cellular integrity and subsequent death induced by damage of organelles, macromolecules, and membranes through ROS are a major threat for aerobic organisms. Well-described enzymatic and non-enzymatic defense systems have evolved that detoxify ROS (Bai et al. 2003). Autophagy is one of the major pathways for the turnover of redundant or damaged organelles and proteins. The described hypersensitivity of autophagy mutants to H_2O_2 could thus be explained by an impaired capability of the mutants to sequester and degrade proteins and organelles damaged by H_2O_2. The increased resistance to menadione, however, came as a surprise, but might be related to an adaptive stress response in autophagy-deficient mutants. In *S. cerevisiae*, it was, for example, shown that disruption of essential autophagy-related genes results in increased oxidative stress and superoxide dismutase activities (Zhang et al. 2007). Furthermore, adaptive responses to oxidative stress induced by sublethal concentrations of exogenous oxidants have been demonstrated to protect yeast cells against higher lethal concentrations (Fernandes et al. 2007; Jamieson 1992). Whether an endogenous adaptive mechanism explains the increased menadione resistance of autophagy-deficient mutants remains to be shown in future studies.

Hyphal population dynamics showed that the transition from old (thick) to young (thin) hyphae in response to carbon starvation during submerged cultivation was accelerated for both Δ*atg1* and Δ*atg8* mutants when compared to the wild type (see Fig. 6). These results suggest that autophagy protects old mycelium from the exponential growth phase under carbon starvation conditions and delays cell death. Fluorescence microscopy of wild type and Δ*atg1* and Δ*atg8* reporter strains with GFP-labeled mitochondria revealed that degradation of the mitochondria in response to carbon starvation is impaired in autophagy-deficient mutants. In yeast, the degradation of excess mitochondria during the stationary phase constitutes a physiological adaptation to the reduced energy requirement of the cells (Kanki et al. 2011). Impairment of autophagy has been shown to lead to mitochondria dysfunction and the accumulation of ROS in yeast stationary phase cultures starved for nitrogen (Suzuki et al. 2011). It is thus tempting to speculate that autophagy impairment in *A. niger* leads to increasing cellular ROS

levels caused by the accumulation of excessive and damaged mitochondria during carbon starvation in submerged cultures, which subsequently causes loss of cellular integrity and, finally, the emergence of empty hyphal compartments. Taken together, the results indicate that an induction of autophagy upon carbon starvation in submerged cultures of *A. niger* is not exclusively required for endogenous recycling, but constitutes an important physiological adaptation by the turnover of excessive mitochondria.

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References

Abràmoff MD, Hospitals I, Magalhães PJ (2004) Image processing with ImageJ. Biophotonics Int 11:36–42

Alic M, Bennett JW, Lasure LL (1991) More gene manipulations in fungi. Academic, San Diego

Andersen MR, Salazar MP, Schaap PJ, van de Vondervoort PJI, Culley D, Thykaer J, Frisvad JC, Nielsen KF, Albang R, Albermann K, Berka RM, Braus GH, Braus-Stromeyer SA, Corrochano LM, Dai Z, van Dijck PWM, Hofmann G, Lasure LL, Magnuson JK, Menke H, Meijer M, Meijer SL, Nielsen JB, Nielsen ML, van Ooyen AJJ, Pel HJ, Poulsen L, Samson RA, Stam H, Tsang A, van den Brink JM, Atkins A, Aerts A, Shapiro H, Pangilinan J, Salamov A, Lou Y, Lindquist E, Lucas S, Grimwood J, Grigoriev IV, Kubicek CP, Martinez D, van Peij NNME, Roubos JA, Nielsen J, Baker SE (2011) Comparative genomics of citric-acid-producing *Aspergillus niger* ATCC 1015 versus enzyme-producing CBS 513.88. Genome Res 21:885–897. doi:10.1101/gr.112169.110

Archer DB (2000) Filamentous fungi as microbial cell factories for food use. Curr Opin Biotechnol 11:478–483

Arnaud M, Chibucos MC, Costanzo MC, Crabtree J, Inglis DO, Lotia A, Orvis J, Shah P, Skrzypek MS, Binkley G, Miyasato SR, Wortman JR, Sherlock G (2010) The *Aspergillus* Genome Database, a curated comparative genomics resource for gene, protein and sequence information for the *Aspergillus* research community. Nucleic Acids Res 38:D420–D427. doi:10.1093/nar/gkp751

Arnaud M, Cerqueira GC, Inglis DO, Skrzypek MS, Binkley J, Chibucos MC, Crabtree J, Howarth C, Orvis J, Shah P, Wymore F, Binkley G, Miyasato SR, Simison M, Sherlock G, Wortman JR (2012) The *Aspergillus* Genome Database (AspGD): recent developments in comprehensive multispecies curation, comparative genomics and community resources. Nucleic Acids Res 40:D653–D659. doi:10.1093/nar/gkr875

Bai Z, Harvey LM, McNeil B (2003) Oxidative stress in submerged cultures of fungi. Crit Rev Biotechnol 23:267–302

Bartoszewska M, Kiel JAKW (2011) The role of macroautophagy in development of filamentous fungi. Antioxid Redox Signaling 14:2271–2287. doi:10.1089/ars.2010.3528

Bartoszewska M, Kiel JAKW, Bovenberg RAL, Veenhuis M, van der Klei IJ (2011) Autophagy deficiency promotes β-lactam production in *Penicillium chrysogenum*. Appl Environ Microbiol 77:1413–1422. doi:10.1128/AEM.01531-10

Bos C, Debets A, Swart K, Huybers A (1988) Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. Curr Genet 14:437–443

Braaksma M, Smilde AK, van der Werf MJ, Punt PJ (2009) The effect of environmental conditions on extracellular protease activity in controlled fermentations of *Aspergillus niger*. Microbiology 155:3430–3439. doi:10.1099/mic.0.031062-0

Carvalho N, Arentshorst M (2011) Effects of a defective ERAD pathway on growth and heterologous protein production in *Aspergillus niger*. Appl Microbiol Biotechnol 89:357–373. doi:10.1007/s00253-010-2916-5

Carvalho N, Jørgensen TR, Arentshorst M, Nitsche BM, van den Hondel CAMJJ, Archer DB, Ram AFJ (2012) Genome-wide expression analysis upon constitutive activation of the HacA bZIP transcription factor in *Aspergillus niger* reveals a coordinated cellular response to counteract ER stress. BMC Genomics 13:350. doi:10.1186/1471-2164-13-350

Cheong H, Yorimitsu T, Reggiori F, Legakis JE, Wang C-W, Klionsky DJ (2005) Atg17 regulates the magnitude of the autophagic response. Mol Biol Cell 16:3438–3453. doi:10.1091/mbc.E04

Cheong H, Nair U, Geng J, Klionsky DJ (2008) The Atg1 kinase complex is involved in the regulation of protein recruitment to initiate sequestering vesicle formation for nonspecific autophagy in *Saccharomyces cerevisiae*. Mol Biol Cell 19:668–681. doi:10.1091/mbc.E07

Codogno P, Meijer AJ (2005) Autophagy and signaling: their role in cell survival and cell death. Cell Death Differ 12:1509–1518. doi:10.1038/sj.cdd.4401751

Emri T, Molnár Z, Pusztahelyi T, Pócsi I (2004) Physiological and morphological changes in autolyzing *Aspergillus nidulans* cultures. Folia Microbiol 49:277–284

Emri T, Molnár Z, Pusztahelyi T, Varecza Z, Pócsi I (2005) The FluG-BrlA pathway contributes to the initialisation of autolysis in submerged *Aspergillus nidulans* cultures. Mycol Res 109:757–763

Emri T, Molnár Z, Veres T, Tünde P, Dudas G, Pócsi I (2006) Glucose-mediated repression of autolysis and conidiogenesis in *Emericella nidulans*. Mycol Res 110:1172–1178. doi:10.1016/j.mycres.2006.07.006

Fernandes P, Mannarino S, Silva C, Pereira M, Panek A, Eleutherio E (2007) Oxidative stress response in eukaryotes: effect of glutathione, superoxide dismutase and catalase on adaptation to peroxide and menadione stresses in *Saccharomyces cerevisiae*. Redox Rep 12:236–244. doi:10.1179/135100007X200344

Inoue Y, Klionsky DJ (2010) Regulation of macroautophagy in *Saccharomyces cerevisiae*. Semin Cell Dev Biol 21:664–670. doi:10.1016/j.semcdb.2010.03.009

Jamieson DJ (1992) *Saccharomyces cerevisiae* has distinct adaptive responses to both hydrogen peroxide and menadione. J Bacteriol 174:6678–6681

Jørgensen TR, Nitsche BM, Lamers GEM, Arentshorst M, van den Hondel CAMJJ, Ram AFJ (2010) Transcriptomic insights into the physiology of *Aspergillus niger* approaching a specific growth rate of zero. Appl Environ Microbiol 76:5344–5355. doi:10.1128/AEM.00450-10

Kabeya Y, Kamada Y, Baba M, Takikawa H, Sasaki M, Ohsumi Y (2005) Atg17 functions in cooperation with Atg1 and Atg13 in yeast autophagy. Mol Biol Cell 16:2544–2553. doi:10.1091/mbc.E04

Kanki T, Klionsky DJ, Okamoto K (2011) Mitochondria autophagy in yeast. Antioxid Redox Signaling 14:1989–2001. doi:10.1089/ars.2010.3762

Kikuma T, Kitamoto K (2011) Analysis of autophagy in *Aspergillus oryzae* by disruption of *Aoatg13*, *Aoatg4*, and *Aoatg15* genes. FEMS Microbiol Lett 316:61–69. doi:10.1111/j.1574-6968.2010.02192.x

Kikuma T, Ohneda M, Arioka M, Kitamoto K (2006) Functional analysis of the *ATG8* homologue *Aoatg8* and role of autophagy in differentiation and germination in *Aspergillus oryzae*. Eukaryot Cell 5:1328–1336. doi:10.1128/EC.00024-06

Lagopodi AL, Ram AFJ, Lamers GEM, Punt PJ, van den Hondel CAMJJ, Lugtenberg BJJ, Bloemberg GV (2002) Novel aspects of tomato root colonization and infection by *Fusarium oxysporum* f. sp. *radicis-lycopersici* revealed by confocal laser scanning microscopic analysis using the green fluorescent protein as a marker. Mol Plant–Microbe Interact 15:172–179. doi:10.1094/MPMI.2002.15.2.172

MacKenzie DA, Guillemette T, Al-Sheikh H, Watson AJ, Jeenes DJ, Wongwathanarat P, Dunn-Coleman NS, van Peij NNME, Archer DB (2005) UPR-independent dithiothreitol stress-induced genes in *Aspergillus niger*. Mol Genet Genomics 274:410–418. doi:10.1007/s00438-005-0034-3

Matsuura A, Tsukada M, Wada Y, Ohsumi Y (1997) Apg1p, a novel protein kinase required for the autophagic process in *Saccharomyces cerevisiae*. Gene 192:245–250

Mattern IE, Punt PJ, van den Hondel CAMJJ (1988) A vector of *Aspergillus* transformation conferring phleomycin resistance. Fungal Genet Newsl 35:25–30

Mattern IE, van Noort JM, van den Berg P, Archer DB, Roberts IN, van den Hondel CAMJJ (1992) Isolation and characterization of mutants of *Aspergillus niger* deficient in extracellular proteases. Mol Gen Genet 234:332–336

McIntyre M, Berry DR, McNeil B (2000) Role of proteases in autolysis of *Penicillium chrysogenum* chemostat cultures in response to nutrient depletion. Appl Microbiol Biotechnol 53:235–242

McNeil B, Berry DR, Harvey LM, Grant A, White S (1998) Measurement of autolysis in submerged batch cultures of *Penicillium chrysogenum*. Biotechnol Bioeng 57:297–305

Meyer V, Ram AFJ, Punt PJ (2010) Genetics, genetic manipulation and approaches to strain improvement of filamentous fungi. In: Davies J, Demain A (eds) Manual of industrial microbiology and biotechnology. Wiley, New York, pp 318–329

Nadal M, Gold SE (2010) The autophagy genes *atg8* and *atg1* affect morphogenesis and pathogenicity in *Ustilago maydis*. Mol Plant Pathol 11:463–478. doi:10.1111/J.1364-3703.2010.00620.X

Nitsche BM, Jørgensen TR, Akeroyd M, Meyer V, Ram AFJ (2012) The carbon starvation response of *Aspergillus niger* during submerged cultivation: insights from the transcriptome and secretome. BMC Genomics 13:380. doi:10.1186/1471-2164-13-380

Peberdy JF (1994) Protein secretion in filamentous fungi—trying to understand a highly productive black box. Trends Biotechnol 12:50–57

Pel HJ, De Winde JH, Archer DB, Dyer PS, Hofmann G, Schaap PJ, Turner G, De Vries RP, Albang R, Albermann K, Andersen MR, Bendtsen JD, Benen JA, van den Berg MA, Breestraat S, Caddick MX, Contreras R, Cornell M, Coutinho PM, Danchin EG, Debets AJ, Dekker P, van Dijck PW, van Dijk A, Dijkhuizen L, Driessen AJ, D'Enfert C, Geysens S, Goosen C, Groot GS, De Groot PW, Guillemette T, Henrissat B, Herweijer M, van den Hombergh JP, van den Hondel CAMJJ, van der Heijden RT, van der Kaaij RM, Klis FM, Kools HJ, Kubicek CP, vanKuyk PA, Lauber J, Lu X, van der Maarel MJEC, Meulenberg R, Menke H, Mortimer MA, Nielsen J, Oliver SG, Olsthoorn M, Pal K, van Peij NNME, Ram AFJ, Rinas U, Roubos JA, Sagt CM, Schmoll M, Sun J, Ussery D, Varga J, Vervecken W, van de Vondervoort PJI, Wedler H, Wösten HA, Zeng AP, van Ooyen AJ, Visser J, Stam H (2007) Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat Biotechnol 25:221–231

Pinan-Lucarré B, Balguerie A, Clavé C (2005) Accelerated cell death in *Podospora* autophagy mutants. Eukaryot Cell 4:1765–1774. doi:10.1128/EC.4.11.1765

Pócsi I, Miskei M, Karányi Z, Emri T, Ayoubi P, Pusztahelyi T, Balla G, Prade RA (2005) Comparison of gene expression signatures of diamide, H_2O_2 and menadione exposed *Aspergillus nidulans* cultures-linking genome-wide transcriptional changes to cellular physiology. BMC Genomics 6:182. doi:10.1186/1471-2164-6-182

Pollack JK, Li ZJ, Marten MR (2008) Fungal mycelia show lag time before re-growth on endogenous carbon. Biotechnol Bioeng 100:458–465. doi:10.1002/bit.21779

Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH (1987) Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. Gene 56:117–124

Richie DL, Fuller KK, Fortwendel J, Miley MD (2007) Unexpected link between metal ion deficiency and autophagy in *Aspergillus fumigatus*. Eukaryot Cell 6:2437–2447. doi:10.1128/EC.00224-07

Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual. Cold Spring Harbor, Cold Spring Harbor

Shoji J-Y, Craven KD (2011) Autophagy in basal hyphal compartments: a green strategy of great recyclers. Fungal Biol Rev 25:79–83. doi:10.1016/j.fbr.2011.04.001

Shoji J-Y, Arioka M, Kitamoto K (2006) Possible involvement of pleiomorphic vacuolar networks in nutrient recycling in filamentous fungi. Autophagy 2:226–227

Suelmann R, Fischer R (2000) Mitochondrial movement and morphology depend on an intact actin cytoskeleton in *Aspergillus nidulans*. Cell Motil Cytoskeleton 45:42–50. doi:10.1002/(SICI)1097-0169(200001)45:1<42::AID-CM4>3.0.CO;2-C

Suzuki SW, Onodera J, Ohsumi Y (2011) Starvation induced cell death in autophagy-defective yeast mutants is caused by mitochondria dysfunction. PLoS One 6:e17412. doi:10.1371/journal.pone.0017412

Thorpe GW, Fong CS, Alic N, Higgins VJ, Dawes IW (2004) Cells have distinct mechanisms to maintain protection against different reactive oxygen species: oxidative-stress-response genes. Proc Natl Acad Sci U S A 101:6564–6569. doi:10.1073/pnas.0305888101

Tsukada M, Ohsumi Y (1993) Isolation and characterization of autophagy-defective mutants of *Saccharomyces cerevisiae*. FEBS Lett 333:169–174

Tucker CL, Fields S (2004) Quantitative genome-wide analysis of yeast deletion strain sensitivities to oxidative and chemical stress. Comp Funct Genomics 5:216–224. doi:10.1002/cfg.391

van Gorcom RF, van den Hondel CAMJJ (1988) Expression analysis vectors for *Aspergillus niger*. Nucleic Acids Res 16:9052

van Hartingsveldt W, Mattern IE, van Zeijl CM, Pouwels PH, van den Hondel CAMJJ (1987) Development of a homologous transformation system for *Aspergillus niger* based on the *pyrG* gene. Mol Gen Genet 206:71–75

Veneault-Fourrey C, Barooah M, Egan M, Wakley G, Talbot NJ (2006) Autophagic fungal cell death is necessary for infection by the rice blast fungus. Science 312:580–583. doi:10.1126/science.1124550

Vinck A, Terlou M, Pestman WR, Martens EP, Ram AFJ, van den Hondel CAMJJ, Wösten HAB (2005) Hyphal differentiation in the exploring mycelium of *Aspergillus niger*. Mol Microbiol 58:693–699. doi:10.1111/j.1365-2958.2005.04869.x

Vishniac W, Santer M (1957) The *Thiobacilli*. Bacteriol Rev 21:195–213

White S, McIntyre M, Berry DR, McNeil B (2002) The autolysis of industrial filamentous fungi. Crit Rev Biotechnol 22:1–14

Xie Z, Klionsky DJ (2007) Autophagosome formation: core machinery and adaptations. Nat Cell Biol 9:1102–1109

Zhang Y, Qi H, Taylor R, Xu W, Liu LF, Jin S (2007) The role of autophagy in mitochondria maintenance: characterization of mitochondrial functions in autophagy-deficient *S. cerevisiae* strains. Autophagy 3:337–346

Zustiak MP, Pollack JK, Marten MR, Betenbaugh MJ (2008) Feast or famine: autophagy control and engineering in eukaryotic cell culture. Curr Opin Biotechnol 19:518–526. doi:10.1016/j.copbio.2008.07.007