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Contents

Outline

Filamentous fungi with a saprophytic lifestyle, like *Aspergillus niger*, commonly encounter limitations in the available carbon sources in their environment. During such conditions, very specific carbon starvation responses are induced aiming at survival and proliferation of the fungus. One of the processes that is highly activated by carbon starvation is autophagy. Autopaghy is an intracellular degradation system which targets cytosolic components to lytic compartments for degradation and recycling of the building blocks of the cell. The process has been described in many species, most importantly in yeast and mammalian cells, and also including filamentous fungi. During autophagy in *A. niger*, cytoplasmic proteins and organelles are sequestered and delivered to vacuoles in double membrane vesicles, termed autophagosomes. Upon fusion of the outer membrane of the autophagosome with the vacuolar membrane, a single membrane vesicle is released into the hydrolytic environment of the vacuole. Following lysis of the autophagosomal membrane and hydrolytic degradation of the vesicular contents, breakdown products are transported back into the cytosol for reuse by the cell. The autophagy pathway is regulated by autophagy-related (Atg) proteins, which specifically support the different steps in the process. The process is highly induced by carbon starvation conditions, during which the recycling of nutrients is highly important for maintenance of the mycelium and to fuel asexual spore formation and cellular differentiation. This thesis aims at evaluating the autophagy process in the filamentous fungus *A. niger*, focusing on its role during carbon starvation, ER stress and unconventional protein secretion.

Chapter 1 reviews the specific responses to carbon starvation in *Aspergillus* species, giving insights into the most important cellular responses which include morphological responses such as asexual spore formation and recycling mechanisms such as the degradation and utilization of inner cell materials by autophagy and the degradation of cell wall constituents by extracellular hydrolytic enzymes.

In **chapter 2** phenotypical and cytological characterizations of *A. niger* autophagy deletion mutants in surface and submerged growth during carbon starvation are provided. The disruption of autophagy-related homologs in fluorescent reporter strains shows that both *atg1* and *atg8* are essential for autophagy, whereas *atg17* is not essential. By using automated image analysis it is demonstrated that cell death and outgrowth of cryptic hyphae is accelerated in autophagy deletion strains.

Chapter 3 describes the results of genome-wide transcriptional analysis comparing the *A. niger* Δ*atg1* mutant strain with the wild-type during submerged carbon starvation. Early

and late carbon starvation responses can be clearly distinguished, as genes related to cell division and DNA repair are higher expressed in the Δ*atg1* mutant at one day post carbon depletion whereas lower expression of metabolic processes is specific for the late phase of carbon starvation.

Chapter 4 shows the effects of applying ER stress in *A. niger* strains defective for functional autophagy and/or ER associated degradation (ERAD), by which the possible link between ER stress and autophagy is investigated. ER stress conditions are induced either by exposure to a chemical ER stressor or by expression of a disulfide bond mutated form of the glucoamylase protein. Fluorescent labeling of this misfolded protein visualizes higher accumulating protein levels in the ER in ERAD-defective background strains as compared to wild-type and autophagy single deletion mutants.

In **chapter 5** the unconventionally secreted aspartic protease PepN is used as a target protein to describe the role of autophagy in unconventional protein secretion. Proteome and immunoblotting analyses demonstrate the presence of PepN in secretomes of wild-type, Δ*atg1* and Δ*atg8* strains, indicating that the secretion of PepN is independent of autophagy-essential Atg1 and Atg8.

Chapter 1

Post-genomic approaches to dissect carbon starvation responses in *Aspergilli*

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Abstract

Most filamentous fungi have a saprophytic lifestyle and proliferate on organic materials from plants. They contain a large arsenal of enzymes that are expressed and secreted in response to available carbon sources. Regulatory networks in which carbon-specific transcription factors and wide-domain transcription factors play essential roles tightly control the expression of these enzymes. While a suitable carbon source is present, the entire metabolism of the fungus is directed to grow as fast as possible via hyphal tip extension. At a certain point however, the available exogenous carbon source will be limited, resulting in a carbon starvation response. Filamentous fungi react to carbon starvation with some very specific responses including the induction of glycosyl hydrolases involved in fungal cell wall degradation (autolysis) and the onset of asexual spore formation. Recent advances in genome-wide transcriptomic and proteomic approaches have revealed new insights into molecular mechanisms involved in these fungal-specific carbon starvation responses. Together with the genetic accessibility of filamentous fungi, the omics technologies have made a large contribution in broadening our understanding of the carbon starvation response in recent years. In this review, we have focussed on summarizing and integrating the most important cellular responses of filamentous fungi towards carbon starvation. As most studies to date have been carried out with *Aspergillus nidulans* and *Aspergillus niger,* the focus will be further on these two species and to compare their responses to carbon starvation.

Introduction

The morphology of filamentous fungi is characterized by the presence of hyphae. Filamentous growth starts with the germination of a spore and the subsequent formation of a germling. Via branching or hyphal fusion events, a network of hyphae is formed which is referred to as a hyphal mycelium. Such a hyphal mycelium can be considered as a multicellular structure. In most filamentous fungi, except the zygomycete fungi, hyphae are divided into individual cells via the formation of internal walls named septa. Each septum contains a septal pore, which is large enough to allow transport of organelles and nutrients from one hyphal compartment to the other. Filamentous fungi can control the flow of cytosol or organelles between compartments by the controlled closure of the septal pore via Woronin bodies. Woronin bodies are peroxisome-derived, spherical organelles that are located near the septal pore. By plugging the septal pores with Woronin bodies, a multicellular structure with individual cells is formed. The opening and closing of septal pores are highly dynamic and well-controlled events (Bleichrodt *et al.*, 2015).

A fungal mycelium is typically formed on solid medium such as agar plates. To analyse growth on agar plates, spores are often inoculated in the centre of the plate from where they will gradually cover the plate through radial extension. During growth, the fungus will utilize the available nutrients until they become limiting (for most of the media this will be the carbon source). At that point, the mycelium starts a developmental program leading to sexual or asexual reproduction. Since nutrient limitation first occurs in the centre of the colony, differentiation will start here also. Whether the sexual or asexual developmental pathway is chosen depends on environmental factors such as light (Bayram and Braus, 2012). Some fungi, such as *Aspergillus niger*, are only known to proliferate asexually. The ability to reproduce sexually has either been lost through evolution or remains undiscovered.

Besides the growth on solid medium, fungi are also cultured in liquid medium, referred to as submerged cultivation. To start a submerged cultivation, spores can be used as an inoculum. Depending on the spore density and ambient pH (and possibly other factors), fungal hyphae develop as a dispersed fungal mycelium (individual hyphal filamentous cells) or as pellets (dense aggregates of filamentous cells). The tendency of a culture to form pellets is mainly caused by aggregation of the spores during the initial stages of spore swelling. Low pH values reduce the aggregation of swollen spores and are therefore used to prevent pellet formation. The macromorphology of the submerged culture (dispersed mycelia or pellets) can have important consequences on the productivity and heterogeneity of the culture and is therefore an important parameter. As on solid medium, nutrients can become limiting during batch growth and filamentous fungi respond to starvation with the activation of asexual reproduction program leading to the formation of conidiospores

(Nitsche *et al.*, 2012; Sarkar *et al.*, 2012). The advantages of studying starvation responses in filamentous fungi using bioreactors are reviewed in the next paragraph.

Controlled cultivation of filamentous fungi to study starvation responses

Responses to carbon starvation have been most elaborately studied in submerged cultures. Batch cultivation in controlled bioreactors is preferred as growth can be monitored on-line and is more reproducible (Nitsche *et al.*, 2012). Batch cultures in shake flasks have also been successfully used to perform C-starvation responses (Szilágyi *et al.*, 2013; de Sousa Lima *et al.*, 2014; van Munster *et al.*, 2014; Xiong *et al.*, 2014). However, more detailed measurements regarding the physiology of the fungus cannot be measured in shake flask cultures. On-line analysis of O_2 consumption and CO_2 production in a bioreactor is a convenient method to monitor carbon limitation as the parameters will dramatically change upon starvation (Nitsche *et al.*, 2012). Yet another advantage of bioreactor cultivation is that when sufficient large vessels are used (e.g. 5L), multiple samples for analysis can be withdrawn without affecting the total volume of the culture to such an extent that it changes the physiology of the culture. Substrate-limited growth has also been studied in chemostat cultures. There is an important difference between carbon starvation in batch growth and C-limited chemostat cultures, as additional carbon source is continuously added during chemostat cultivations. However, at low dilution rates (resulting in low growth rates), starvation-like responses such as sporulation have been observed (Bushell and Bull, 1999; Jørgensen, personal communication). An extreme form of chemostat cultivation which leads to a very low growth rate is retentostat cultivation. In a retentostat system, the biomass is retained in the bioreactor while keeping the dilution rate of the medium constant, leading to a severe reduction of the energy source per biomass unit. Cultivation of *A. niger* in retentostats also results in the onset of conidiation (Jørgensen *et al.*, 2010) indicating that limited metabolic fluxes might be sufficient to activate the sporulation program. Based on the phenotype of conidial mutants both on solid plates and in submerged conditions, it is likely that the same genetic regulatory program is involved under both conditions (Nitsche and Jørgensen, personal communication). Although there are obvious benefits, maintaining the environmental parameters (*e.g.* pH, oxygen levels and medium composition) at a constant/controlled level may alter the response of the fungus. In nature, fungi are continuously modifying their environment by secreting enzymes and metabolites during carbon starvation and these changes (*e.g.* alkalizing pH, liberation of nutrients from the hydrolyzing cell wall) can alter their behaviour. When controlling conditions in the bioreactor, we can gain precise data on chosen stress responses, but may lose information on the complexity of the whole stress response.

Carbon starvation responses

Carbon starvation responses in filamentous fungi have been studied both on surface cultures (mostly agar plates) and in submerged conditions. Several transcriptomic and

proteomic studies have been performed in the last few years to describe the carbon starvation responses and are discussed in the next two paragraphs.

Carbon starvation responses on surface cultures

The Wösten group developed an elegant ring system to analyse differential gene expression in a fungal colony and defined five zones, representing differently aged fungal mycelium. The differential gene expression between the different zones as well as the different extracellular proteomes demonstrates the multicellular nature of a fungal colony (Levin *et al.*, 2007; Krijgsheld *et al.*, 2012). Comparison of mRNA profiles between the central zone of an *A. niger* colony and the most outer zone revealed major differences in expression of genes from various functional classes (Levin *et al.*, 2007). Only the broad functional categories 01 (metabolism) including 01.01.10 (amino acid degradation), 01.05.01 and 01.05.07 (C-compound and carbohydrate utilization and transport) and 01.06.01 (lipid, fatty acid and isoprenoid biosynthesis), 06 (protein fate) including 06.07.03 (modifications by (de)-phosphorylation), 06.07.07 (modifications by (de)-ubiquitination) and 06.13.04 (lysosomal and vacuolar degradation), were consistently enriched in the central zones independent of the carbon source used (Levin *et al.*, 2007). It should be noted that in the experiments by Levin et al., the *A. niger* colony was covered with a perforated polycarbonate membrane to prevent sporulation in the centre of the colony. Therefore genes related to conidiation were not induced in these experiments.

mRNA profiling to dissect colony development has also been studied in *Neurospora crassa* (Kasuga and Glass, 2008) and despite the relatively large evolutionary distance between *A. niger* and *N. crassa* a comparative study between the zonal transcriptomes between the two filamentous fungi has been performed. Even though such a global comparison has its limitations, as it is highly dependent on the annotation and the classification in GO-terms or functional categories, the cross-species examination indicated conserved patterns of gene expression during colony development. These conserved patterns were mostly found in the outer parts of the colony and included genes involved in metabolism of lipids and fatty acids, which were predicted to be involved in membrane biosynthesis, and genes involved in biogenesis of cellular components which included genes involved in actin cytoskeleton organisation (Kasuga and Glass, 2008).

Carbon starvation responses under submerged conditions

Several genome-wide transcriptomic or proteomic studies have been reported in the last few years to study the response of filamentous fungi towards carbon starvation under submerged growth conditions. Initial genome-wide transcriptomic studies were performed with *A. niger* for which a genome sequence and microarrays were available (Pel *et al.*, 2007; Nitsche *et al.*, 2012; van Munster *et al.*, 2015b). Since then, other reports have been published, including carbon starvation responses in the medically relevant fungus

Paracoccidioides spp (de Sousa Lima *et al.*, 2014) and a transcriptome study including qRT-PCR for 99 selected genes in *Aspergillus nidulans* to analyse the response to carbon starvation (Szilágyi *et al.*, 2013). Because of the different experimental designs in each of the studies, a detailed comparison between the studies is challenging. Whereas in *A. niger* three different time points were analysed (16h, 60h and 140h after carbon starvation), studies in *Paracoccidioides* and *A. nidulans* included a single time point (6h and 4h after starvation, respectively). Starvation leads to important morphological changes, including vacuolization, the formation of empty compartments, hyphal fragmentation, the formation of thin hyphae and conidiation. These morphological changes are accompanied by massive changes in gene expression patterns. About 50% of the genes (7,292 out of 13,989) are differentially expressed during starvation compared to the exponential growth phase in *A. niger* (Nitsche *et al.*, 2012). Analysis of the differentially expressed genes during carbon starvation in submerged conditions identified several commonly induced functional categories or GO terms. As expected, many of these terms were related to dealing with the carbon limitation and the use of endogenous or reserve carbon sources. Obviously, these endogenous carbon sources include extracellular sources such as the fungal cell wall, but also intracellular sources. Therefore in all three genome-wide studies performed (Nitsche *et al.*, 2012; Szilágyi *et al.*, 2013; de Sousa Lima *et al.*, 2014) terms related to amino acid metabolism, fatty acid oxidation, C-compound and carbohydrate metabolism were upregulated in response to starvation. Terms related to cell biological processes such as cell cycle arrest *(A. niger*), protein synthesis and secretion (*A. nidulans*), autophagy (*A. niger* and *A. nidulans*), vacuolar transports (*A. niger* and *A. nidulans*) were enriched in the upregulated genes in response to carbon starvation. The energy and building blocks obtained from recycling its own mycelium, allow the fungus to respond to the starvation condition whereby the energy is used for maintenance and to fuel conidiation. In the next parts, we will further focus on the morphological responses observed in carbon starved cultures of filamentous fungi.

Morphological responses during C-limitation

Carbon starvation induces hyphal fragmentation in submerged *A. nidulans* cultures (Emri *et al.*, 2004, 2005; Pócsi *et al.*, 2009; Szilágyi *et al.*, 2010a, 2013). During this process, hyphal fragments are liberated from the surface of pellets and as a consequence the pellet size is decreased until it is completely disintegrated (Emri *et al.*, 2004). The disintegration of hyphae is followed by the complete degradation of the released hyphal branches until only small numbers of "yeast like cells" (hyphal fragments of one to two cells) are present in the fermentation broth (Emri *et al.*, 2004). These short fragments can grow out and form hyphae after glucose supplementation (Emri *et al.*, 2004). Carbon starvation in *A. niger* is also accompanied by several morphological changes which include: the formation of empty compartments (starting to emerge after 16 h of glucose depletion), the outgrowth of thin hyphae or thin hyphae within empty hyphae (16 h after glucose depletion), and conidiation

(60 h after glucose depletion) (Nitsche *et al.*, 2012). These morphological changes are similar to the morphological changes upon starvation which have been described before (White *et al.*, 2002). The formation of thin hyphae in response to carbon starvation was reported also for *Aspergillus oryzae* (Pollack *et al.*, 2008). An important difference in comparison to morphological changes upon starvation in *A. nidulans* (Emri *et al.*, 2004) is that in *A. niger* no mycelial fragmentation was observed. Additional unpublished studies have shown that the lack of fragmentation in *A. niger* is probably due to the low pH value (pH3.0) which is maintained in the bioreactor during the experiments. Fragmentation of the mycelium upon starvation in *A. niger* is observed when the pH is not controlled and reaches values around 6.0 during starvation (Nitsche, unpublished results). Likely, the low pH (3.0) conditions lower the activity of cell wall hydrolytic enzymes that are involved in hyphal fragmentation. In *A. nidulans*, fragmentation and formation of empty hyphae is also accompanied by the production of cell wall glycosyl hydrolases (Emri *et al.*, 2004, 2005; Pócsi *et al.*, 2009; Szilágyi *et al.*, 2010a, 2013). *A. nidulans* mutants with defects in cell wall glycosyl hydrolase production show reduced hyphal fragmentation but these mutations do not inhibit vacuolization and formation of empty hyphae (Emri *et al.*, 2005; Pócsi *et al.*, 2009; Szilágyi *et al.*, 2010a). It clearly demonstrates that hyphal fragmentation is an enzymatic process and not the passive consequence of mechanic forces occurring in shaken cultures.

In addition to the formation of empty compartments and hyphal fragmentation, some hyphal cells remain viable and these surviving cells differentiate and can produce conidiospores (Schrickx *et al.*, 1993; Emri *et al.*, 2004; Jørgensen *et al.*, 2010; Nitsche *et al.*, 2012; Szilágyi *et al.*, 2013). BrlA encodes a transcription factor which is essential for asexual reproduction in Aspergilli both in surface cultures as well as in submerged cultures (Adams *et al.*, 1998; Twumasi-Boateng *et al.*, 2009; van Munster *et al.*, 2015b). The regulation of conidiation in submerged conditions as well as the regulation of conidiation at surface cultures is dependent on BrlA and therefore BrlA is likely to be a key regulator for conidiation under both conditions. Asexual development (conidiogenesis) is supposed to be initiated by internal signals and/or several stress conditions including carbon starvation (Adams *et al.*, 1998; van Munster *et al.*, 2013). The stress-dependent activation of conidiogenesis is mandatory during submerged cultivation but thought to be secondary in surface cultures (Skromne *et al.*, 1995; Adams *et al.*, 1998; Krijgsheld *et al.*, 2013). In surface cultures, asexual development generally starts well before carbon depletion and continues during starvation (Adams *et al.*, 1998). Autolytic processes (see below) induced by carbon starvation are supposed to support asexual development with nutrients during this period (Emri *et al.*, 2008; Pócsi *et al.*, 2009).

In addition to asexual development (formation of conidiophores and conidia), developmental processes also involve sexual development (formation of cleistothecia and ascospores) in aspergilli with a sexual cycle such as *A. nidulans* (Adams *et al.*, 1998; Etxebeste

et al., 2010; Dyer and O'Gorman, 2012; Park and Yu, 2012; Krijgsheld *et al.*, 2013). Carbon starvation induces sexual development in several pezizomycete fungi (Dyer *et al.*, 1992; Dyer and O'Gorman, 2012). *A. nidulans* is among the exceptions where sufficient nutrition is needed for the initiation of cleistothecia formation (Han *et al.*, 2003; Dyer and O'Gorman, 2012). However, further research is needed to elucidate whether carbon starvation can block sexual development if it has already started. If not, autolytic processes could support sexual development with nutrients as was suggested in the case of asexual development.

Recycling responses to deal with carbon starvation

Under carbon starvation, fungi have two options to cope with this type of environmental stress: i) to recycle the biopolymers of old hyphal parts to provide energy for surviving cells (autolysis) or ii) to use other, non-easily degradable substrates as carbon source (*e.g.* plant materials). The recycling of cell contents is coupled tightly to the (controlled) death of old cells and proliferation of other cells, and includes the utilization of inner cell material by autophagy as well as the degradation of cell wall constituents by extracellular hydrolytic enzymes. In the next few paragraphs we will discuss in more detail the role of autophagy during starvation and the roles of glycosyl hydrolases (GH) during cell wall recycling and cell wall active enzymes involved in conidiation. We will also discuss the strategies employed by fungi to scout for non-easily degradable substrates that could be used as alternative C-sources.

Recycling responses: The role of autophagy in the carbon starvation response

To survive carbon starvation conditions and support the formation of reproductive structures and substrate exploring hyphae, endogenous recycling of nutrients has been proposed to be an important strategy (White *et al.*, 2002; Shoji *et al.*, 2006; Richie *et al.*, 2007; Shoji and Craven, 2011). As such, the autophagic pathway is one of the most dominantly induced processes during carbon limitation in filamentous fungi (Nitsche *et al.*, 2012; Szilágyi *et al.*, 2013; Krohn *et al.*, 2014; Ellström *et al.*, 2015). Autophagy is a non-selective catabolic pathway which targets cytoplasmic contents to the vacuole for degradation and recycling of the building blocks of the cell. It includes the sequestering of cytosolic components and organelles in double-membrane vesicles which subsequently fuse with the vacuole, releasing their contents.

The autophagy machinery is negatively regulated by the kinase activities of the target of rapamycin (Tor) and the cAMP-dependent protein kinase (PKA), which independently act through control of the interaction between the autophagy-related proteins Atg1 and Atg13 (Kamada *et al.*, 2000; Stephan *et al.*, 2009). Under nutrient-rich conditions, autophagy is blocked by Tor-mediated hyperphosphorylation of Atg13, which reduces its affinity for Atg1. Inhibition of Tor by nutrient starvation conditions reduces the phosphorylation state of Atg13, enabling it to form a complex with Atg1 and thereby initiating the autophagy

process. Both Atg1 and Atg13 are direct substrates for PKA, but its phosphorylation sites are distinct from those targeted by Tor (Stephan *et al.*, 2009). The concerted regulation of autophagy by Tor and PKA is rather complex and currently not fully understood. Responses are likely dependent on the nature of the limiting nutrients, providing the cell with more flexibility to adapt to changing environments (Dechant and Peter, 2008).

To date, more than 30 *atg* genes have been identified in yeast and filamentous fungi, which control the autophagic machinery (Feng *et al.*, 2014). Genome-wide transcriptional profiling of *A. niger* and *Paxillus involutus* has shown that the majority of these genes is upregulated under carbon starvation conditions (Nitsche *et al.*, 2012; Ellström *et al.*, 2015). In addition, extensive transcriptome analysis demonstrated the transcriptional induction of specific *atg* genes in *A. nidulans* (Szilágyi *et al.*, 2013). Analysis of *atg* deletion mutants in *Aspergillus* and *Penicillium* showed that phenotypic effects vary among different species. While the deletion of *atg1* severely reduced conidiation in *A. fumigatus* (Richie *et al.*, 2007) and *P. chrysogenum* (Bartoszewska *et al.*, 2011), conidiospore formation was only mildly reduced in the *A. niger* Δ*atg1* mutant (Nitsche *et al.*, 2013). Similarly, the effect on radial growth and conidiation upon disruption of *atg8* was less pronounced in *A. niger* (Nitsche *et al.*, 2013) than in *A. oryzae* (Kikuma *et al.*, 2006). In *A. nidulans*, the deletion of either *atg1, atg4, atg5, atg7* or *atg9* resulted in a deficiency in conidiation at 42°C (Pinar *et al.*, 2013). During submerged carbon starvation, *A. niger* Δ*atg1* and Δ*atg8* mutants showed growth profiles similar to wild-type, but the emergence of empty compartments and the formation of thin hyphae was clearly accelerated (Nitsche *et al.*, 2013). This suggests that autophagy promotes survival during carbon starvation by endogenous recycling of nutrients and protecting older hyphal compartments from cell death.

Recycling responses: GH specific for cell wall degradation

During carbon starvation, the fungal cell wall is thought to undergo drastic remodelling or degradation. Fungal carbohydrate-active enzymes are key effectors in these cell wall changes. The production of such hydrolytic activities in response to carbon starvation has been observed in a number of fungi (Jaroszuk-Scisel *et al.*; Lahoz *et al.*, 1976; Perez-Leblic *et al.*, 1982; McIntyre *et al.*, 2000; White *et al.*, 2002). Recent investigations of the transcriptome and proteome of carbon starving *A. niger* (Nitsche *et al.*, 2012; van Munster *et al.*, 2015b) and *A. nidulans* (Szilágyi *et al.*, 2013) cultures have provided an overview of the response of fungal cell wall active carbohydrate hydrolases.

The cell wall is a rigid, complex structure, which maintains the shape of the cell and is responsible for counteracting turgor pressure and for protecting the plasma membrane against mechanical damage. The fungal cell wall accounts for a considerable percentage of cellular biomass (about 20 %) and thus it represents a rich carbon source during carbon starving conditions. The fungal cell wall consists mainly of polysaccharides. For almost all

fungi, the central core of the cell wall is a ß-1,3-glucan with ß-1,6 branch points. Chitin is covalently bound to the non-reducing end of ß-1,3-glucan side chains via ß-1.4-linkages. The *Aspergillus* cell wall also contains α-1,3-glucan, galactomannan (α-1,2/α-1,6-linked mannan with short β-1,5-galactofuranose side chains), and galactosaminogalactan (a linear heterogenous polymer containing α-1,4-galactose/galactosamine) (Latgé, 2007). Cell walls also contain several heavily glycosylated proteins including GPI-modified cell wall proteins or hydrophobins (De Groot *et al.*, 2005).

Cell wall homeostasis undergoes a dramatic change during carbon starvation. For example, processes responsible for the synthesis of cell wall components are down-regulated (Szilágyi *et al.*, 2013). In *A. nidulans*, *fksA* encoding the catalytic subunit of ß-1,3-glucan synthase (Kelly *et al.*, 1996); *chsB* and *chsF* coding for III type chitin synthases (Horiuchi, 2009) and *agsB* encoding α-1,3-glucan synthase (Fujioka *et al.*, 2007) were down-regulated in carbon starving cultures (Szilágyi *et al.*, 2013). In both *A. niger* and *A. nidulans*, genes coding for GPI-anchored enzymes, which may function in the remodeling of rigid cell wall to a looser structure during growth–*e.g. chiA* in the case of chitin (Yamazaki *et al.*, 2008) or *eglC* in the case of ß-1,3-glucan (Choi *et al.*, 2005)–were also down-regulated during carbon starvation (Szilágyi *et al.*, 2013; van Munster *et al.*, 2015b).

In contrast to the down-regulation of cell wall synthesis, cell wall-degrading processes are upregulated in carbon starving cultures (Szilágyi *et al.*, 2013). In *A. nidulans* and *A. niger*, elevated levels of extracellular protease, ß-1,3-glucanase, ß-glucosidase, α-1,3-glucanase and chitinase were detected during carbon starvation (Zonneveld, 1974; Emri *et al.*, 2004; Szilágyi *et al.*, 2010a; Nitsche *et al.*, 2012) and genes encoding such autolysis-related hydrolytic enzymes were up-regulated (Nitsche *et al.*, 2012; Szilágyi *et al.*, 2013; van Munster *et al.*, 2015b). However, only a number of these enzymes have been investigated in detail.

The *A. nidulans* EngA plays a role in biomass decrease during autolysis (Szilágyi *et al.*, 2010a). Its ortholog in *A. fumigatus* has been characterized as an endo-acting β-1,3-glucanase, and was found to be associated with the fungal cell wall (Fontaine *et al.*, 1997; Mouyna *et al.*, 2002). *A. nidulans* EngA also has ß-1,3-endoglucanase activity, and its activity and stability at a broad pH range means that EngA preserves its activity even at alkaline pH values typical of late carbon starving cultures. EngA is the main secreted ß-1,3-glucanase in carbon starving cultures of *A. nidulans* and the deletion of *engA* confirmed the prominent role of EngA in cell wall degradation and autolysis. However, gene deletion did not abolish the ß-1,3-glucanase enzyme production (Szilágyi *et al.*, 2010a). Nuero et al. purified three enzymes with ß-1,3-glucanase activity from autolyzing cultures of *A. nidulans* confirming the existence of several enzymes with ß-1,3-glucanase activity in carbon-depleted cultures of *A. nidulans* (Nuero *et al.*, 1993).

High α-1,3-glucanase activities are characteristic for carbon starving cultures of *A. nidulans* (Zonneveld, 1974). Correspondingly, genes encoding enzymes with putative α-1,3-glucanase activity were up-regulated during carbon starvation in *A. niger* and *A. nidulans* (Nitsche *et al.*, 2012; Szilágyi *et al.*, 2013). One of these, *A. niger* AgnB, was characterized as α-1,3-glucanase which releases glucose from α-1,3-glucan substrates. However, no direct role for this enzyme in cell wall degradation could be identified (van Munster *et al.*, 2015a). In *A. nidulans*, which in contrast to *A. niger* has a sexual cycle, the α-1,3-glucanase MutA hydrolyzes cell wall material during sexual reproduction, possibly to generate energy that is needed for the production of the cleistothecia (fruiting bodies) (Zonneveld, 1972; Wei *et al.*, 2001). The complete hydrolysis of glucans is achieved by the synergistic action of endo- and exo-glucanases. The up-regulation of high affinity glucose transporters also supports the hypothesis that glucan monomers are taken and utilized by the surviving cells upon carbon starvation (Szilágyi *et al.*, 2013).

Chitinase ChiB is rate-limiting in biomass degradation during carbon starvation in *A. nidulans.* Deletion of *chiB* resulted in the loss of extracellular endochitinase producing capability and caused a non-autolysing phenotype, which was characterized by the lack of hyphal fragmentation and pellet disorganization in ageing carbon starving cultures (Yamazaki *et al.*, 2007; Pócsi *et al.*, 2009). Deletion of the homologous *A. niger cfcA* and *A. fumigatus chiB1* – also encoding the main chitinases induced during starvation – does not result in a change in biomass loss during starvation (Jaques *et al.*, 2003; van Munster *et al.*, 2015a); the physiological role of these enzymes thus appears to be somewhat different. However, the *A. niger* enzyme is responsible for cell wall degradation as deletion of the corresponding gene reduces hyphal fragmentation during starvation. *A. fumigatus* ChiB and *A. niger* CfcA hydrolyze chitin and chitin oligosaccharides by cleaving mainly chitobiose from the non-reducing substrate end, but can also perform a transglycosylation reaction (Escott *et al.*, 1998; Xia *et al.*, 2001; Jaques *et al.*, 2003; Lü *et al.*, 2009; van Munster *et al.*, 2015a). The *A. nidulans* ChiB appears to have similar activity (Erdei *et al.*, 2008). Chitobiose can be further hydrolysed to monomers by *N*-acetyl-ß-D-glucosaminidase enzymes. The *A. niger* and *A. nidulans nagA,* both encoding an *N*-acetyl-ß-D-glucosaminidase, were induced by carbon starvation (Pusztahelyi *et al.*, 2006; Nitsche *et al.*, 2012; Szilágyi *et al.*, 2013). The *A. nidulans nagA* disruptant strain was not able to grow on medium containing chitobiose as sole carbon source (Kim *et al.*, 2002), but there was no effect on autolysis. Instead, deletion reduced cell death (Shin *et al.*, 2009). Similar viability effects were observed for the *ΔengAΔchiB* strain (Szilágyi *et al.*, 2012), showing a possible link between cell wall-hydrolyzing enzymes to the initiation of cell death processes in old carbon starving cultures.

High extracellular hydrolase activities were demonstrated to have a strong antifungal activity against the producer itself (Szilágyi *et al.*, 2012). It means that living fungal cells need to protect themselves against the deleterious effects of these enzymes. To shed light

on the details of such self-protection remains a challenge for future research in this field. The transcriptionally regulated *mpkA* and *rlmA* genes, which encode key elements of the cell wall integrity stress signaling pathway (Fujioka *et al.*, 2007), were not up-regulated during carbon starvation. The clear overall repression of cell wall biosynthetic genes also rejects the idea that the cell wall integrity pathway would have a prominent role in self-protection (Szilágyi *et al.*, 2013). An alternative way for protection could be an increased production of the pigment melanin in the surviving cells. This could be an effective response against the deleterious effects of cell wall hydrolysing enzymes as melanin itself can inhibit autolytic chitinase and glucanase activities and, as a consequence, the enzymatic lysis of cells in *A. nidulans* (Kuo and Alexander, 1967).

Recycling responses: GH specific for conidiation

During periods of stress, such as during prolonged starvation, *Aspergillus* can survive by forming stress resistant spores. The formation of these spores requires energy and building blocks. In case of nutrient limitation, fungal autolysis and autophagy may make these nutrients available by recycling the cell wall or intracellular components. The regulatory machinery responsible for the initiation of sporulation is linked to the regulation of autolysis and is discussed in the following section on genetic control of the responses to carbon starvation.

Extensive morphological changes are taking place during sporulation, and the fungal cell wall needs to be adapted to enable these changes. This can be observed by changes in appearance (Adams *et al.*, 1998), as well as by the differences in carbohydrate composition of vegetative mycelium and spores (Maubon *et al.*, 2006). Spore walls of *A. niger* and *A. fumigatus* contain, compared to walls of vegetative hyphae, more mannose (25 % instead of 4-6 %) and galactose (12-14 % instead of 4-8 %), and less (N-acetyl)-glucosamine (6 % instead of 13 %) (Maubon et al., 2006; van Munster et al., 2013). In addition to a carbohydrate backbone, the spore cell walls contain an outer layer of melanin, which gives the spores their color and increases its resistance to environmental stress (Latgé *et al.*, 2005). CAZymes build and remodel this cell wall during sporulation. Sporulation-specific chitin synthases (Fujiwara *et al.*, 2000; Lee *et al.*, 2004) have been identified that contribute to these cell wall changes. Recently, sporulation-specific CAZymes that are predicted to be active on the fungal cell wall, were identified by comparing the expression profiles of glycoside hydrolases in wild-type and a strain carrying a deletion of the developmental regulator *brlA* (van Munster *et al.*, 2015b). These are a glycoside transferase from GT family 1, a protein containing only a CBM14 domain, the three β-glucan acting enzymes BgtD, An02g00850 and GelG, and the chitin acting CfcI, CtcB and an AA11 member.

Transcriptome analysis shows that the sporulation-specific glycoside hydrolases are strongly induced in sporulating aerial mycelium compared to vegetative mycelium (van Munster *et*

al., 2013). Using promoter fusion strains, activities of the promoters of *cfcI* and *ctcB* were located to the conidophores. These genes encode the chitinases CfcI and CtcB. Deletion of both genes resulted in accumulation of chitin in the spore cell wall, indicating that both enzymes have a function in modification of the cell wall during sporulation (van Munster *et al.*, 2013). CfcI is capable of hydrolyzing chitotriose and longer chitin oligosaccharides by cleaving off monomers, possibly in a processive mode. The enzyme acts on the reducing end of the oligosaccharide substrates. CtcB belongs to the phylogenetic subgroup of GH18 that only encodes endo-chitinases. Together these enzyme activities would thus be capable of degrading the chitin polymer to N-acetyl-glucosamine monomers (van Munster *et al.*, 2012, 2015c).

The *A. fumigatus* β-1,3-glucanosyltransferase Gel7, the ortholog of the sporulation-specific *A. niger* GelG, was found to be important during sporulation. Deletion of the *gel7* gene resulted in abnormal conidia formation. In addition, the enzyme is capable of compensating for cell wall effects generated by *gel1* and *gel2* deletion (Zhao *et al.*, 2014). Orthologs of the *A. niger* sporulation-specific An02g00850 have been characterized, the *A. fumigatus eng2* and *A. nidulans xgeA* hydrolyze soluble β-1,3-glucan with β-1,6-glucan branches as well as β-1,3:1,4-glucan (lichenan) (Bauer *et al.*, 2006; Hartl *et al.*, 2011). Deletion of *eng2* in *A. fumigatus* gave no phenotype during vegetative growth consistent with a possible (unexplored) role during sporulation (Hartl *et al.*, 2011).

Recycling responses: scouting for new C-sources

Next to recycling fungal cell components, another possibility for carbon-starved fungi is to find an external carbon source. One of the substrates that may be available is the recalcitrant cell wall from dead plants. Plant cell walls can contain the carbohydrates cellulose, hemicelluloses and pectins, as well as other components such as lignin and structural proteins. The exact plant cell wall composition is dependent on the plant species, but also varies between different plant structures and wall types. For example, the primary cell walls of grasses contain low amounts of pectin and relatively high amounts of the hemicelluloses glucuronoarabinoxylans and mixed-linkage glucans. For gymnosperms, the cell wall contains more xyloglucan, pectin and structural proteins (Vogel, 2008; Sarkar *et al.*, 2009). The structure of individual plant cell wall carbohydrates is also dependent on their origin (Scheller and Ulvskov, 2010). Plant cell walls thus constitute a carbon source that is very variable in composition, but in all cases, plant cell wall components are cross-linked and form a complex network that may be quite resistant to enzymatic degradation.

A. niger and *A. nidulans* are both metabolically versatile and are capable of growing on a large number of different carbon sources. Their genome encodes a large suite of CAZymes that are active on plant-derived polysaccharides, and a recent catalogue of the *A. niger* genome showed that its genome encodes enzymes that are predicted to be active on at

least 14 different plant cell wall polysaccharides (Andersen *et al.*, 2012). The response of *A. niger* to different lignocellulosic substrates seems to require a core set of enzymes, that is complemented by a set of genes that seem differentially expressed on different substrates (Pullan *et al.*, 2014), a strategy similar to that observed in *N. crassa* (Benz *et al.*, 2014).

The expression of the genes encoding all these enzymatic activities requires a big investment by the fungus, and thus needs to be carefully regulated. This regulation is mediated by the coordinated action of transcriptional repression and inducer-dependent transcriptional activation.

In the presence of sufficient amounts of easily metabolisable carbon sources such as glucose or xylose, carbon catabolite repression (CCR) inhibits transcription of genes that encode enzymes required for complex carbon sources (Ruijter and Visser, 1997; de Vries *et al.*, 1999). Similar to other filamentous fungi and yeast, in *A. niger* and *A. nidulans* CCR is mediated by the global transcriptional repressor CreA. In nutrient rich conditions, glucose is phosphorylated after uptake by the kinases GlkA and HxkA (Flipphi *et al.*, 2003). In *A. nidulans*, similar to in *S. cerevisiae*, a kinase can phosphorylate CreA, causing it to shuttle from the nucleus to the cytoplasm under de-repressing conditions. The presence of phosphorylated sugar inhibits this kinase via RAS-signaling, allowing non-phosphorylated CreA to remain in the nucleus under repressive conditions (Brown *et al.*, 2013). There, CreA can bind to two palindromic consensus sequences (5'-SYGGRG-3) in the promoter of genes (Cubero and Scazzocchio, 1994; Takashima *et al.*, 1996) and represses gene transcription. CreA influences other transcription factors, for example the XlnR transcriptional activators of genes encoding plant polysaccharide active CAZymes (de Vries *et al.*, 1999), as well as the transcription of individual CAZyme encoding enzymes.

To be transcribed to a high level, genes encoding plant polysaccharide active enzymes require activation by inducer-dependent transcriptional regulators, such as XlnR for hemicellulase and cellulase genes, or ClbR, ClrA, ClrB for cellulase genes (Glass *et al.*, 2013).

In carbon starvation conditions, low level transcription of a set of genes that encode plant polysaccharide-degrading enzymes has been detected in *A. niger* as well as in other fungi (Foreman *et al.*, 2003; Martens-Uzunova and Schaap, 2009; Coradetti *et al.*, 2012; Szilágyi *et al.*, 2013; van Munster *et al.*, 2014). These genes include for example *A. niger* endo-α-1,5-arabinanase encoding *abnA* and *abnC*, α-arabinofuranosidase encoding *abfB* and *abfC*, exo-polygalacturonase encoding *pgaX*, *pgxB*, several genes encoding beta-glucosidases and the exo-inulinase encoding *inuE*. The encoded enzyme activities are mainly predicted to be active on terminal residues of plant polysaccharides, or their corresponding oligosaccharides. The regulatory mechanism for the up-regulation of genes during carbon starvation is not known completely. However, for the cellobiohydrolase encoding *cbhB*, its

neighboring gene encoding a GH5 cellulase and also the α-arabinofuranosidase encoding *abfB*, low level up-regulation was detected not only under carbon starvation conditions, but also in a *creA* deletion mutant grown on glucose (Delmas *et al.*, 2012). This suggests that carbon catabolite derepression via CreA is responsible for the observed up-regulation.

In the culture filtrate of carbon-starved cultures, proteins corresponding to such transcripts have also been found (Braaksma *et al.*, 2010; Nitsche *et al.*, 2012; van Munster *et al.*, 2014, 2015b). The concerted action of these enzymes on plant polysaccharides results in the release of products that include monomers as well as small linear and branched oligosaccharides. These reaction products include known inducers for lignocelluloses degrading enzymes such as arabinose (van Munster *et al.*, 2014). This supports a model where carbon starvation allows low-level up-regulation of a subset of genes, which encode enzymes that are thought to perform a scouting role. These enzymes are thought to release inducers from any substrates detected in the environment, either alone or in coordination with any constitutively expressed plant polysaccharide-degrading enzymes. These inducers may be taken up by the fungus and induce the transcription of a full set of plant carbohydrate degrading enzymes through dedicated transcription factors. Correspondingly, the early responses of *A. niger* and *N. crassa* to carbon starvation overlap with their early response to wheat straw and Avicel respectively (Coradetti *et al.*, 2012; van Munster *et al.*, 2014). This indicates that an initial phase of carbon starvation is required, during which scouting enzymes may generate inducers and dedicated transcription factors are allowed to respond to inducers. Subsequently, gene transcription is activated to generate the full set of enzymes required for the degradation of lignocellulose.

Co-regulation of conidiogenesis and cell wall hydrolase production

In the previous sections we have shown that carbon stress induces complex morphological, developmental and physiological changes both in *A. nidulans* and *A. niger*. Many of these stress responses (*e.g.* hyphal fragmentation and cell wall hydrolase production on one hand and conidiophore formation and survival on the other) seem to be contradictory. The diversity of the stress responses as well as their antagonistic nature suggests the existence of a complex regulatory network in which overlaps and intensive cross talks among the different signaling pathways are responsible for their proper orchestration in these multicellular organisms.

A key step of conidiation in *A. nidulans* is the activation of *brlA* (Adams *et al.*, 1998; Park and Yu, 2012). BrlA is a transcription factor which is responsible for the induction of AbaA which further induces a third transcription factor WetA (Adams *et al.*, 1998; Park and Yu, 2012). These three transcription factors (the so-called central regulatory pathway of conidiogenesis) coordinates all the conidiation-specific gene expressions during the development of conidiophores and the maturation of spores (Adams *et al.*, 1998; Park

and Yu, 2012). BrlA (but not AbaA or WetA) is necessary for induction of *engA* and *chiB* during carbon stress (Pócsi *et al.*, 2009; Szilágyi *et al.*, 2010a). Deletion of or loss-of-function mutations in *brlA* inhibits the accumulation of extracellular chitinase and ß-1,3-glucanase activities in the fermentation broth and hinder hyphal fragmentation in carbon starved cultures (Pócsi *et al.*, 2009; Szilágyi *et al.*, 2010a). It is assumed that in some cells induction of *brlA* is followed by the activation of the whole central regulatory pathway of conidiogenes resulting in conidiophore development and conidia formation. In other cells, BrlA does not induce *abaA* (as was observed in submerged carbon starved cultures (Pócsi *et al.*, 2009)) and these cells are responsible for cell wall hydrolase production. Identification of factors preventing the complete activation of the central regulatory pathway after *brlA* induction needs further investigations. Successful escape of hyphae into the air with the help of hydrophobins induced by BrlA is one possible regulator of conidiogenesis versus cell wall hydrolase production at least in surface cultures.

Two heterotrimeric G-protein mediated signaling pathways, the FadA and GanB pathways, negatively influence *brlA* expression (Yu, 2006; Park and Yu, 2012). Activation of the FadA(Gα)-SfaD(Gβ)-GpgA(Gγ) heterotrimeric G protein modulate cAMP-protein kinase A signaling, repress *brlA*, negatively regulate secondary metabolism and maintain vegetative growth (Shimizu and Keller, 2001; Yu, 2006; Park and Yu, 2012). Dissociation of the GanB(Gα)-SfaD(Gβ)-GpgA(Gγ) complex also mediates cAMP-protein kinase A signaling, negatively regulates the central pathway of conidiogenesis and positively regulates germination and stress responses (Yu, 2006; Park and Yu, 2012). FluG is proposed to be a key positive activator of *brlA* in *A. nidulans* (Lee and Adams, 1994). Its activity somehow belongs to the extracellular formation of a diorcinol – dehydroaustinol adduct which triggers the activation of the pathway (Lee and Adams, 1994; Rodríguez-Urra *et al.*, 2012). Accumulation of FluG beyond a certain level inhibits the negative regulation imposed by SfgA on Flb (including FlbA) proteins and as a consequence activates *brlA* (Park and Yu, 2012). FlbA is a key regulator of the FadA pathway (Adams *et al.*, 1998; Park and Yu, 2012; Pusztahelyi and Pócsi, 2013), therefore activation of FluG signaling not only induces *brlA* but also decreases the negative effect of FadA signaling on *brlA* transcription (Adams *et al.*, 1998; Park and Yu, 2012; Pusztahelyi and Pócsi, 2013). Activity of FadA and GanB G-protein signaling is proposed to depend on nutritional conditions therefore, carbon starvation decreases their activity, hinders vegetative growth and allows *brlA* to be induced. Carbon stress-dependent activation of *brlA* cannot be explained exclusively by the decreased activity of heterotrimeric G-protein pathways. Mutations in the genes involved in FadA or GanB signaling had only negligible effect on chitinase production and hyphal fragmentation in carbon stressed cultures (Molnár *et al.*, 2004, 2006). Since loss of function mutation in *fluG* (but not in *flbA*) inhibited the carbon starvation induced activation of *engA* and *chiB* (Molnár *et al.*, 2004; Emri *et al.*, 2005; Pócsi *et al.*, 2009; Szilágyi *et al.*, 2010a) a carbon stress-dependent behavior of FluG signaling is assumed.

Elements of the sexual development are also known to influence *brlA* expression, *e.g.* NsdD; an activator of sexual development (Lee *et al.*, 2014) and MpkB MAP kinase; an upstream regulator of NsdD (Kang *et al.*, 2013), both negatively regulate *brlA* expression. At the same time, BrlA has also been reported to represses *steA,* which encodes another activator of sexual development (Vallim *et al.*, 2000), indicating that BrlA has a central role in the developmental choices. Moreover, Kang *et al.,* (Kang *et al.*, 2013) showed the negative effect of MpkB not only on conidiogenesis but also on cell wall hydrolase production during long cultivation times which might also be controlled via BrlA.

Atoui et al. (Atoui *et al.*, 2010) demonstrated that appropriate glucose concentration can modify the light-dependent intracellular localization of VeA which is an important element of the light dependent regulation of conidiogenesis (including *brlA* and *fluG* transcription), sexual development, and secondary metabolism (Etxebeste *et al.*, 2010; Bayram and Braus, 2012; Park and Yu, 2012). This observation suggests a further possible way for carbon source-dependent modulation of differentiation, secondary metabolism and cell wall hydrolase production.

RlmA is also a negative regulator of *brlA* and represses *engA* and *chiB* transcription right after glucose depletion (Pusztahelyi and Pócsi, 2013). Since RlmA is supposed to be involved in cell wall stress response (Fujioka *et al.*, 2007), this connection between hydrolase production and cell wall stress may represent a negative feedback regulation of cell wall hydrolase production preventing overproduction of these enzymes which would be dangerous for the producer cells.

Regulation of cell wall hydrolase production depends not only on BrlA, but other regulatory mechanism including the carbon catabolite repressor CreA (Emri *et al.*, 2006). Positive regulatory effect of mono- and oligosaccharides released during cell wall hydrolysis is also emphasized (Szilágyi *et al.*, 2010a). The CreA-dependent repression of certain cell wall hydrolases (e.g. *chiB*, but not *engA*) in the presence of repressive carbon sources during carbon starvation may explain why cell wall hydrolase production depends strongly on carbon stress while in case of conidiogenesis nutritional stress is only one and probably not the most important signal for induction (Park and Yu, 2012).

Regulation of extracellular protease formation during carbon starvation

The induction of proteases has also been proposed as a key event in aging fungal cultures (White *et al.*, 2002). The production of extracellular proteases is a part of a general stress response aimed at scavenging new energy sources for survival in carbon starving cultures. The proteases may be involved in the degradation of extracellular proteins and/or the protein content of the cell wall. For industrial relevant fungi, such as *A. niger*, this is relevant in relation to the production of (recombinant) protein in this fungus. High protease activity

during starvation has a negative effect on the yield of product formation because of the unwanted proteolytic degradation of the product (Braaksma *et al.*, 2009). In *A. niger*, Nitsche et al., identified several proteases that are highly induced during starvation (Nitsche *et al.*, 2012). These proteases included *pepA* and *pepB* which encode the major extracellular proteases. Regulation of protease expression in *A. niger* involves the Zn(II)2Cys6 transcription factor PrtT (Punt *et al.*, 2008), but the expression of protease encoding genes is also under control of carbon catabolite repression (van den Hombergh *et al.*, 1997a). The expression of the PrtT transcription factor is also strongly induced upon starvation (Nitsche *et al.*, 2012). PrtT-homologues are only present in a few other *Aspergilli*. Interestingly, *A. nidulans* does not have a PrtT homolog and the protease regulation seems to be fundamentally different in comparison with *A. niger.*

The genome of *A. nidulans* contains several genes encoding putative extracellular proteases which are up-regulated by carbon starvation (Szilágyi *et al.*, 2013; Katz *et al.*, 2013). These include *prtA* and *pepJ* encoding the two major extracellular proteases of this fungus (Katz *et al.*, 1994; Emri *et al.*, 2009; Szilágyi *et al.*, 2011). The p53-like transcription factor XprG was identified as a major positively acting transcription factor of extracellular protease formation in carbon starved cultures of *A. nidulans* (Katz *et al.*, 1996, 2006). Microarray data demonstrates that XprG regulates (directly or indirectly) a large number of genes related to carbon stress including secondary metabolism, development or high-affinity glucose uptake (Katz *et al.*, 2013). It also activates hydrolases other than proteases: It proved to be important in the carbon starvation dependent activation of *engA* and *chiB* most likely via the activation of BrlA (Katz *et al.*, 2013), regulates the carbon stress dependent induction of *agdB* encoding a putative α-glucosidase (Katz *et al.*, 2013) and the production of acidic phosphatase in response to phosphate limitation (Katz *et al.*, 2006). These data suggest that XprG can be a key regulator of responses to starvation (Katz *et al.*, 2013). Little is known of the regulation of XprG. It is well documented that carbon starvation induces it (Szilágyi *et al.*, 2013; Katz *et al.*, 2013). HxkC, HxkD and AtmA are negative regulators of XprG (Bernardo *et al.*, 2007). HxkC and HxkD are regulatory type hexokinases; HxkC is associated with mitochondria, while HxkD is a nuclear protein (Bernardo *et al.*, 2007). AtmA is a serine/threonine protein kinase, an ortholog of mammalian ataxia-telangiectasia mutated (ATM) kinase which acts as a redox sensor controlling mitochondrial function (Ditch and Paull, 2012). Deletion of *atmA* in *A. nidulans* has very pleiotropic effects. AtmA is involved in the DNA damage response and required for polarized hyphal growth (Malavazi *et al.*, 2006, 2007, 2008). Among carbon starving conditions AtmA is necessary for normal transcription of genes involved in autophagy (Krohn *et al.*, 2014), it may contribute in the selective clearance of damaged mitochondria which prevent accumulation of reactive oxygen species (Krohn *et al.*, 2014), genetically interacts with XprG inhibiting XprG functions (Krohn *et al.*, 2014) and it is necessary for induction of certain carbohydrate active enzymes (CAZy) but acts independently to CreA (Brown *et al.*, 2013). CreA also negatively regulates

extracellular protease formation during carbon starvation (Emri *et al.*, 2006; Katz *et al.*, 2008). It is suggested that CreA regulates *xprG* expression or interacts directly with the XprG protein (Katz *et al.*, 2008).

FluG is necessary for extracellular protease formation in carbon starved cultures; no extracellular protease activity was detected in a loss-of-function *fluG* mutant (Emri *et al.*, 2005; Szilágyi *et al.*, 2011). BrlA is also a positive regulator of protease formation, however its action depended on the experimental design: when growing cultures were transferred into carbon source free media, inactivation of *brlA* (or *fluG*) decreased markedly the extracellular protease activity. In contrast, when carbon starvation was induced simply by long time cultivation, *brlA* deletion had only a minor effect, while in the *fluG* loss-of-function mutant no proteinase activity was detected (Szilágyi *et al.*, 2011). These data suggest that FluG may act not only by regulating *brlA* transcription. Since mutations in FadA and GanB signaling have modulatory effect on protease production, it is possible that FluG, at least in parts, regulates the protease genes via FlbA and heterotrimeric G protein signaling (Molnár *et al.*, 2004, 2006). A connection between FluG and XprG is also possible but this hypothesis needs further investigation.

Although the presence of extracellular proteins is not needed for the induction of protease genes in carbon starving cultures (Katz *et al.*, 2008), the substrate of these enzymes can influence indirectly their production. High amount of organic nitrogen sources can repress the formation of proteases (as well as cell wall hydrolases) which depends on the MeaB transcription factor (Szilágyi *et al.*, 2010b; Spitzmüller *et al.*, 2015). The alkalization of pH by the release of ammonia during carbon starvation also supports the role of protein degradation in carbon-starving cultures (Emri *et al.*, 2004). It is worth mentioning that PrtA, PepJ, EngA and ChiB preserves their activity in alkaline conditions that shows that the fungus is adapted to the alkaline environment during carbon starvation (Katz *et al.*, 1994; Erdei *et al.*, 2008; Emri *et al.*, 2009; Szilágyi *et al.*, 2010a). Moreover alkaline pH is important in the up-regulation of extracellular protease and secondary metabolite production partly by the activity of PacC (Brakhage *et al.*, 2004; Szilágyi *et al.*, 2011).

Conclusions and future trends

The lack of available carbon source can be life-threatening for fungi as maintenance of crucial cellular processes such as ion homeostasis requires ATP. A lack of ATP could consequently result in rapid cell death. Endogenous recycling (autolysis) and extracellular hydrolysis are ways to liberate energy-rich building blocks to generate ATP under carbon starvation conditions. In filamentous fungi, the energy obtained from recycling processes is not only used for maintenance, but also used to fuel asexual development (Figure 1).

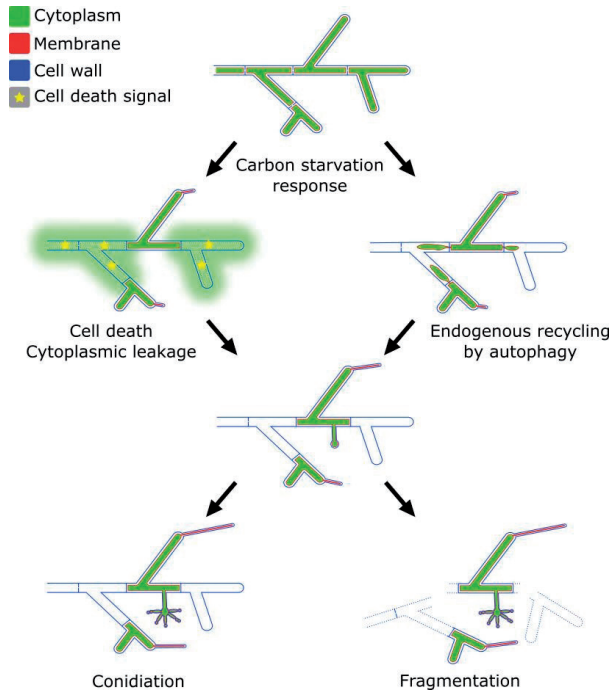

Figure 1 | Model for carbon starvation responses in filamentous fungi. Schematic representation of morphological starvation responses during submerged carbon starvation. Upon carbon starvation, empty hyphal compartments emerge as a consequence of cell death and/or endogenous recycling of neighbouring compartments by autophagy. Cell death of compartments results in the leakage of cytoplasm into the culture broth, whereas this is prevented when cellular contents are endogenously recycled by autophagic processes. Liberated carbon sources are reused to fuel the outgrowth of thin hyphae. Towards late phases of carbon starvation, only a few surviving compartments are left in the culture broth, often bearing reproductive structures and elongated thin hyphae. Depending on the species and the cultivation conditions, the largely empty mycelial network either remains intact or is being fragmented by the activity of glycosyl hydrolases weakening the cell walls (amended and adopted from Nitsche *et al.* 2012).

The formation of conidiospores might assist in the propagation of the fungal species as conidia are much more resistant to unfavourable conditions (including starvation) and allow spreading of the species. Many authors have referred to carbon starvation response as autolysis (White *et al.*, 2002). However, autolysis suggests a rather uncontrolled and destructive process. In fact, the carbon starvation response seems to be a sophisticated manner to deal with the limitation and to search for alternative sources and to maintain viability of the species via reproduction. Several studies have indicated that the process of autophagy plays an important role in the starvation response. Preliminary studies have shown that autophagy is important to delay cell death and as such allows adaptation of the fungus to the carbon starvation condition (Nitsche *et al.*, 2013). However, the carbon

starvation-related phenotypes of autophagy defective mutants are subtle, indicating that additional processes or pathway are also contributing.

An important challenge for future research will be to understand the molecular mechanisms driving the multi-cellular nature of a carbon starved mycelium. When exposed to carbon starvation, the fungal mycelium starts to differentiate and initially thin hyphae are formed (Figure 1). These hyphae might be considered as scouting hyphae and might produce new enzymes with interesting biochemical properties to look for alternative carbon sources. During starvation, some compartments in the mycelium are protected from cell death, while other compartments seem to be sacrificed. It is currently unknown whether the cell content of empty compartments leaks into the medium or that neighbouring compartments can directly take up the cell content via the septal pore. Further understanding the destination and function of different cell types (thin hyphae, sacrificed compartment, surviving hyphae and hyphae that form conidiospores) may be assisted by recent tool developments for single cell/compartment transcriptomics and by identifying molecular signals and proteins related to programmed cell death or cell surviving. Identification of these molecular signals to promote fungal cell death or protect cells from dying in response to starvation conditions are also very interesting targets for antifungal drug development.

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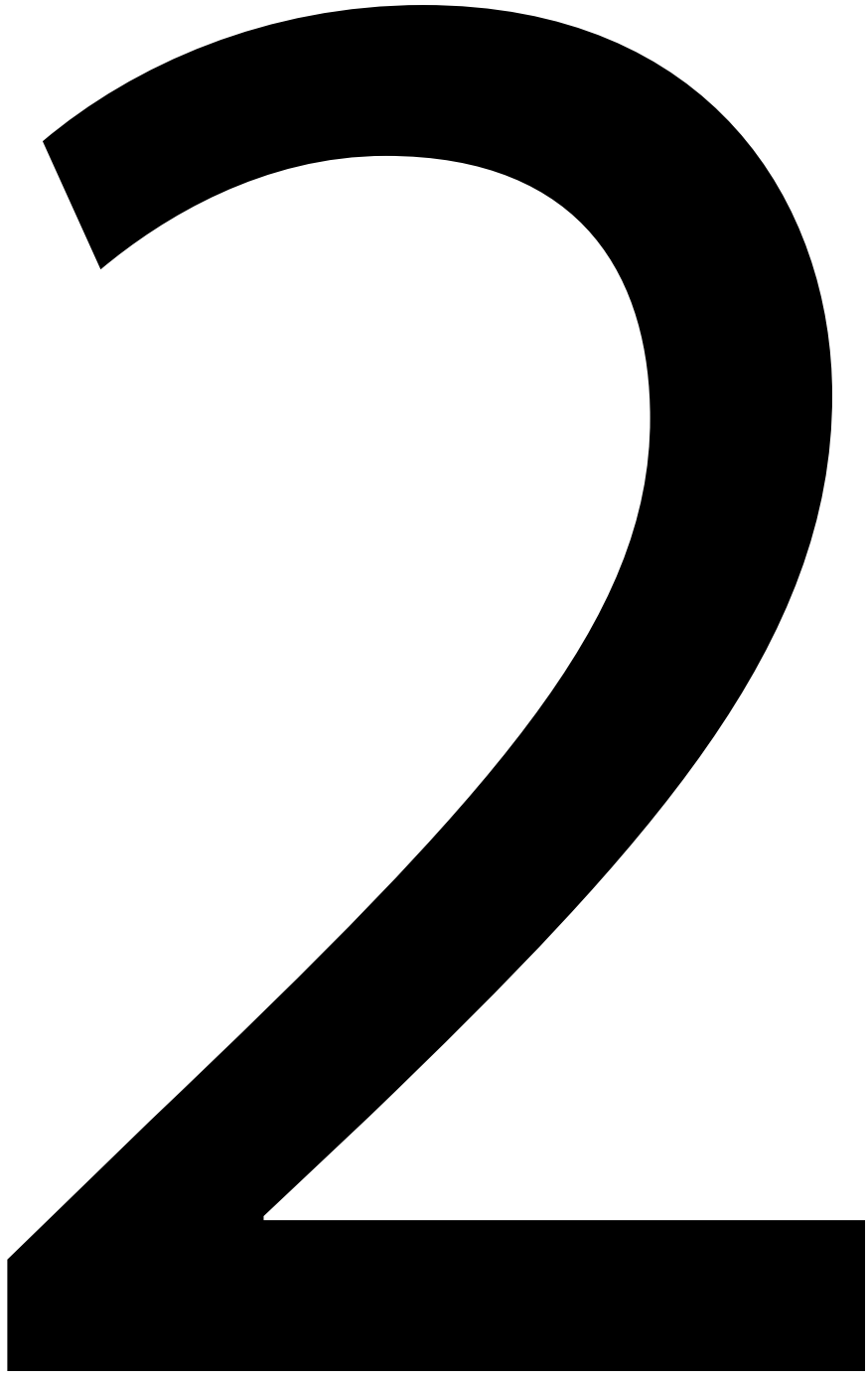

Chapter 2

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 targeting of cytoplasmic content and organelles to vacuoles. Autophagy is strongly induced by 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 (Nitsche 2012). 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.

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 (Archer, 2000; Pel *et al.*, 2007; Andersen *et al.*, 2011). 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 (Mattern *et al.*, 1992; Peberdy, 1994; MacKenzie *et al.*, 2005; Braaksma *et al.*, 2009; Carvalho *et al.*, 2012), 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 (Inoue and Klionsky, 2010; Bartoszewska and Kiel, 2011). During autophagy, cellular components are sequestered and transported to lytic compartments in double-membrane vesicles, termed autophagosomes. 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 (Xie and Klionsky, 2007; Kanki *et al.*, 2011). One key player controlling the levels of autophagy is the autophagy-related protein Atg1, which is a serine/threonine protein kinase (Inoue and Klionsky, 2010; Bartoszewska and Kiel, 2011). 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 (Kabeya *et al.*, 2005; Cheong *et al.*, 2008) (Cheong 2008; Kabeya 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 (PE) forming an essential component of autophagic vesicle membranes (Inoue and Klionsky, 2010; Bartoszewska and Kiel, 2011). The *atg8* gene has also been deleted in the filamentous fungus *A. oryzae* resulting in mutants that were defective in autophagy and did not form aerial hyphae 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 (Shoji *et al.*, 2006; Richie *et al.*, 2007; Shoji and Craven, 2011). The hyphae that are formed during this starvation-induced (cryptic) re-growth 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.

Materials and Methods

Strains, media and molecular techniques

Aspergillus strains used (see Table 1) were grown at 30°C on solidified (20 $g \cdot l^{-1}$ agar) nitrate minimal medium (MM) (Bennett and Lasure, 1991) or complete medium (CM) containing 0.5% (w/v) yeast extract and 0.1% (w/v) casamino acids in addition to MM. The pH of 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, 0.5 g $MgSO_4 \cdot 7H_2O$ 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. Transformation of *A. niger* was performed as previously described (Meyer *et al.*, 2010). Hygromycin resistant transformants were isolated from plates supplemented with 200 $\mu g \cdot ml^{-1}$ hygromycin and 50 $\mu g \cdot ml^{-1}$ caffeine and subsequently purified on plates with 100 $\mu g \cdot ml^{-1}$ hygromycin. Phleomycin resistant transformants were isolated and purified on plates supplemented with 100 $\mu g \cdot ml^{-1}$ phleomycin. Southern analysis was performed as described by Sambrook and Russell (2001)

Table 1 | *Aspergillus* strains used in this study

Strain name[1]	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*::*N-citA*::*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)

[1] Accession numbers for public strain repositories are indicated in brackets

using either [α-32P]dATP labeled probes synthesized with the Rediprime II DNA labelling System (Amersham Pharmacia Biotech) or DIG labeled probes generated by PCR with the PCR DIG Probe Synthesis Kit (Roche Applied Science). 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 constitutive expression of mitochondrially targeted GFP was constructed as follows. A 1.1 kb *NcitA*::*gfp* fragment was PCR amplified from genomic DNA of the *A. nidulans* strain SRS29 (Suelmann 2000), blunt-end ligated into pJET1.2 (Fermentas) and sequenced. Subsequently, the fragment was excised using *Bgl*II and *BamH*I restriction enzymes and ligated into the 3.5 kb *Bgl*II-*BamH*I 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 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 (Hartingsveldt 1987). Single copy integration at the *pyrG* locus was confirmed by Southern analysis according to

the method previously described (Meyer *et al.*, 2010) (see Figure S1). 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, 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) 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 *Nhe*I-*Xba*I fragment 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 analysis (see Figure S2) giving the stains 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 template and primer pairs as indicated in Table S1. 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 *et al.* (2011a) using primers indicated in Table S1, blunt-end ligated into pJet1.2 and sequenced. Subsequently, the *amdS* cassette was isolated as an *Xho*I-*Hind*III 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(+) harbouring 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 analysis (Figure S4) 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 mitochondrial 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 analysis (see Figure S3) 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) holding 5 L sterile synthetic medium were inoculated with $5 \cdot 10^9$ conidia. During cultivation, the temperature was set to 30°C and pH 3 was maintained by addition of titrants (2 M NaOH, 1 M HCl). The supply of sterile air was set to 1 $l \cdot min^{-1}$. 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 six hours 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 (PPG) 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, Netherlands). Dissolved oxygen tension (DOT) and pH were measured electrochemically with autoclavable sensors (Mettler Toledo). 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 (Whatmann). 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 Lactophenolblue solution (Fluka). Per sample, a minimum of 40 micrographs were taken using a 40x objective and an ICC50 camera (Leica). 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 2012) for the open source program ImageJ (Abràmoff 2004) was used. DIC and fluorescence images were taken with a Zeiss axioplan 2 imaging microscope equipped with DIC optics. For the GFP settings, an epi-fluorescence filter cubeXF 100-2 with excitation 450-500 nm and emission 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 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, orthologs of three genes known to encode essential components of the autophagic machinery in *S. cerevisae* (Tsukada and Ohsumi, 1993; Matsuura *et al.*, 1997; Kabeya *et al.*, 2005; Cheong *et al.*, 2005) 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 2010; Arnaud 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 analysis (see Figure S2) 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 and Kiel, 2011) have demonstrated 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 vacuolar localization of cytosolic GFP (see Figure 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 Figure 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 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.

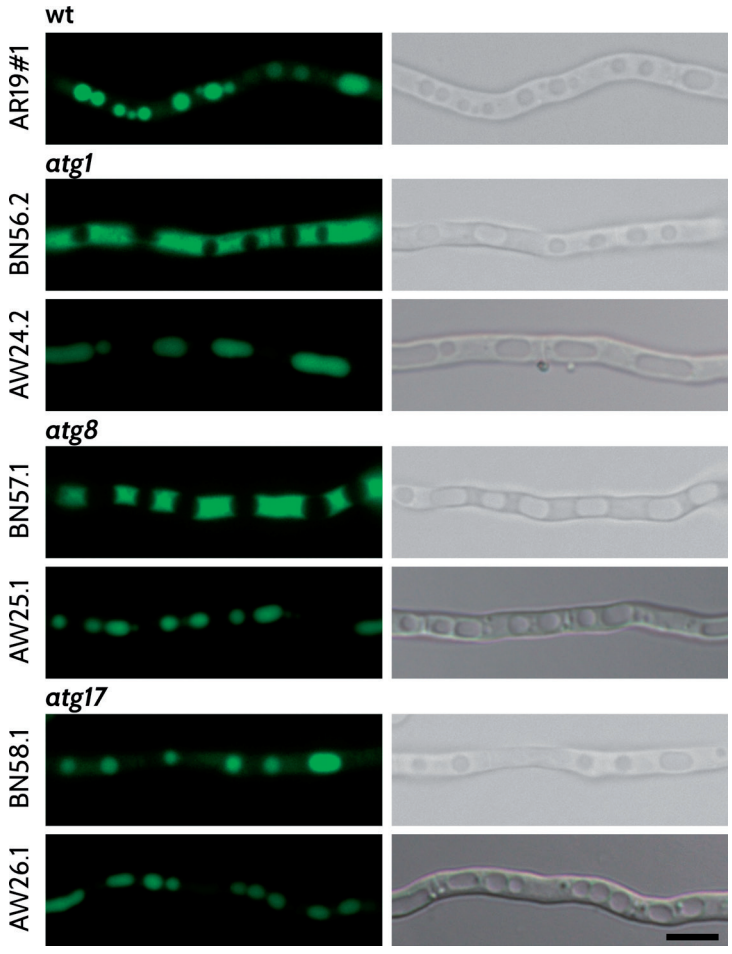

Figure 1 | Localization of cytosolically expressed GFP during carbon starvation. The strains were pre-grown for 8 hours at 30°C on coverslips in Petridishes with liquid MM. Subsequently, coverslips with adherent hyphae were washed and transferred to MM without carbon source. Micrographs were taken 40 hours 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.

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 for visualization of mitochondria. 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 (data not shown). During carbon starvation however,

microscopic analysis indicated considerable differences in mitochondrial morphology (see Figure 2). The phenotypes correspond to those observed for the localization of cytosolically targeted GFP (see Figure 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 (Kikuma *et al.*, 2006; Richie *et al.*, 2007; Bartoszewska and Kiel, 2011). 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

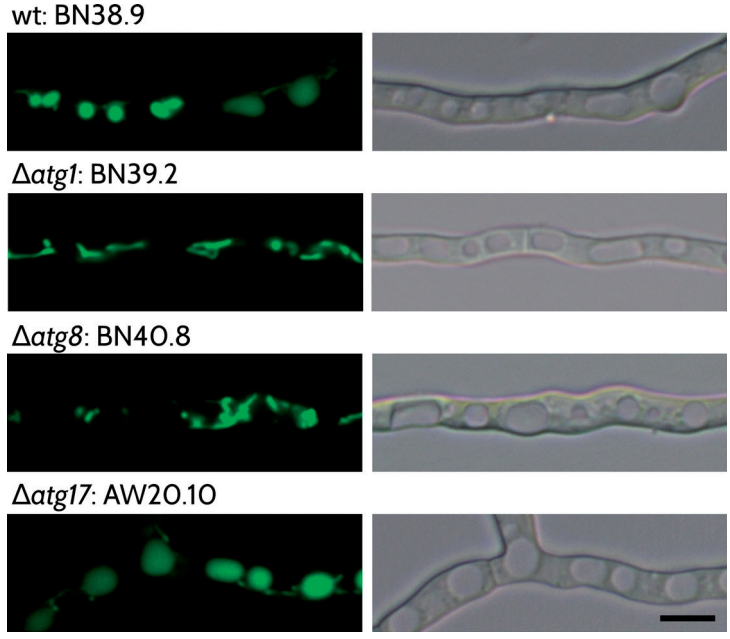

Figure 2 | Localization of mitochondrially expressed GFP during carbon starvation. The strains were grown as described in Figure 1. Wild-type (strain BN38.9) and Δ*atg17* mutant (strain AW20.10) showed vacuolar localization of mitochondrially targeted GFP upon carbon starvation, whereas both Δ*atg1* 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) (data not shown). Scale bar: 5 µm.

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 Figure 3A) indicating reduced spore densities and/or differences in melanization of spores. Indeed, the amount of spores recovered from the colonies was significantly reduced (see Figure 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 Figure 1) or mitochondrial GFP (see Figure 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 to studies in *A. oryzae* (Kikuma *et al.*, 2006), colonies of the Δ*atg8* strain showed slower radial growth on MM (see Figure 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 Figure 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.

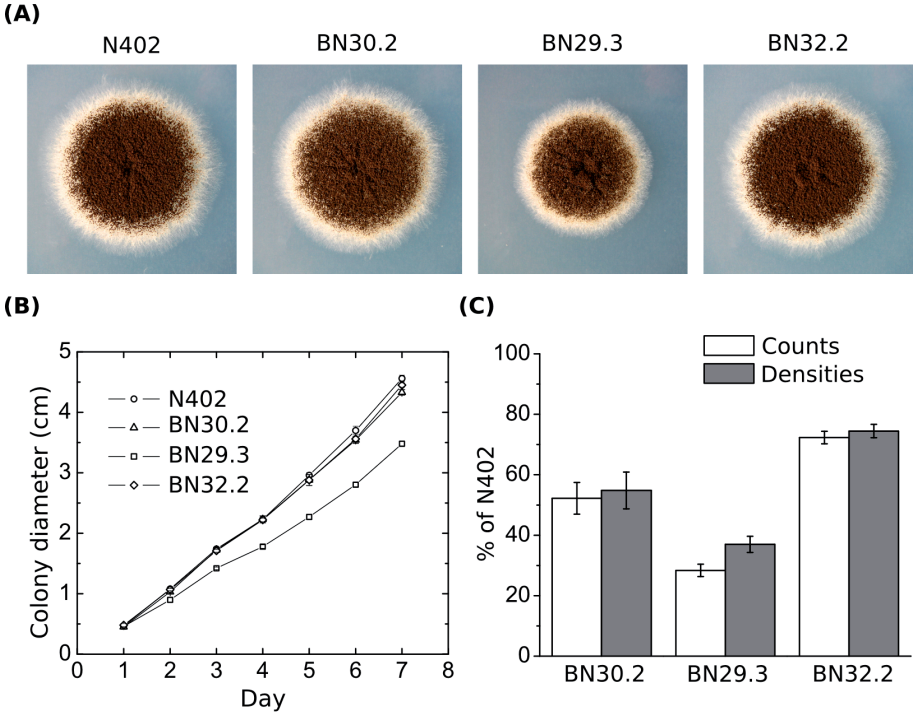

Figure 3 | Conidiation and colony expansion. Wild-type (strain N402) and the Δ*atg1*, Δ*atg8* and Δ*atg17* mutants (strains BN30.2, BN29.3 and BN32.2, respectively) were grown for 7 days on solid MM at 30°C. (A) Colony appearance; (B) Colony diameters (n=3); (C) Recovered conidia (n=3) including spore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

In filamentous fungi, autophagy has been suggested to contribute to nutrient recycling along the mycelial network promoting foraging of individual substrate exploring hyphae and conidial development (Shoji *et al.*, 2006; Richie *et al.*, 2007; 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 investigated in comparison to the wild-type under nitrogen and carbon limitation on solid MM (see Figure 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 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 results shown in Figures 1, 2 and 3A, deletion of *atg17* results in a phenotype that is indistinguishable from that of the wild-type strain N402.

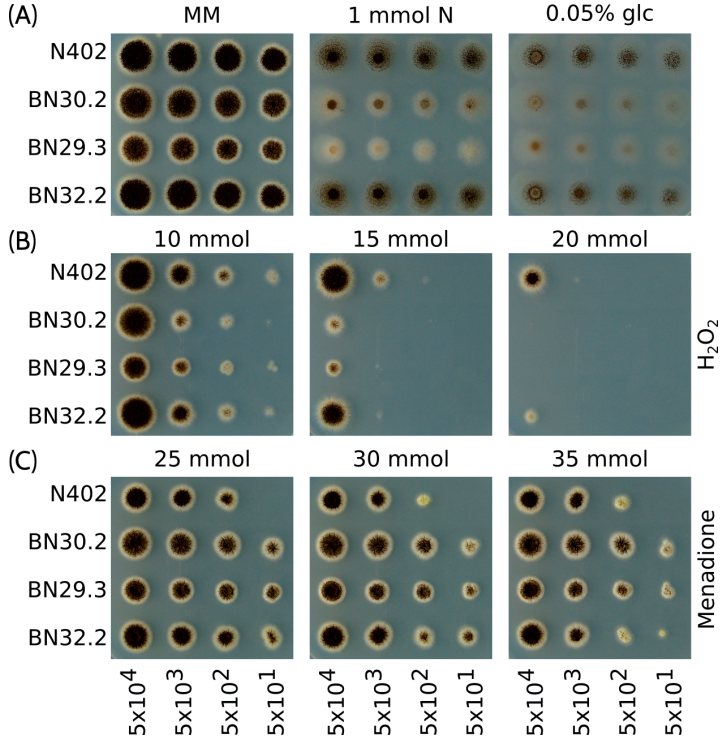

Figure 4 | Sensitivity assay of Δ*atg1*, Δ*atg8*, Δ*atg17* and wild-type strains (strains BN30.2, BN29.3, BN32.2 and N402, respectively). 10-fold dilutions ($5{\cdot}10^4$–50 conidia) were spotted on plates with (A) MM and MM with N (nitrate) or C (glucose) limitation as well as MM supplemented with (B) H_2O_2 or (C) menadione. Plates were incubated for 4 days at 30°C.

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; Thorpe *et al.*, 2004; Tucker and Fields, 2004; Pócsi *et al.*, 2005), are shown in Figure 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. Complementation studies showed that all described oxidative stress related phenotypes could by partly restored upon expression of the corresponding wild-type gene (Figure S5).

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.

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 (Shoji *et al.*, 2006; Richie *et al.*, 2007; 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 six days after carbon depletion (see Figure 5).

The application of bioreactor technology allowed highly reproducible culture conditions. Monitoring of 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

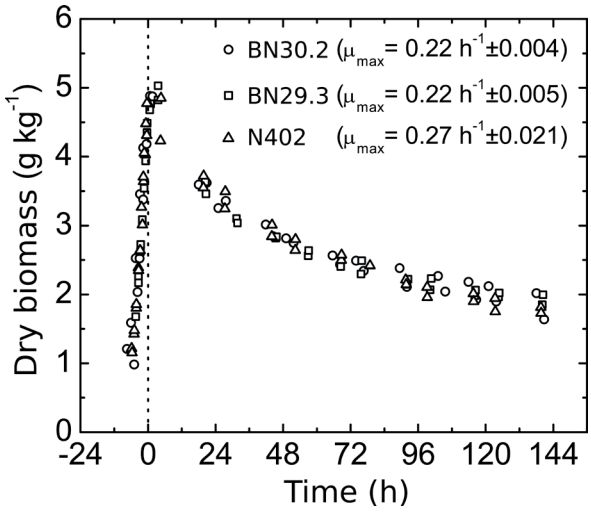

Figure 5 | Carbon starvation in submerged batch cultures. Duplicate biomass profiles of the wild-type (strain N402), the Δ*atg1* and Δ*atg8* mutants (strains BN30.2 and BN29.3, respectively) are shown. The time points of carbon depletion were set to 0 hours and used for synchronization of (replicate)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

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 Figure 3A-B), the maximum specific growth rates for both mutants during exponential growth were affected to the same extend. The mutants grew slower (μ_{max}=0.22h^{-1} ± 0.005) than the wild-type (μ_{max}=0.27h^{-1} ± 0.021). The biomass profiles did not show any considerable differences during the post-exponential phase (see Figure 5).

Flow chamber experiments with *A. oryzae* (Pollack *et al.*, 2008) have shown that carbon depletion induces 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 of 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, the Δ*atg1* and Δ*atg8* strains from carbon limited bioreactor batch cultures were analyzed accordingly (see Figure 6). During exponential growth (day 0), all three strains displayed single populations of thick 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,

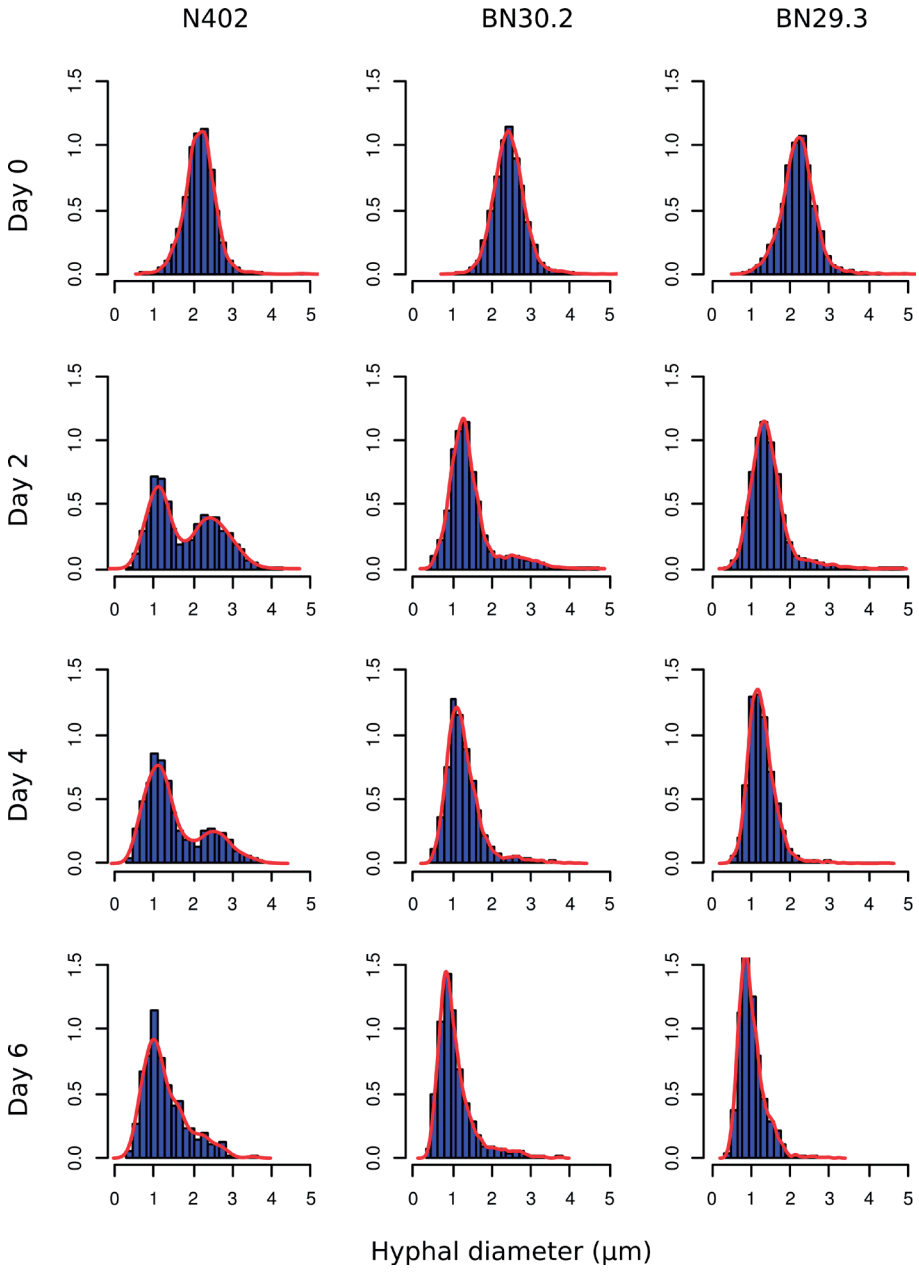

Figure 6 | Hyphal diameter populations. Population dynamics of hyphal diameters for the wild-type (strains N402), the Δ*atg1* and Δ*atg8* mutants (strains BN30.2 and BN29.3, respectively) are shown. Micrographs (≥40) of dispersed hyphae were analyzed by automated image analysis per strain and time point.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

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*atg*1) and BN40.8 (Δ*atg8*) with constitutive expression of mitochondrially targeted GFP (see Figure 2) were cultured in carbon limited bioreactor batch cultures and monitored by fluorescent microscopy. Hyphae from the exponential growth phase showed fluorescent tubular structures resembling those described by Suelmann et al. (2000) and no difference in mitochondrial morphology was observed between the three strains (see Figure 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 Figure 7B-C. The density of mitochondrial structures decreased in the wild-type hyphae but accumulated in the intervacuolar space for both mutants. Further, there were considerable differences in the mitochondrial morphology. Remaining mitochondrial structures were largely tubular in the wild-type, while they appeared as fragmented and punctuated structures in the mutants.

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 (Zustiak *et al.*, 2008; Bartoszewska and Kiel, 2011). For different filamentous fungi, several studies have described the phenomena of carbon starved submerged cultures (White *et al.*, 2002; Emri *et al.*, 2004, 2005, 2006). 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 (McNeil *et al.*, 1998; McIntyre *et al.*, 2000; Emri *et al.*, 2005) 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

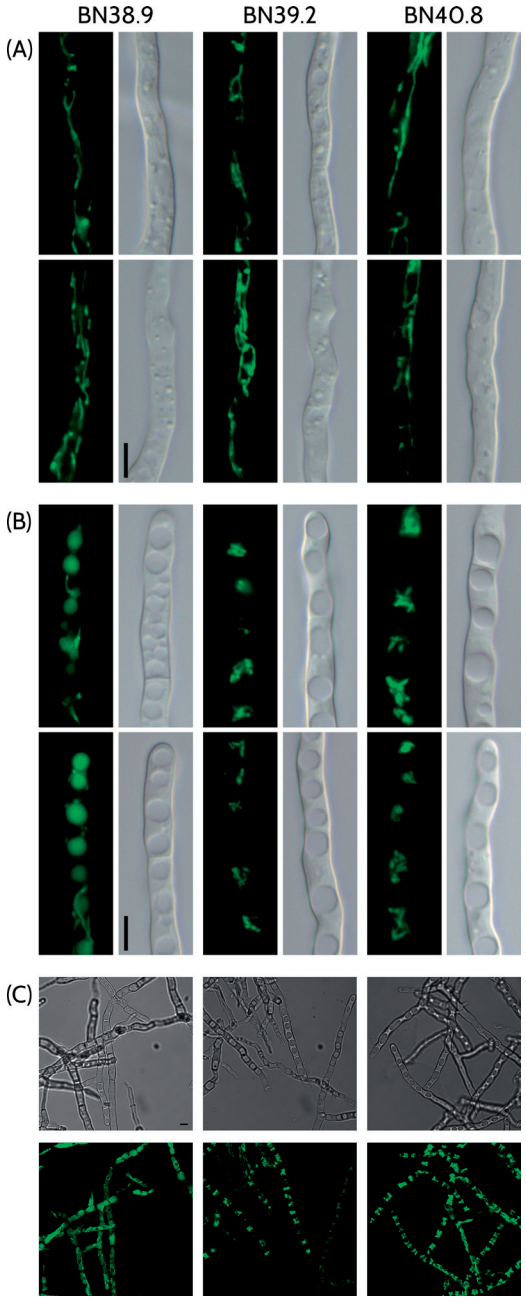

Figure 7 | Localization of mitochondrially targeted GFP during submerged carbon starvation. Constitutive expression of mitochondrially targeted GFP in the wild-type (strain BN38.9), the Δ*atg1* and Δ*atg8* mutants (strains BN39.2 and BN40.8, respectively) during carbon limited batch cultures. Differential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

attained much attention yet. In a recent systems 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 to 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* (Kabeya *et al.*, 2005; Cheong *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 Figure 3) and intermediate phenotypes in response to oxidative stress (see Figure 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 (Shoji *et al.*, 2006; Richie *et al.*, 2007; Shoji and Craven, 2011). Similar to studies with other filamentous fungi (Kikuma *et al.*, 2006; Richie *et al.*, 2007; Bartoszewska and Kiel, 2011), impairment of autophagy in *A. niger* considerably reduced conidiation (see Figure 3), a phenotype, which was much enhanced by carbon and nitrogen starvation (see Figure 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 *A. 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 condidiophore stalks are short.

In addition to its role in nutrient recycling, autophagy has been shown to be associated with programmed cell death (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 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 reactive oxygen species (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 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 at 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). Further, adaptive responses to oxidative stress induced by sublethal concentrations of exogenous oxidants have been demonstrated to protect yeast cells against higher lethal concentrations (Jamieson, 1992; Fernandes *et al.*, 2007). 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 Figure 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, Δ*atg1* and Δ*atg8* reporter strains with GFP-labeled mitochondria revealed that degradation of 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 turnover of excessive mitochondria.

Acknowledgements

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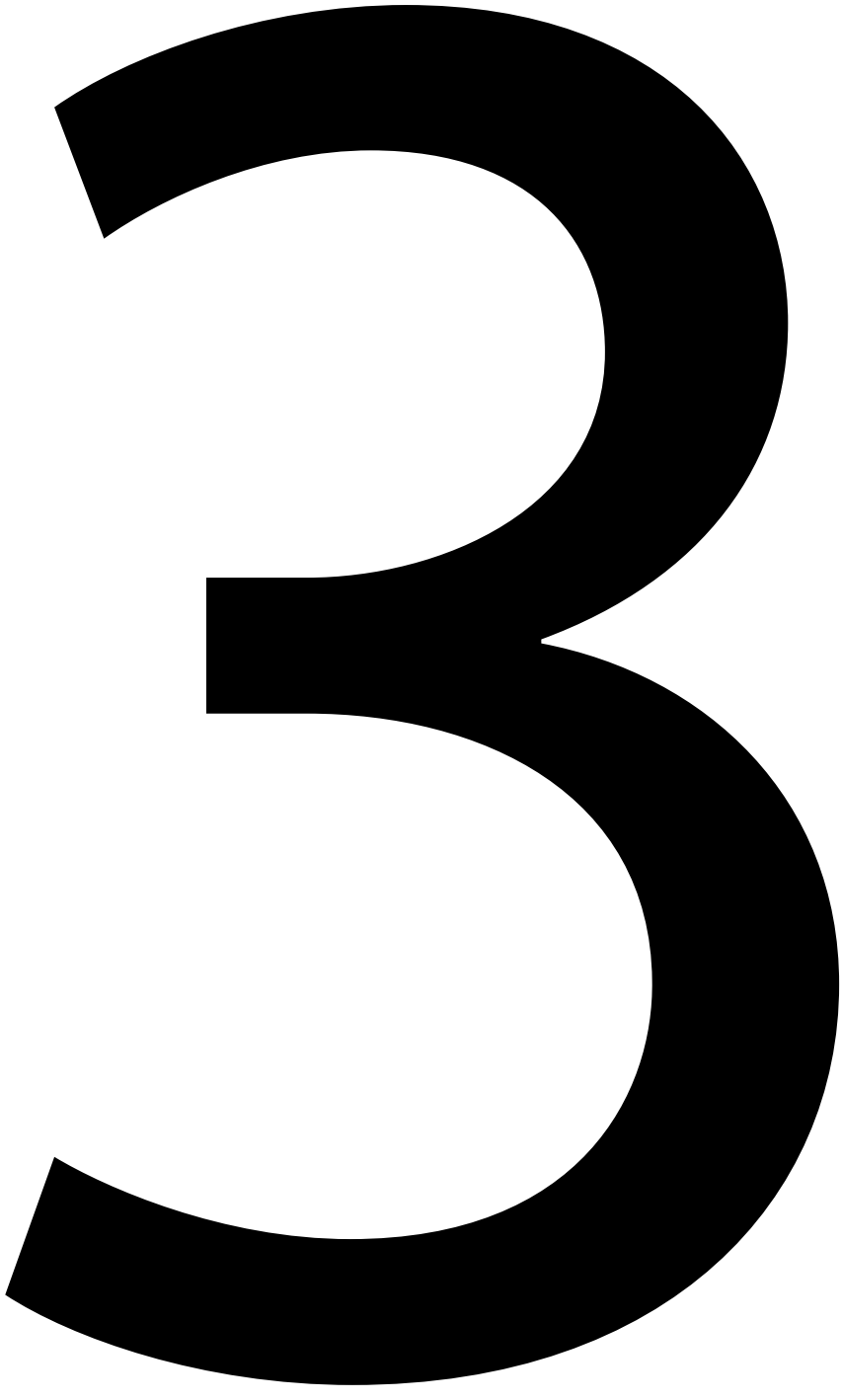

Chapter 3

Transcriptional profiling of the *Aspergillus niger* Δ*atg1* mutant during submerged carbon starvation

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Abstract

During limitations in the available nutrient sources, *Aspergillus niger* modifies the expression of specific sets of genes in order to respond to these stress conditions. Among them, autophagy-related (*atg*) genes are concertedly induced. Autophagy is a catabolic process during which organelles and cellular content are delivered to vacuoles for degradation and recycling. Deleting genes essential for autophagy hardly affected growth during the exponential phase, but the mutants did show morphological deviations during submerged carbon starvation as the formation of thin hyphae was accelerated in comparison with the wild-type. This present study aimed to obtain more insight in the underlying processes causing these changes by performing genome-wide transcriptional analysis comparing the *A. niger* Δ*atg1* mutant with the wild-type during submerged carbon starvation. Gene enrichment analysis demonstrated that in the *Δatg1* mutant bioprocesses related to DNA repair and cell division were higher expressed during the early post-exponential phase compared to the wild-type strain while at the later starvation time points ribosomal genes as well as metabolic processes showed lower expression. The data further support that autophagy is important during carbon starvation in order to control cell cycle arrest, degrade harmful cellular components and to support morphological and metabolic changes by endogenous recycling.

Introduction

Filamentous fungi with a saprophytic lifestyle, such as *Aspergillus niger* commonly encounter limitations in the available exogenous carbon source when growing in their natural environment. In response to such conditions, specific processes are induced leading to complex morphological and physiological changes which help the fungus to adapt to starvation stress. Important aspects of the carbon starvation response include the recycling of nutrients and the formation of asexual spores (Nitsche *et al.*, 2012; Szilágyi *et al.*, 2013; Ellström *et al.*, 2015; van Munster *et al.*, 2015b). Genome-wide transcriptomics studies have revealed that autophagy is one of the most dominantly induced processes upon starvation conditions in filamentous fungi (Nitsche *et al.*, 2012; Krohn *et al.*, 2014), which indicates that recycling of cell contents via autophagy plays an important role in order to survive nutrient limitation conditions. The autophagy process involves sequestering of cellular components in double membrane vesicles, which fuse with vacuoles releasing their contents for degradation by hydrolytic enzymes. The autophagy machinery is negatively regulated by the target of rapamycin (Tor) and the protein kinase A (PKA), which sense the nutritional state of a cell and inactivate the autophagy-related protein 13 (Atg13) during nutrient-rich conditions by phosphorylation. During starvation conditions, Atg13 is able to interact with Atg1 to form the Atg1 kinase complex, which initiates the autophagy process (Kamada *et al.*, 2000; Stephan *et al.*, 2009).

The (endogenous) recycling of nutrients by autophagy is important for maintenance of the mycelium, but also to fuel asexual development and cellular differentiation. Both in *A. niger* and *A. oryzae* it has been shown that carbon recycling fuels the secondary growth of hyphae with decreased hyphal diameters on the expense of older hyphal compartments, which become empty (Pollack *et al.*, 2008; Nitsche *et al.*, 2012). As the deletion of genes essential for autophagy accelerated the relative abundance of thin (young) hyphae in comparison with thick (old) compartments during submerged carbon starvation (Nitsche *et al.*, 2013), it has been proposed that autophagy protects older hyphae from cell death, balancing between survival of the mycelium and the formation of foraging hyphae. Studying of autophagy mutants further showed that deletion of autophagy-essential genes affected conidiation in a number of *Aspergilli* (Kikuma *et al.*, 2006; Richie *et al.*, 2007; Kikuma and Kitamoto, 2011; Nitsche *et al.*, 2013; Pinar *et al.*, 2013), although the effects varied between a mild reduction in sporulation in *A. fumigatus* and *A. niger* to a complete impairment of formation of aerial hyphae and conidia in *A. oryzae*.

To gain more insights in the underlying processes driving starvation-induced physiological and morphological changes, we performed genome-wide transcriptional analyses of glucose-starved submerged cultures of *A. niger* wild-type and Δ*atg1* strains using RNA sequencing. Gene expression of wild-type and mutant was compared during the

exponential growth phase and three carbon starvation time points, and enrichment analysis was performed to interpret the complex transcriptional changes. The results showed that after one day of carbon starvation the expression of genes related to cell division and DNA repair was higher in the Δ*atg1* mutant compared to the wild-type. Furthermore, protein synthesis and metabolic processes were significantly lower in the Δ*atg1* mutant during the later stages of carbon starvation. Taken together, the data suggest that the autophagy process plays an important role in order to support cellular integrity and metabolic adaptation during nutrient-limited conditions.

Materials and Methods

Cultivation, RNA isolation and quality control

Duplicate bioreactor batch cultivations of *A. niger* wild-type (N402) and Δ*atg1* (BN30.2) strains under prolonged carbon starvation conditions were performed and described previously (Nitsche *et al.*, 2013). Mycelium intended for gene expression analysis was separated from culture medium and frozen in liquid nitrogen within 15-20 s from sampling and stored at -80°C. Total RNA was extracted from mycelium in liquid nitrogen using Trizol reagent (Invitrogen). Frozen ground mycelium (≈200 mg) was directly suspended in 800 µl Trizol reagent and vortexed vigorously for 1 min. After 5 min of incubation at room temperature, 150 µl of chloroform was added. The samples were mixed well and incubated at room temperature for another 3 min, followed by centrifugation. The upper aqueous phase was transferred to a new tube to which 500 µl of isopropanol was added, followed by a 10 min incubation at room temperature and centrifugation for 15 min at maximum speed. The pellet was washed with 75% (v/v) ethanol and finally dissolved in 100 µl H_2O. RNA samples for RNA sequencing were additionally purified on NucleoSpin RNA II columns (Machery-Nagel) according to manufacturer's instructions. RNA quantity and quality were determined using a Nanodrop ND1000 spectrophotometer.

RNA sequencing analysis

RNA sequencing was outsourced to ServiceXS (Leiden, The Netherlands). cDNA library constructions were performed using the Illumina mRNA-Seq Sample preparation kit according to the instructions of the supplier. In brief, mRNA was isolated from total RNA using oligo-dT magnetic beads. After fragmentation of the mRNA, cDNA synthesis was performed and the cDNA was ligated with the sequencing adapters before PCR amplification of the resulting product. The quality and yield after sample preparation were measured with a DNA 1000 Lab-on-a-Chip. Clustering and mRNA sequencing using the Illumina cBot and HiSeq 2000 were performed according to the manufacturer's protocol. For each RNA sample at least 3.2 Gb of sequence data were obtained that passed the quality control (% ≥ Q30). Image analysis, base calling and quality checks were performed

with the Illumina data analysis pipeline RTA v1.13.48 and/or OLBv1.9 and CASAVA v1.8.2. Quality-filtered sequence tags are available upon request.

Transcriptomic data analysis

RNA sequencing analysis was performed essentially as described previously (Wang *et al.*, 2015) except for the transfer of genome annotations. Annotations were transferred from the CBS 513.88 genome to the N402 genome based on bidirectional alignments created with LAST (version 417) (Kiełbasa *et al.*, 2011), with tandem repeat sections masked with TRF (Benson, 1999). Alignments were combined and chained using CLASP (Otto *et al.*, 2011). Gaps in the global alignment were improved by performing realignment using either LAST (for large gaps, in sensitive mode) or Needleman-Wunsch (for smaller gaps). Chains with scores > 150 were accepted for alignment transfer. In total, 13412 (of 14070) genes were transferred. The normalized, RNAseq-based expression data are summarized in Supplemental Table S1.

Annotation enrichment analyses

Overrepresented Gene Ontology (GO) terms in sets of differentially expressed genes (FDR q-value < 0.005) were determined by using the Fisher's exact test Gene Ontology tool FetGOat (Nitsche *et al.*, 2011). R statistical computing software (R Foundation for statistical computing) was used to perform k-means clustering analysis. Normalized fragment counts were transformed to log space using log2(fragment count + 16) and standardized (mean = 0 and standard deviation = 1). The optimal number of clusters was determined from a total within-cluster sum of squares plot and standardized gene expression values were subsequently clustered accordingly.

Results

Comparison of the transcriptomic response to submerged carbon starvation of *A. niger* wild-type and Δ*atg1* strains

Autophagy is one of the most dominantly induced processes during carbon starvation in *A. niger* (Nitsche *et al.*, 2012). In a previous study, we examined the effects of deleting genes essential for autophagy during carbon starvation and showed that growth profiles of wild-type and *atg* mutants were comparable yielding similar amounts of dry biomass (Nitsche *et al.*, 2013). However, the accelarated formation of carbon starvation-induced thin hyphae and earlier cell death of old hyphae suggested a role for autophagy in controlling cell cycle progression and cellular maintenance during starvation. To gain more insights in the cellular processes in cells defective for autophagy during carbon starvation, transcriptome changes were analyzed using paired-end mRNA sequencing analysis. Total RNA was extracted from biomass samples harvested from batch cultures of both wild-type and Δ*atg1* mutant strains

during the exponential growth phase and during carbon starvation. In order to compare the exponential phase with early and later stages of carbon starvation, three different time points during carbon starvation were assessed: 20 hours (day 1), 70 hours (day 3) and 140 hours (day 6) post carbon depletion. As shown in Figure 1 growth profiles of wildtype and Δ*atg1* mutant strains were very similar. Differentially expressed genes between wild-type and Δ*atg1* strains were identified applying a critical FDR q-value of 0.005, resulting in a total of 2815 genes that were found to be differentially expressed during at least one of the four time points (Table S1). During the exponential growth phase, only small sets of genes were either higher or lower expressed in the Δ*atg1* mutant compared to the wild-type (121 and 25 genes, respectively) and a majority of them showed higher or lower expression only during the exponential phase (69% and 68% respectively). Considering the post-exponential starvation phase, 238 genes were conjointly higher expressed in the Δ*atg1* mutant, while 118 genes were conjointly lower expressed during all three starvation time points (Figure 2). The number of differentially expressed genes shared between day 3 and day 6 was even higher (401 and 206 genes higher and lower expressed, respectively), in contrast to relatively low overlaps of genes between day 1 and day 3, and day 1 and day 6. This indicates that the differences in gene expression between wild-type and Δ*atg1*

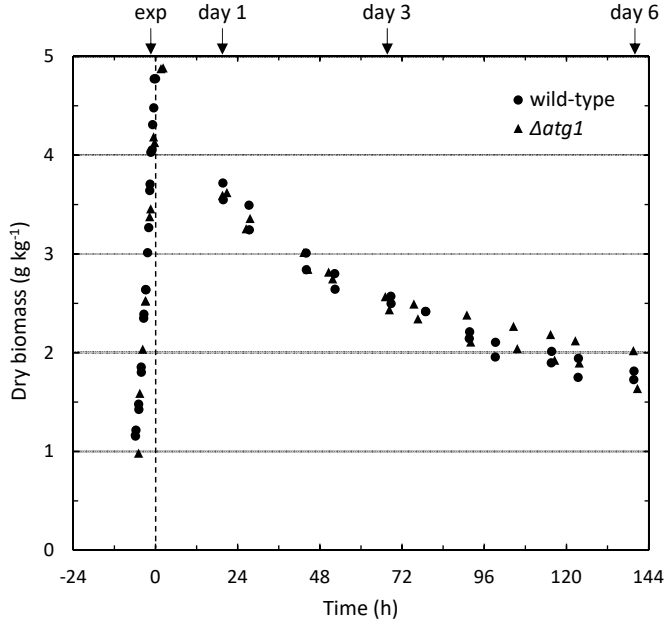


Figure 1 | Growth curves of duplicate submerged batch cultures of *A. niger* wild-type (N402) and Δ*atg1* strains. Replicate cultures were synchronized by setting the carbon depletion time points to 0. RNA was extracted from biomass samples taken during the exponential growth phase (exp) and 20 hours (day 1), 70 hours (day 3) and 140 hours (day 6) post carbon depletion, as indicated by arrows.

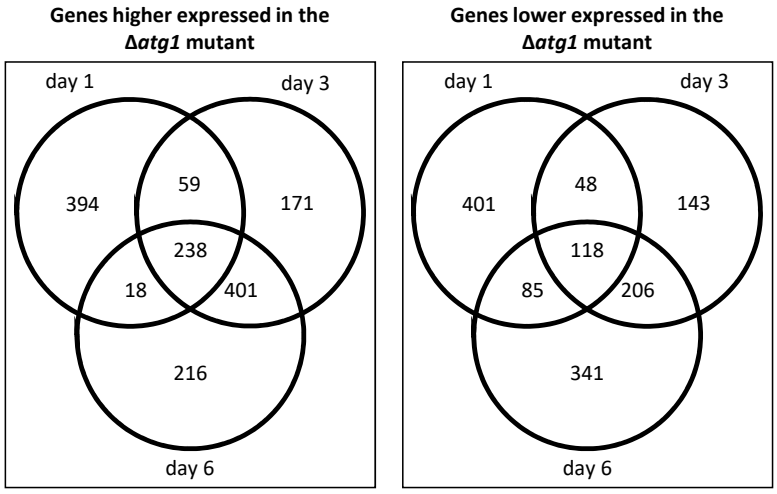


Figure 2 | Venn diagrams showing genes differentially expressed in the Δ*atg1* strain compared to the wild-type. Numbers of genes that are significantly higher (left) or lower (right) expressed in the Δ*atg1* strain compared to the wild-type during submerged carbon starvation. Differential expression (FDR q-value < 0.005) was determined during the exponential growth phase and at 1 day, 3 days and 6 days post carbon depletion. Numbers of differentially expressed genes during the exponential phase are not included in the figure for clarity reasons.

mutant are specific for the duration of carbon depletion, which is in accordance with the finding of distinct early and late carbon starvation responses in *A. niger* (Nitsche *et al.*, 2012). Subsequently, enrichment analysis was performed to identify overrepresented bioprocesses among the gene sets using the Gene Ontology tool FetGOat (Nitsche *et al.*, 2011). A summary of the results is shown in Figure 3 (see Table S2 for complete GO enrichment results). Remarkably, enriched GO-terms among differentially higher expressed genes in the Δ*atg1* mutant compared to the wild-type were identified during the exponential phase (although not more than one GO-term was found) and on day 1, but not on day 3 and day 6. Considering gene sets differentially lower expressed in the Δ*atg1* mutant compared to the wild-type, overrepresentation of GO-terms could be found on all starvation time points, but not during the exponential growth phase.

The early carbon starvation response: higher expression of cell division and DNA repair processes in the Δ*atg1* mutant

In order to deduce biologically relevant information from the GO enrichment analysis, we first focused on the set of enriched GO-terms identified from genes higher expressed in the Δ*atg1* mutant on day 1. Investigation of the GO-terms in this set of 709 genes (394 + 59 + 238 + 18) revealed that all of them could be subdivided into two main categories: cell division and DNA repair (Figure 3), indicating that genes related to cell division and DNA repair are higher expressed in the Δ*atg1* mutant compared to the wild-type during early carbon starvation. The corresponding genes, which are listed in Table 1, include orthologues

GO enrichment results for genes higher expressed in the Δ*atg1* mutant					
Exp	Day 1	Day 3	Day 6	GO-term	Description
Specific					
●				GO:0006083	acetate metabolic process
	●			GO:0000726	non-recombinational repair
	●			GO:0000727	double-strand break repair via break-induced replication
	●			GO:0006302	double-strand break repair
	●			GO:0010695	regulation of spindle pole body separation
	●			GO:0032465	regulation of cytokinesis
	●			GO:0042148	strand invasion
	●			GO:0045787	positive regulation of cell cycle
	●			GO:0090068	positive regulation of cell cycle process

GO enrichment results for genes lower expressed in the Δ*atg1* mutant					
Exp	Day 1	Day 3	Day 6	GO-term	Description
Common					
	●	●	●	GO:0000462	maturation of SSU-rRNA from tricistronic rRNA transcript (SSU-rRNA, 5.8S rRNA, LSU-rRNA)
	●	●		GO:0042273	ribosomal large subunit biogenesis
		●	●	GO:0000027	ribosomal large subunit assembly
		●	●	GO:0000028	ribosomal small subunit assembly
		●	●	GO:0006407	rRNA export from nucleus
Specific					
	●			GO:0019388	galactose catabolic process
		●		GO:0006412	translation
		●		GO:0006525	arginine metabolic process
		●		GO:0044238	primary metabolic process
			●	GO:0005980	glycogen catabolic process
			●	GO:0005982	starch metabolic process
			●	GO:0005996	monosaccharide metabolic process

Figure 3 | Summary of the GO enrichment results. GO enrichment analysis was performed with sets of genes differentially expressed (FDR q-value < 0.005) between wild-type and Δ*atg1* during the exponential growth phase as well as 1, 3, and 6 days post carbon depletion. Dots indicate common most-specific bioprocesses that are statistically significant overrepresented (FDR < 0.05) within a gene set.

of the *A. nidulans* cell cycle regulator genes *nimA, nimE* and *nimX* (An12g08100, An01g07420 and An11g02960, respectively) (Ye *et al.*, 1999) as well as the ku70 and ku80 orthologues *kusA* and *kueA* (An15g02700 and An07g05980) involved in non-homologous end-joining. It is important to note that the observed higher expression of genes involved in cell-division and DNA repair in the Δ*atg*1 mutant on day 1 does not allow conclusions about the mutant-specific response of these genes to early carbon starvation. More detailed examination of the expression values of the corresponding genes showed that only a minority of the genes (25%) was lower expressed in the wild-type and higher expressed in the Δ*atg1* mutant when comparing day 1 of the post-exponential phase with the exponential phase (Table

1). For a majority of the genes an increase or decrease in expression at day 1 compared to the exponential phase was found simultaneously in both wild-type and Δ*atg1* mutant, indicating that the observed significant differential expression resulted mostly from a difference in magnitude of the response to early carbon starvation. Both cell division and DNA repair processes were generally lower expressed upon carbon starvation in both strains as determined from lower expression values of corresponding genes at day 1 compared to the exponential phase, but the decrease was found to be less in the Δ*atg1* mutant compared to the wild-type. Analysis of the individual expression values further confirmed that the significant difference between wild-type and *atg1* deletion mutant considering cell division and DNA repair processes was specific to early carbon starvation only, as from the corresponding genes that were significantly higher expressed in the Δ*atg1* mutant at day 1, only 21% was found to be also significantly higher at day 3 and/or day 6 (Table 1). Also given the fact that both strains show identical growth curves (Figure 1), the results indicate that during early carbon starvation genes related to cell division and DNA repair are decreased in expression to a lesser extent in the Δ*atg1* mutant than in the wild-type, suggesting that these processes remain more active in the mutant during the early post-exponential phase.

Bioprocesses related to ribosomal function are lower expressed in the Δ*atg1* mutant during the post-exponential phase

During the exponential growth phase, only 25 genes were lower expressed in the Δ*atg1* mutant compared to the wild-type. Most likely as a result of this low number of genes, no overrepresented GO-terms were found in GO-enrichment analysis. In contrast, significant overrepresentation of a total of 49 unique bioprocesses was found among the genes lower expressed in the Δ*atg1* mutant compared to the wild-type during the post-exponential phase. As shown in Figure 3, GO-terms analysis revealed that both common as well as time point specific overrepresented GO-terms were identified among the genes lower expressed in the Δ*atg1* mutant. Bioprocesses related to ribosome biogenesis and assembly were enriched during all starvation time points, suggesting that ribosomal function might be decreased in the Δ*atg1* mutant during carbon starvation possibly as a result of less available building blocks for protein synthesis.

To investigate and compare the response to starvation of the corresponding ribosomal genes (n = 100) both in the wild-type and in the autophagy deletion mutant, individual gene expression profiles were assessed. Based on the expression profiles at all different time points, the genes were assigned to three different clusters by applying K-means clustering analysis to standardized expression values. The resulting clusters contained unequal number of genes (Figure 4) and the distribution of the genes over the clusters could not be related to functional nor genomic clustering (data not shown). The results show that 92 out of 100 genes were assigned to clusters 1 and 2, which contain genes with lower expression

Table 1 | Transcriptome data of genes corresponding to bioprocesses that are enriched among higher expressed genes in the Δatg1 mutant at day 1.

		Fold change Δ*atg1*/WT[a,b]				Fold change day 1/exp	
Identifier	**Description**	**Exp[c]**	**Day 1**	**Day 3**	**Day 6**	**WT**	**Δ*atg1***
Cell division							
An01g03090	strong similarity to 1,3-beta-glucanosyltransferase *gel1*–*A. fumigatus*	0.9	2.5	3.0	2.4	21.5	58.4
An02g11920	strong similarity to fizzy-related protein Fzr1–*Mus musculus*	1.0	2.1	2.2	1.6	1.6	3.7
An12g08530	similarity to Swe1 regulating protein kinase Hsl1–*S. cerevisiae* [truncated ORF]	0.9	2.5	0.8	0.5	1.1	2.9
An08g10320	strong similarity to checkpoint protein kinase chk1p–*Schizosaccharomyces pombe*	1.1	2.4	2.3	2.1	0.8	1.7
An11g11100	strong similarity to cell cycle protein kinase hsk1p–*S. pombe*	1.0	1.7	1.2	0.9	0.6	1.0
An01g07420	strong similarity to cyclin B *nimE*–*A. nidulans*	1.0	2.0	0.8	0.8	0.4	0.7
An01g12750	strong similarity to cell cycle protein CDC20–*Homo sapiens*	1.0	2.4	0.8	0.6	0.3	0.7
An05g00100	strong similarity to aurora/IPL1-related kinase AIK–*H. sapiens*	1.1	2.2	0.7	0.6	0.3	0.6
An05g00280	similarity to protein kinase Swe1–*S. cerevisiae*	1.0	1.8	0.8	0.9	0.4	0.8
An07g08210	strong similarity to cytoskeleton regulator IQGAP1–*H. sapiens*	0.9	2.1	0.9	0.7	0.4	0.8
An09g05760	similarity to hypothetical protein SPAC9G1.06c–*S. pombe*	1.0	1.8	1.2	1.3	0.3	0.5
An09g06400	Glycosylphosphatidylinositol-anchored chitinase *ctcA*–*A. niger*	1.0	6.8	3.9	2.2	0.1	0.9
An11g02000	strong similarity to spindle assembly checkpoint protein SldA–*A. nidulans*	1.0	2.0	0.7	0.5	0.4	0.8
An11g02960	strong similarity to protein kinase functional homolog of cdc2 NimX–*A. nidulans*	1.0	1.8	0.9	0.7	0.3	0.6
An12g08100	strong similarity to serine/threonine protein kinase NimA–*A. nidulans*	1.1	2.3	1.1	1.0	0.4	0.9
An14g00660	strong similarity to chitin synthase *chsA*–*A. nidulans*	1.1	2.3	0.9	0.7	0.4	0.8
An14g05530	Rho GTPase *rhoD*–*A. niger*	1.0	3.3	0.9	0.7	0.2	0.6
An16g07040	similarity to beta-1,3-glucanosyltransferase *bgt1*–*A. fumigatus* [truncated ORF]	1.0	2.3	0.7	0.9	0.3	0.8
DNA repair							
An07g05980	Ku80 ortholog involved in non-homologous end-joining *kueA*–*A. niger*	1.3	1.8	1.7	1.3	1.1	1.6
An01g05260	strong similarity to DEAH protein Mph1–*S. cerevisiae*	1.0	1.9	0.8	0.7	0.6	1.2
An03g02640	strong similarity to DNA ligase CaLIG4–*Candida albicans* [putative frameshift]	1.2	2.0	2.1	1.7	0.8	1.4
An04g01290	strong similarity to Rad52 homologue MUS11–*Neurospora crassa* [truncated ORF]	1.2	2.4	1.4	1.2	0.7	1.4

Continue

Continued

Identifier	Description	Fold change Δ*atg1*/WT[a,b]				Fold change day 1/exp	
		Exp[c]	Day 1	Day 3	Day 6	WT	Δ*atg1*
An07g08490	similarity to ATP-dependent helicase pcrA–*Bacillus stearothermophilus*	1.2	2.3	1.4	0.8	0.9	1.7
An15g02700	Ku70 ortholog involved in non-homologous end-joining kusA–*A. niger*	1.2	1.9	1.2	1.0	0.7	1.1
An01g13280	strong similarity to RAD54B – *H. sapiens*	1.3	1.9	1.2	0.8	0.3	0.5
An08g02350	strong similarity to protein uvsC–*A. nidulans*	1.2	1.9	1.0	0.8	0.4	0.6
An08g02440	similarity to DNA polymerase lambda POL lambda–*H. sapiens*	1.2	1.9	1.3	1.4	0.5	0.8
An08g10560	strong similarity to Rad54 homolog mus-25–*N. crassa*	1.1	1.8	1.3	1.4	0.4	0.7

[a] Significant differentially expressed genes are shaded in grey (FDR q-value < 0.005)
[b] WT, wild-type
[c] Exp, exponential growth phase

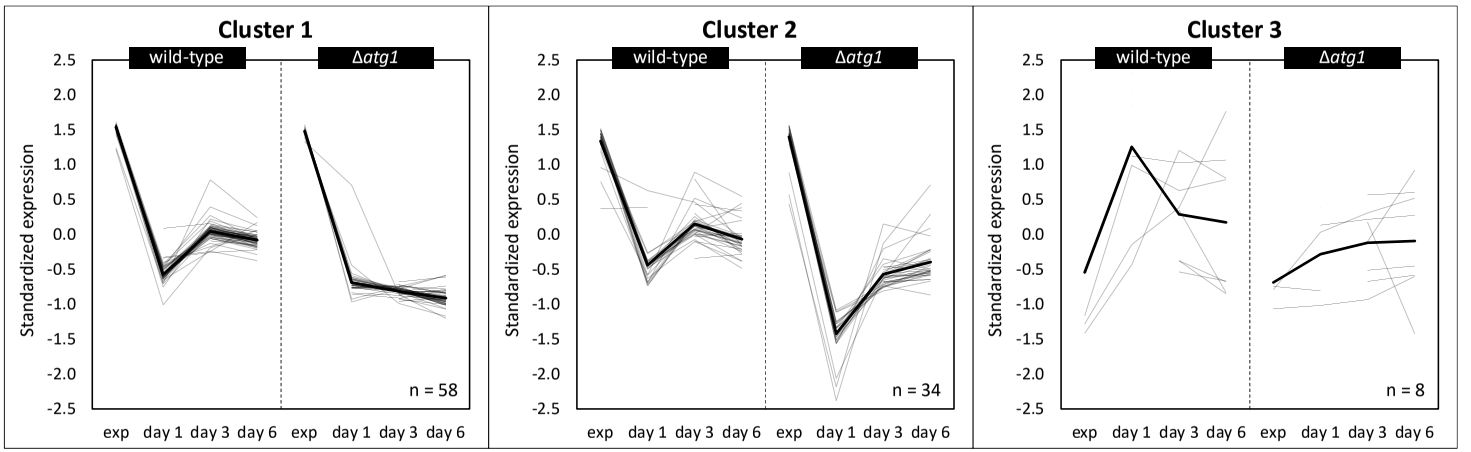


Figure 4 | Clustering of gene expression profiles for a subset of genes related to ribosomal function. The k-means clustering analysis was performed with a total of 100 genes, which were identified from GO enrichment analyses among genes that are lower expressed in the Δ*atg1* mutant during carbon starvation. Enriched GO-terms related to ribosomal processes were selected and the corresponding genes were used for clustering analysis. Normalized fragment counts were transformed to log space, standardized and subsequently clustered. Bold lines indicate mean expression profiles per cluster.

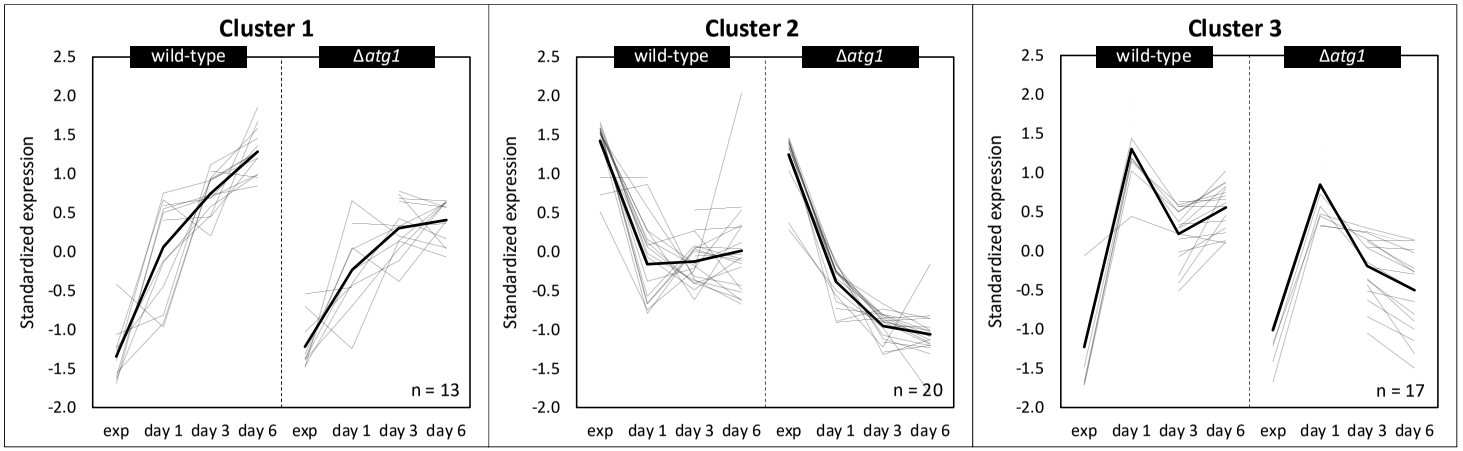


Figure 5 | Clustering of gene expression profiles for a subset of genes related to catabolic and metabolic bioprocesses as identified from GO enrichment results. From the GO enrichment analyses among lower expressed genes in the Δ*atg1* mutant during carbon starvation, overrepresented bioprocesses related to catabolism and metabolism were selected and the corresponding genes (n = 50) were used for k-means clustering analysis. Normalized fragment counts were transformed to log space, standardized and subsequently clustered. Bold lines indicate mean expression profiles per cluster.

values during carbon starvation compared to the exponential growth phase. For a vast majority of the genes (89 in the wild-type and 92 in the Δ*atg1* mutant) this downregulation was significant during at least one of the starvation time points with an FDR q-value below 0.005. The difference between cluster 1 and cluster 2 was determined by the Δ*atg1* gene expression profiles as gene expression profiles for the wild-type were similar between cluster 1 and cluster 2. The autophagy mutant expression profiles showed an initial decrease in gene expression upon starvation in both clusters, followed by increasing expression in cluster 1 and a decrease in expression in cluster 2. The third cluster contains the remaining eight genes. Significant upregulation for at least one of the starvation time points was observed for 7% and 3% of the genes in the gene set in wild-type and mutant, respectively. Starvation-induced upregulation of a gene in the wild-type and downregulation in the mutant at the same time point in the post-exponential phase was also observed from the expression profiles, but never significant. Taken together, the gene expression profiles and differential expression analysis show that ribosomal genes are generally significantly downregulated upon carbon starvation. Furthermore, increases and decreases in ribosomal gene expression mostly happen simultaneously in wild-type and *atg1* mutant, but the magnitude of the response is different. As autophagy plays an important role in cellular maintenance during starvation by the recycling of cellular products to be reused by the cell, lower expression of genes related to ribosome function in the autophagy mutant could be the consequence of lower availability of building blocks. This implicates that the cell is less capable to adapt to carbon starvation conditions by efficient turnover of macromolecules, which is in agreement with our previous observation that cell death of older hyphae is accelerated in autophagy mutants (Nitsche *et al.*, 2013).

The late carbon starvation response: lower expression of metabolic processes in the Δ*atg1* mutant

The majority of the overrepresented GO-terms identified from lower expressed genes during the post-exponential phase in the *Δatg1* mutant was related to protein synthesis and was commonly found on all three starvation time points. In addition, enrichment analysis also identified time-dependent responses specific to early, intermediate and late carbon starvation (Figure 3). Most of them were related to certain metabolic processes e.g. galactose catabolic process (day 1), arginine metabolic process (day 3), glycogen catabolic process, starch and monosaccharide metabolic processes (day 6). None of the identified metabolic pathways showed overlap between the time points. Investigation of the expression values of genes corresponding to the enriched GO-terms revealed that the vast majority of the genes was lower expressed in the Δ*atg1* mutant for all starvation time points, and especially for day 3 and day 6. However, as this was mostly not significant, no conclusions can be drawn for specific metabolic pathways on time points different than resulting from the enrichment analysis.

During late carbon starvation (120h post carbon depletion, day 6) multiple metabolic and catabolic processes were lower expressed in the Δ*atg1* mutant (Figure 3). The set of genes corresponding to these particular bioprocesses identified on day 6 consisted of 50 unique genes. K-means clustering analysis performed with this gene set resulted in three distinct clusters showing similar profiles of gene expression during the exponential phase and during carbon starvation (Figure 5). Clusters 1 and 2 consisted of genes that show respectively an increase or a decrease in gene expression during all starvation time points compared to the exponential phase, whereas genes in cluster 3 showed an increase in gene expression during the early post-exponential phase (day 1) followed by a decrease in expression during later starvation time points (day 3 and day 6). All bioprocesses were represented in all three clusters, except for GO:0005980 (glycogen catabolic process) and GO:0005982 (starch metabolic process). Enriched genes corresponding to these two particular GO-terms were found in clusters 2 and 3, but not in cluster 1 (data not shown). Among the genes related to starch metabolism were the members of the amylolytic cluster (*amyR-agdA-amyC*), which were all assigned to cluster 2 showing decreasing expression values following carbon starvation.

Discussion

The transcriptomic profiling of *A. niger* wild-type and Δ*atg1* strains during submerged carbon starvation uncovered significant differences approving that autophagy plays an important role specifically during these conditions. The data showed that during the exponential growth phase only small sets of genes were differential expressed between the wild-type and the mutant. This is in accordance with our previous study, in which we demonstrated that for autophagy mutants only a mild reduction in growth rate could be observed both in surface and in submerged growth, while no morphological differences were seen during exponential growth (Nitsche *et al.*, 2013).

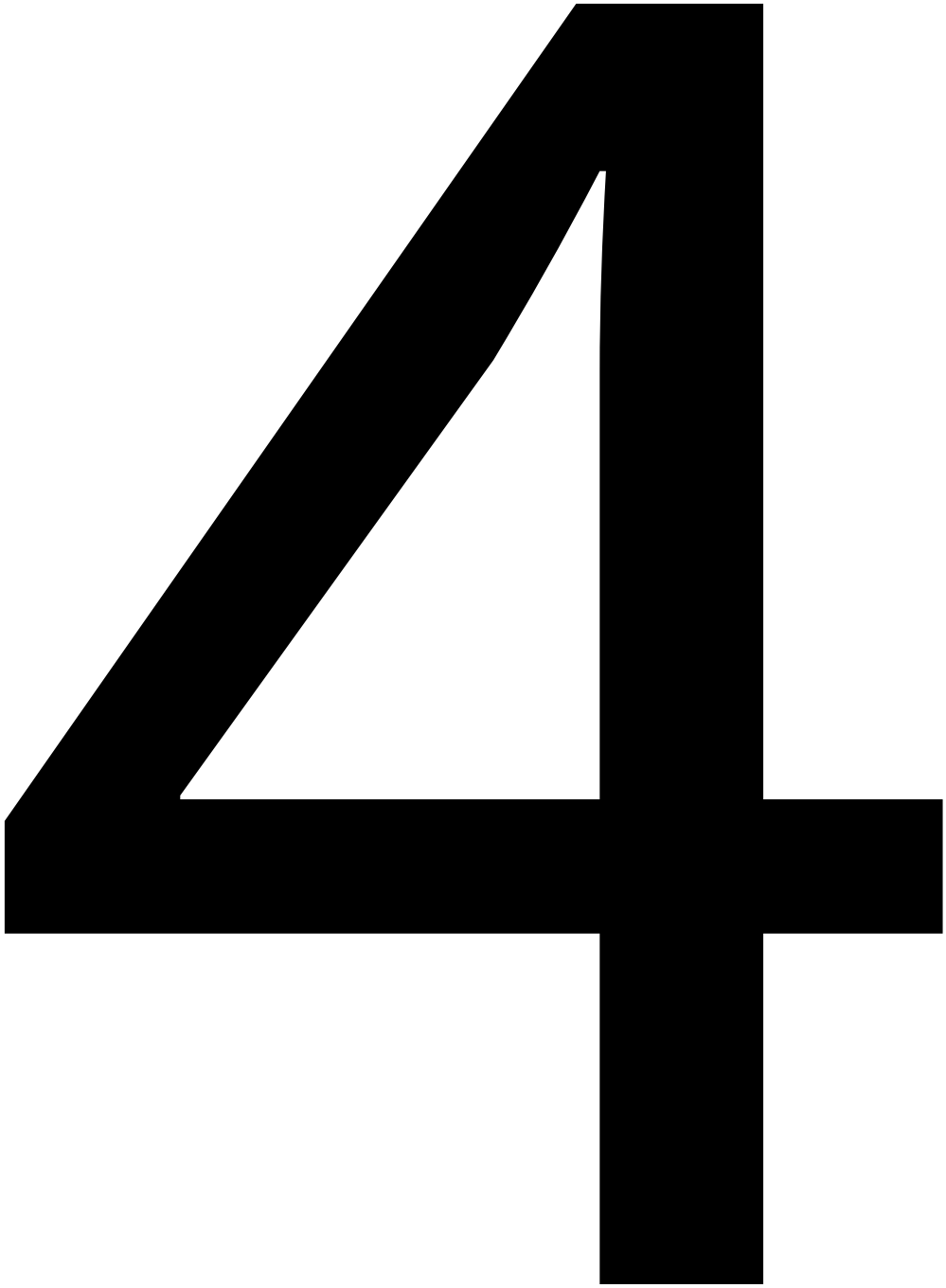
During early starvation (20 hours after glucose depletion) bioprocesses related to cell cycle regulation and DNA repair were significantly higher expressed in the Δ*atg1* mutant compared to the wild-type (Table 1). This suggests a higher cell division rate in autophagy mutants, which might increase the need for DNA repair mechanisms. It has been shown that TOR-mediated autophagy is involved (albeit not required) in cell cycle arrest awaiting the repair of severe DNA damage (Klermund *et al.*, 2014). Alternatively, the higher expression of genes related to DNA repair mechanisms could be resulting from the absence of autophagic mechanisms resolving DNA damage, consequently inducing other DNA repair pathways. Evidence for the involvement of autophagy in genome stability mainly results from studies in yeast and mammalian cells showing that autophagy has a strong impact in multiple ways. First of all, autophagy plays a role in mitigating DNA damage by controlling the

production of hazardous reactive oxygen species (ROS) through the degradation of injured mitochondria (Nitsche *et al.*, 2013; Kanki *et al.*, 2015). Secondly, autophagy can influence the dynamics of DNA repair by recycling key proteins involved in these mechanisms (Dyavaiah *et al.*, 2011; Robert *et al.*, 2011). Finally, autophagy contributes to the maintenance of nuclear function and genome stability by degrading damaged nuclei or nuclear components (Park *et al.*, 2009; Shoji *et al.*, 2010; Kikuma *et al.*, 2017a). Compromising the autophagy pathway during nutrient-limited conditions resulted in e.g. impaired mitochondrial turnover, aberrant nuclear division, increased DNA damage and aneuploidy (Mathew *et al.*, 2007; Matsui *et al.*, 2013; Nitsche *et al.*, 2013). But although a number of studies have demonstrated a link between autophagy and DNA integrity, its role is far from understood, as it can either contribute to or prevent cell death.

The expression of genes related to ribosome biogenesis and ribosome assembly was significantly lower in the Δ*atg*1 mutant on all starvation time points, whereas catabolic and metabolic processes were lower expressed specifically during the late post-exponential phase. Taking into account the importance of autophagy in the recycling of nutrients, this suggests that the level of metabolism is lower in the mutant as a consequence of a shortage for available building blocks. As an important catabolic program being required for maintaining cellular nutrient homeostasis, autophagy has been described to be involved in amino acid and lipid metabolism as well as in glycogen breakdown (Onodera and Ohsumi, 2005; Deng and Naqvi, 2010; Ha *et al.*, 2015; Müller *et al.*, 2015). It has been reported that *atg5* knockout mice have reduced amino acid concentrations in plasma and tissue during nutrient limitations in their early neonatal state (Kuma *et al.*, 2004), while an even more dramatic reduction in free amino acid pools was observed in Δ*atg7* yeast cells (Onodera and Ohsumi, 2005). Furthermore, consistent with its key role in amino acid recycling, autophagy-deficient *S. cerevisiae* Δ*atg7* and Δ*atg8* mutants showed decreased levels of protein synthesis compared to wild-type cells during nitrogen starvation (Onodera and Ohsumi, 2005; Müller *et al.*, 2015). In this respect it is interesting to note that the non-classically secreted protease, PepN (An01g00370) and a presumably non-classical secreted hemolysin (An19g00210) were overexpressed in the Δ*atg1* mutant at all time points respectively in the exponential phase (Table S1), corroborating our earlier finding that non-classical protein secretion is not decreased in *atg* deletion mutants (Burggraaf *et al.*, 2016). Although a role of autophagy in secondary metabolism was not clearly identified, it is also interesting to note that the fumonisin biosynthetic gene cluster (An01g06820-An01g06930) was highly overexpressed in the Δ*atg1* mutant during the exponential phase (Table S1). Another gene cluster, with amylolytic genes (An04g06910-An04g06930) showed decreased expression upon carbon starvation with significant lower expression values in the Δ*atg1* mutant as compared to the wild-type during starvation. In addition, significant lower expression in the autophagy deletion mutant was also observed for acid α-amylase (*aamA*) and glucoamylase (*glaA*). These particular amylolytic genes have been associated

with repression under secretion stress (RESS), a mechanism activated during secretion stress which selectively downregulates genes encoding secreted proteins (Al-Sheikh *et al.*, 2004; Guillemette *et al.*, 2007; Carvalho *et al.*, 2012). Hence, lower expression of these genes in the Δ*atg1* mutant might indicate that secretion stress is elevated in the absence of functional autophagy. However, the ER stress marker genes *bipA* and *pdiA* were not significantly higher expressed in the Δ*atg1* mutant compared to the wild-type (Table S1). Furthermore, it has been demonstrated that ER-accumulation of the constitutively expressed misfolded mutant GlaA::GFP protein was not increased in the autophagy mutant background compared to the wild-type background (Burggraaf and Ram, 2016), indicating that ER stress levels were comparable between mutant and wild-type and the diminishing of ER stress via the degradation of misfolded proteins was independent of autophagy.

A. niger autophagy deletion mutants grown in carbon-starved submerged cultures showed accelerated cell death of older hyphal compartments accompanied by a faster emergence of thin, unbranched hyphae when compared to wild-type (Nitsche *et al.*, 2013). On the phenotypic level as well as from the transcriptome it becomes clear that autophagy plays an important role in the adaptation to nutrient-limited conditions by affecting multiple cellular processes. Our data suggest that autophagy is protective against cell death though the degradation of excessive or harmful cellular products (including ROS) while providing new building blocks supporting changes in metabolism and protein synthesis.



Chapter 4

Autophagy is dispensable to overcome ER stress in the filamentous fungus *Aspergillus niger*

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Abstract

Secretory proteins are subjected to stringent quality control systems in the endoplasmic reticulum (ER) which include the targeting of misfolded proteins for proteasomal destruction via the ER-associated degradation (ERAD) pathway. Since deletion of ERAD genes in the filamentous fungus *Aspergillus niger* had hardly any effect on growth, this study investigates whether autophagy might function as an alternative process to eliminate misfolded proteins from the ER. We generated *A. niger* double mutants by deleting genes essential for ERAD (*derA*) and autophagy (*atg1* or *atg8*), and assessed their growth both under normal and ER stress conditions. Sensitivity towards ER stress was examined by treatment with Dithiothreitol (DTT) and by expressing a mutant form of glucoamylase (mtGlaA::GFP) in which disulfide bond sites in glucoamylase were mutated. Misfolding of mtGlaA::GFP was confirmed, as mtGlaA::GFP accumulated in the ER. Expression of mtGlaA::GFP in ERAD and autophagy mutants resulted in a two-fold higher accumulation in *ΔderA* and *ΔderAΔatg1* strains compared to *Δatg1* and wild-type. As *ΔderAΔatg1* mutants did not show increased sensitivity towards DTT, not even when mtGlaA::GFP was expressed, the results indicate that autophagy does not act as an alternative pathway in addition to ERAD for removing misfolded proteins from the ER in *A. niger*.

Introduction

Folding and post-translational modification of secretory and transmembrane proteins in eukaryotic cells takes place in the endoplasmic reticulum (ER), with the assistance of chaperones, foldases and lectins. Stringent quality control mechanisms tightly regulate the folding process, only allowing correctly folded, completely assembled proteins to exit the ER for subsequent delivery to the site of action (Ruggiano *et al.*, 2014). The presence of improperly folded proteins in the ER induces a set of signalling pathways known as the unfolded protein response (UPR) to alleviate the stress and restore homeostasis. Efficient clearance of improperly folded proteins is indispensable for cellular function, since persistent accumulations of unfolded proteins are potentially harmful for the cell as they impair ER function and homeostasis and eventually lead to the activation of apoptotic pathways. The decline in activity of the ER folding machinery with age has been shown to contribute to multiple aging-related neurodegenerative human diseases, including Alzheimer's, Parkinson's and Huntington's disease (Vilchez *et al.*, 2014).

Activation of the UPR upon ER stress results in induced expression of genes encoding chaperones and foldases which enhance protein folding and thereby increase folding capacity. Simultaneously, the UPR regulates the reduction of the ER folding load by the attenuation of protein synthesis on the one hand and the increase of misfolded protein clearance via the ER-associated degradation (ERAD) system on the other hand (Travers *et al.*, 2000; Ron and Walter, 2007). Proteins that are targeted for destruction via ERAD are retrotranslocated to the cytosol for degradation by the 26S proteasome. Studies in yeast and mammalian cells indicate that recognition, retrograde transport and ubiquitination of misfolded proteins that accumulate in the ER lumen are mediated by the Hrd1 membrane complex, which includes the E3-ubiquitin ligase Hrd1 itself as well as Hrd3, Der1 and other regulatory and scaffold proteins (Vembar and Brodsky, 2008; Ruggiano *et al.*, 2014). Homologs of ERAD genes were also identified in filamentous fungi and studying deletion mutants of a number of those in *Aspergilli* species showed that a functional ERAD pathway is not required for growth both under normal and ER stress conditions (Carvalho *et al.*, 2011a; Krishnan *et al.*, 2013). Deletion of the *der1* homolog *derA* in a heterologous protein overexpression strain of *Aspergillus niger* resulted in a 6-fold increase in the intracellular accumulation of the protein accompanied by the induction of UPR target genes, but had no effect on growth and conidiation (Carvalho *et al.*, 2011a).

Clearance of aberrant proteins from the ER can alternatively involve vacuolar targeting via the autophagic pathway (Cheng, 2011; Deegan *et al.*, 2013; Pu and Bassham, 2013; Senft and Ronai, 2015). Autophagy is a highly conserved, catabolic process responsible for the delivery of proteins, cytoplasmic components and organelles to lytic compartments such as lysosomes in mammalian cells or vacuoles in plant and fungal cells. It involves the

sequestering of cytoplasmic contents in double membrane vesicles which subsequently fuse with the lysosome or vacuole, releasing their cargo. Hydrolytic enzymes facilitate the degradation of the vesicle membrane and its contents, whereupon the breakdown products are transported back into the cytoplasm to be reused by the cell (Yang *et al.*, 2006). Autophagic activity is controlled by a set of more than 30 autophagy-related (Atg) proteins (Feng *et al.*, 2014) and we previously showed that at least two of them (Atg1 and Atg8) are essential for autophagy in *A. niger* (Nitsche *et al.*, 2013). The serine/threonine protein kinase Atg1 forms a part of the regulatory Atg1 kinase complex in yeast which initiates the autophagic process, while Atg8 is a structural component of autophagic vesicle membranes (Cheong *et al.*, 2008; Inoue and Klionsky, 2010). Although deletion of either one of these genes results in severe or complete impairment of conidiation in several filamentous fungal species (Pinan-Lucarré *et al.*, 2005; Kikuma *et al.*, 2006; Richie *et al.*, 2007; Bartoszewska *et al.*, 2011), phenotypic effects were only modest in *A. niger* (Nitsche *et al.*, 2013). Basal levels of autophagy contribute to the maintenance of cellular homeostasis by eliminating old or damaged organelles and long-lived proteins. Autophagy is strongly induced by conditions of intracellular or extracellular stress, including nutrient starvation, pathogen invasion and oxidative stress. Increasing evidence indicates that autophagy can also be induced by ER stress, suggesting that it is functioning in the clearance of aggregated and misfolded proteins. Studies in yeast and in mammalian cells show transcriptional induction of genes related to autophagy upon ER stress (Travers *et al.*, 2000; Kouroku *et al.*, 2007) and autophagy-mediated removal of aggregated mutant proteins from the ER (Kruse *et al.*, 2006; Fujita *et al.*, 2007; Ishida *et al.*, 2009).

In filamentous fungi the possible link between ER stress and autophagy has been hardly studied. For *Aspergillus oryzae* it was shown that mutant proteins accumulating in the ER are transported to vacuoles upon starvation in an autophagy-dependent manner (Kimura *et al.*, 2011). In this study we investigated whether autophagy is involved in the removal of misfolded proteins from the ER in *A. niger* by deleting genes essential for autophagy in an ERAD-defective background. Growth of the double knockout strains was compared to single deletion mutants of ERAD and autophagy both under normal conditions and after applying ER stress. Intracellular localization of misfolded proteins was visualized by expressing a disulfide bond deleted mutant of the secretory protein glucoamylase and compared between wild-type, single deletion and double knockout strains. The results indicate that deleting autophagy genes in strains defective for ERAD did not have an additional effect on growth and the level of accumulation of misfolded proteins in the ER.

Materials and Methods

Strains, culture conditions and molecular techniques

Aspergillus niger strains used in this study are listed in Table 1. Strains were cultivated in minimal medium (MM) (Bennett and Lasure, 1991) or in complete medium (CM) containing 0.1% casamino acids and 0.5% yeast extract in addition to MM. Growth media were supplemented with 10 mM uridine when required. Hygromycin resistant transformants were isolated from plates supplemented with 200 µg ml^{-1} hygromycin and 500 µg ml^{-1} caffeine and subsequently purified on plates containing 100 µg ml^{-1} hygromycin.

Table 1 | *A. niger* strains used in this study

Strain	Genotype	Reference
N402	*cspA1* derivative of ATCC9029	Bos *et al.* (1988)
MA78.6	Δ*kusA::amdS* in N402	Carvalho *et al.* (2010)
MA169.4	*kusA*::DR-*amdS*-DR in AB4.1	Carvalho *et al.* (2010)
AW12.1	Δ*atg1::hyg* in MA78.6	This study
AW13.1	Δ*atg8::hyg* in MA78.6	This study
MA97.2	Δ*kusA*, Δ*derA::amdS*	Carvalho *et al.* (2011a)
AW14.2	Δ*atg1::hyg* in MA97.2	This study
AW15.1	Δ*atg8::hyg* in MA97.2	This study
MA134.64	Δ*kusA*, multicopy P*gpdA-gla*$_{514}$*-gus*	Carvalho *et al.* (2011a)
AW16.1	Δ*atg1::hyg* in MA134.64	This study
AW17.2	Δ*atg8::hyg* in MA134.64	This study
MA136.18	Δ*kusA*, multicopy *PgpdA-gla*$_{514}$*-gus*, Δ*derA::amdS*	Carvalho *et al.* (2011a)
AW18.6	Δ*atg1::hyg* in MA136.18	This study
AW19.9	Δ*atg8::hyg* in MA136.18	This study
AW27.10	FOA-resistant derivative of MA97.2	This study
AW28.12	FOA-resistant derivative of AW12.1	This study
AW30.3	FOA-resistant derivative of AW14.2	This study
AW47.2	P*gpdA*-wt*glaA::sgfp*-T*trpC*, *pyrG*** in MA169.4	This study
AW48.2	P*gpdA*-mt*glaA::sgfp*-T*trpC*, *pyrG*** in MA169.4	This study
AW49.1	P*gpdA*-wt*glaA::sgfp*-T*trpC*, *pyrG*** in AW27.10	This study
AW50.1	P*gpdA*-mt*glaA::sgfp*-T*trpC*, *pyrG*** in AW27.10	This study
AW51.1	P*gpdA*-wt*glaA::sgfp*-T*trpC*, *pyrG*** in AW28.12	This study
AW52.1	P*gpdA*-mt*glaA::sgfp*-T*trpC*, *pyrG*** in AW28.12	This study
AW53.2	P*gpdA*-wt*glaA::sgfp*-T*trpC*, *pyrG*** in AW30.3	This study
AW54.1	P*gpdA*-mt*glaA::sgfp*-T*trpC*, *pyrG*** in AW30.3	This study
MA141.1	P*gpdA-glaAG2::sgfp-HDEL*-T*trpC*, *pyrG** in MA70.15	Carvalho *et al.* (2011b)

All PCR and cloning steps were performed according to standard procedures (Sambrook and Russell, 2001). Total RNA was extracted using Trizol reagent (Invitrogen) according to manufacturer's instructions. Obtainment of uridine-requiring strains by counter selection on FOA, transformation of *A. niger* and genomic DNA extraction were conducted as described by Myer *et al* (2010). Northern and Southern blot analyses were performed as described by Sambrook and Russell (2001), using [α-32P]dATP-labeled probes synthesized with the DecaLabel DNA labeling kit (Thermo Scientific).

Plate growth assays were performed on MM solidified by the addition of 1.5% agar and growth at 30°C and 42°C was monitored for 3 days. Sensitivity of the deletion strains towards ER-stress was determined by spotting 1 x 10^4 spores on solid MM supplemented with 1, 5 or 10 mM Dithiothreitol (DTT).

Generation of double knockout strains

Plasmids carrying the *atg1* or *atg8* deletion cassettes were described previously (Nitsche *et al.*, 2013). Linearized constructs were transformed to *A. niger* strains MA78.6 (*ΔkusA*), MA97.2 (*ΔkusA, ΔderA*), MA134.64 (multi-copy GlaGus, *ΔkusA*) and MA136.18 (multi-copy GlaGus, *ΔkusA, ΔderA*) (Carvalho *et al.*, 2010, 2011a). Homologous integration of the constructs was confirmed by Southern analysis (Figure S1).

Generation of strains constitutively expressing GFP fusion proteins

The pAN52-1*Not* vector was used to facilitate constitutive expression of *glaA* and mutant *glaA* fusion proteins from the *A. nidulans gpdA* promoter. An extra *Not*I restriction site was introduced in the *Xba*I site of pAN52-1Not to enable the isolation of the fusion constructs via *Not*I digestion in a later stage of the cloning procedure. A phosphorylated oligonucleotide adaptor (5'-CTAGAGCGGCCGCT-3') was heated at 95°C for two minutes and slowly cooled down to room temperature. Three volumes of ice-cold 70% ethanol were added and after centrifugation the pellet was air-dried and dissolved in MQ water. The adaptor was then directly ligated in *Xba*I-opened pAN52-1*Not*-gfp (Arentshorst et al., unpublished), giving rise to plasmid pAW56.

The *glaA::sgfp* fragment was PCR amplified from plasmid pAN56-2sGFP (Gordon *et al.*, 2000), using primers which introduced restriction sites *Nco*I and *Bam*HI (5'-CATGCCATGGGCTTCCGATCTCTACTCGCCCTG and 5'-CGGGATCCTTACTTGTACAGCTCGTCCATG). The mutated form of *glaA::sgfp* (mt*glaA::gfp*) was synthetically produced at GeneArt® Life Technologies (Carlsbad, CA, USA). Both the wild-type and the mutated fusion constructs were blunt-end ligated in the pJET1.2 cloning vector, sequenced and subsequently isolated as two *Nco*I-*Nhe*I and *Nhe*I-*Bam*HI fragments in two double digestions to circumvent the extra *Bam*HI site which is present in the fusion

construct. The fragments were cloned between the *gpdA* promoter and *trpC* terminator of the *Nco*I – *Bam*HI-opened pAW56 plasmid in a three-way ligation.

The constructs, containing the *gpdA* promoter and *trpC* terminator, were subsequently isolated by *Not*I digestion and cloned into pMA334 (Arentshorst *et al.*, 2015a). The pMA334 plasmid has been designed such that (reporter) constructs can be inserted between parts of the *pyrG* partial open reading frame and 3' flanking region, facilitating efficient targeting of the construct to the *pyrG* locus. The constructs were linearized by *Asc*I digestion before transformation to *A. niger* strains MA169.4 (Δ*kusA, pyrG*$^-$), AW27.10 (Δ*kusA*Δ*derA, pyrG*$^-$), AW28.12 (Δ*kusA*Δ*atg1, pyrG*$^-$) and AW30.3 (Δ*kusA*Δ*derA*Δ*atg1, pyrG*$^-$). Single integration of the construct on the *pyrG* locus was confirmed by Southern analysis (Figure S3).

Microscopy, image processing and statistical analysis

Confocal images were obtained using a confocal laser scanning microscope (Zeiss Imager, Zeiss, Jena, Germany) equipped with a LSM 5 exciter. For fluorescence intensity measurements, five biological replicate experiments were performed for each individual strain and 10 micrographs were taken per experiment using equal microscope settings. Intersections of hyphae were excluded and the fluorescence intensity within the hyphae was measured using the open source image processing program ImageJ (Abramoff *et al.*, 2004). R statistical computing software (R Foundation for statistical computing, Vienna, Austria) was used for statistical analysis. The data was first tested for normality and for homogeneity of variance with Levene's Test. Differences in means were analyzed with ANOVA and subsequent post-hoc Tukey Honest Significant Difference test when significant differences were observed.

Results

ERAD/autophagy double knockout mutants do not show increased sensitivity towards ER stress conditions

To prevent persistent accumulation of misfolded proteins in the ER, improperly folded proteins are efficiently removed and degraded by the ER-associated degradation (ERAD) system. Surprisingly, compromising the ERAD pathway in the filamentous fungus *Aspergillus niger* only had a modest effect on growth (Carvalho *et al.*, 2011a), indicating that other mechanisms might be of importance to help the cells cope with ER stress.

In order to investigate the possible involvement of the autophagy process in the removal of misfolded proteins from the ER, we deleted the autophagy-essential genes *atg1* and *atg8* in ERAD-defective *A. niger* Δ*derA* background strains expressing multiple copies of the heterologous Glucoamylase-β-Glucuronidase (GlaGus) gene fusion. It has been shown

previously that in this strain, to which we will refer to as the multi-copy GlaGus (mcGlaGus) strain, both genes related to ERAD and UPR were transcriptionally induced, which was accompanied by intracellular accumulation of GlaGus (Carvalho *et al.*, 2011a).

The two autophagy genes were deleted in four different background strains: the control strain MA78.6 (Δ*kusA*), the *derA* deletion strain MA97.2 (Δ*kusA*, Δ*derA*), the mcGlaGus strain MA134.64 (Δ*kusA*, mcGlaGus) and the mcGlaGus strain with *derA* deletion MA136.18 (Δ*kusA,* mcGlaGus, Δ*derA*). Transformants for each strain were purified on hygromycin containing medium and homologous integration was confirmed by Southern hybridization (Figure S1).

To study the growth effects of deleting genes related to autophagy and ERAD and compare the double mutants with the single knockout strains, mutants were grown for three days on solid minimal medium (MM) at 30°C and 42°C (Figure 1). The mutants were also tested on their sensitivity towards ER stress by exposing them to increasing concentrations of the ER stressor Dithiothreitol (DTT), an inhibitor of disulfide bond formation. Deletion of the *atg* genes in wild-type and Δ*derA* backgrounds did not result in severe growth defects or increased sensitivity to DTT, although, in accordance with our previous observations, growth and conidiation of the Δ*atg1* and Δ*atg8* mutants was reduced compared to the parental strains (Nitsche *et al.*, 2013). Importantly, no growth reduction was observed in

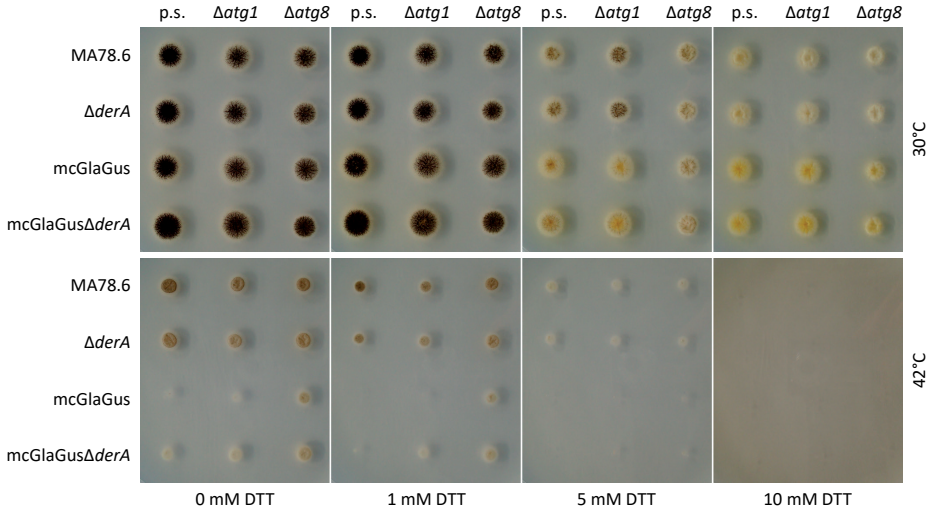

Figure 1 | Sensitivity of ERAD and autophagy mutants towards elevated temperature and increasing concentrations of DTT. A total of 10^4 spores from the parental strains (p.s.) MA78.6, Δ*derA*, mcGlaGus and mcGlaGusΔ*derA* together with the respective autophagy deletion mutants (Δ*atg1* or Δ*atg8*) was point inoculated on MM agar plates containing 0, 1, 5 or 10 mM DTT and incubated for 3 days at 30°C or 42°C.

the double knockout mutants compared to the single *atg* deletion strains. Both wild-type and mutant strains showed sensitivity to elevated temperature and DTT, but no differences were observed among the strains.

Considering the mcGlaGus strains, growth was reduced at 42°C in comparison with the strains that did not produce the heterologous GlaGus fusion protein. It was anticipated that the deletion of both ERAD and autophagy in the background of a heterologous GlaGus protein producing *A. niger* strain would result in serious growth defects. However, no differences considering growth and conidiation between the double mutants and the single mutants could be observed. Even the induction of ER stress through the addition of DTT did not have an effect on the phenotype, as all mutants showed a similar sensitivity to DTT. This indicates that impairment of functional ERAD and autophagy does not affect growth in *A. niger*, not even in heterologous protein overproducing strains.

The expression of misfolded Glucoamylase::GFP in *A. niger* mildly induces UPR

To study further the role of ERAD and autophagy in the degradation of misfolded proteins in *A. niger*, we investigated whether impairment of these processes causes protein accumulation in the ER by visualizing a wild-type and a mutant form of the major secreted protein in *A. niger*, Glucoamylase (GlaA) with GFP. The mutant GlaA (mtGlaA) was constructed such, that formation of disulfide bridges is prevented in the protein, resulting in improper folding. Crystallographic analysis reveals that the catalytic domain of GlaA contains three disulfide bridges (Lee and Paetzel, 2011), which we removed by replacing all the cysteines linked by disulfide bonds with alanines to generate mtGlaA (Figure S2). Both wild-type *glaA* (wt*glaA*) and mt*glaA* fused to *sgfp* were constitutively expressed from the *gpdA* promoter and constructs were targeted to the *pyrG* locus in the wild-type (MA169.4) and in strains defective for ERAD (Δ*derA*) and/or autophagy (Δ*atg1*, Δ*derA*Δ*atg1*). Single integration at the *pyrG* locus was confirmed by Southern analysis (Figure S3).

The accumulation of misfolded proteins in the ER induces an ER stress response, characterized by the upregulation of UPR marker genes, such as *bipA* and *pdiA* (Carvalho *et al.*, 2011a). To investigate whether the expression of wtGlaA::GFP or mtGlaA::GFP leads to ER stress, the expression of *bipA* and *pdiA* was determined by Northern analysis. In addition, the expression of *atg8* was assessed to see whether autophagy was induced. The results showed that *bipA* and *pdiA* expression levels were comparable between wild-type (N402) and strains expressing wt*glaA::gfp*, but were upregulated in mt*glaA::gfp* strains, especially in ERAD defective Δ*derA* backgrounds (Figure 2). This indicates that the presence of misfolded mtGlaA::GFP leads to ER stress, which is further enhanced by the deletion of *derA*. Since *atg8* was not upregulated in strains expressing either wt*glaA::gfp* or mt*glaA::gfp*, it was concluded that autophagy was not activated under these conditions.

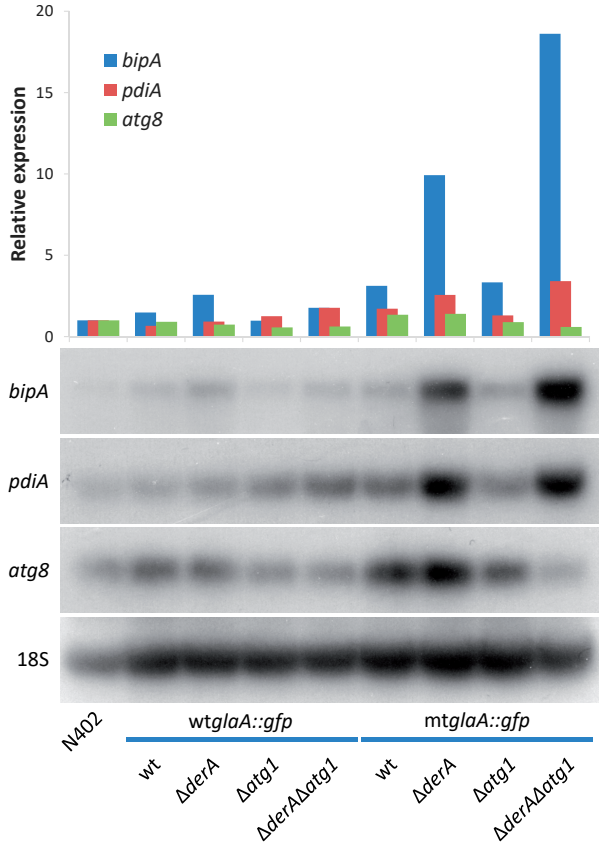

Figure 2 | Northern blot analysis of UPR- and autophagy-marker genes in wt*glaA::gfp* and mt*glaA::gfp* expressing strains. Total RNA was extracted after 16 hours of growth in liquid CM at 30°C. The gene expression levels were normalized using N402 as a reference. Values were corrected for loading differences by comparison with 18S ribosomal RNA. Strains expressing the misfolded protein mtGlaA::GFP showed an UPR. Wild-type (wt, MA169.4).

Removal of mtGlaA::GFP from the ER involves ERAD, but is independent of autophagy

Plate growth on MM showed no differences between the strains expressing either wt*glaA::gpf* or mt*glaA::gfp* and their respective parental strains (data not shown).The strains were subsequently subjected to confocal microscopy to investigate the localization of the GFP signal. In the wt*glaA::gfp* strains, fluorescence signal was observed in the cell wall and septa (Figure 3A), which is typical for secretory proteins and similar as reported before (Gordon *et al.*, 2000). In contrast, mtGlaA::GFP showed an intracellular localization pattern (Figure 3B), which, by comparison to an *A. niger* strain expressing ER-targeted GFP (P*glaA*::GFP-HDEL) (data not shown), was designated as ER localization. This indicates that

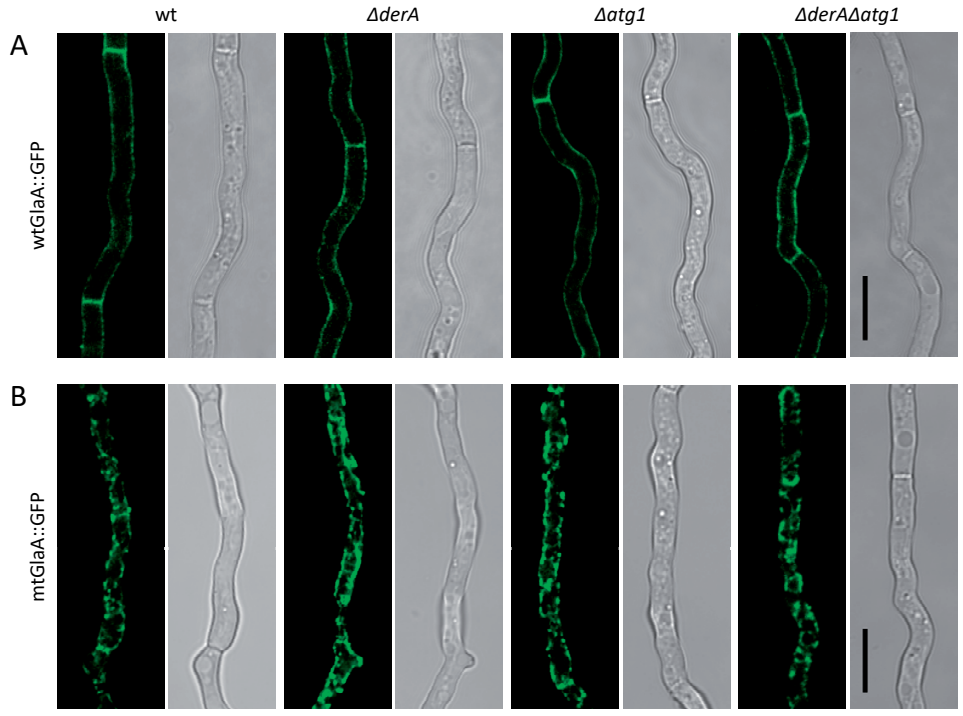

Figure 3 | Localization of wtGlaA::GFP and mtGlaA::GFP in wild-type, Δ*derA*, Δ*atg1* and Δ*derA*Δ*atg1* background strains. The strains were grown on coverslips in Petri dishes with MES-buffered MM (pH 6.0) for 16 hours at 30°C. Strains expressing wtGlaA::GFP showed localization of GFP in the cell walls and septa (A), whereas GFP localized mainly to the ER in mtGlaA::GFP expressing strains (B). Wild-type (wt, MA169.4). Scale bar: 10 µm.

misfolded GlaA proteins are not transported towards the membrane to be secreted, but at least temporarily retained in the ER to be refolded or degraded.

The degradation of misfolded proteins from the ER is important to prevent harmful accumulations impairing cellular functions. To investigate whether the degradation of misfolded proteins is dependent on functional ERAD and/or autophagy systems, the accumulation of misfolded GlaA proteins in the ER was determined by quantifying the intensity of GFP fluorescence signal from images taken after 16 hours of growth. For comparison, strains expressing wtGlaA::GFP proteins were taken along in the analysis and it was observed that all of these strains exhibited the same level of fluorescence (Figure 4A). However, significant differences were observed among mt*glaA::gfp* strains, with a more than twice as intense signal in the Δ*derA* and the Δ*derA*Δ*atg1* background strains compared to wild-type and Δ*atg1* (Figure 4B). Increased GFP levels in Δ*derA* background strains suggest that lacking a functional ERAD system hampers the efficient degradation of mtGlaA::GFP, leading to accumulation of the misfolded protein in the ER. In contrary, fluorescence

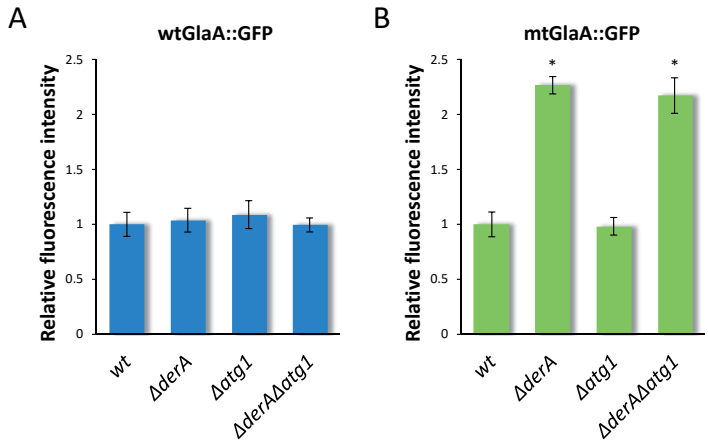

Figure 4 | Quantification of wtGlaA::GFP and mtGlaA::GFP fluorescence intensity. The strains were grown on coverslips in Petri dishes with MES-buffered MM (pH 6.0) for 16 hours at 30°C. For each individual strain, the average fluorescence intensity was determined from five independent replicate experiments (n=5) in which 10 images were taken per sample. The intensity levels were normalized to the respective wild-type values. Strains expressing wtGlaA::GFP showed similar levels of fluorescence (A), while mtGlaA::GFP showed two-fold higher accumulation in Δ*derA* and Δ*derA*Δ*atg1* compared to wild-type (wt, MA169.4) and Δ*atg1* backgrounds (B). Error bars represent standard deviation. *Significantly different from respective wild-type ($p < 0.05$).

intensity of mtGlaA::GFP in the Δ*atg1* background was not different from mtGlaA::GFP in wild-type, indicating that degradation of misfolded proteins is independent of functional autophagy. Moreover, even in the absence of ERAD, autophagy is not redundantly taking over its degradation function, since Δ*derA* and Δ*derA*Δ*atg1* displayed similar fluorescence intensities.

Carbon starvation-induced transport of wtGlaA::GFP and mtGla::GFP is mediated by autophagy

Plate growth assays and fluorescence microscopy described above showed that autophagy is not essential for the degradation of mtGlaA::GFP under normal growth conditions or conditions disturbing protein folding in *A. niger*. We next examined the role of autophagy during carbon starvation. Previously, we demonstrated that both cytosolically and mitochondrially targeted GFP are localized to vacuoles under starvation conditions in *A. niger* wild-type strains, but not in Δ*atg1* and Δ*atg8* mutants (Nitsche *et al.*, 2013). In order to assess whether wtGlaA::GFP and mtGlaA::GFP are transported to vacuoles upon starvation, the fluorescent strains were subjected to microscopy after 40 hours of starvation in MM without a carbon source. Vacuolar localization of both properly folded (wtGlaA::GFP) and misfolded GlaA::GFP proteins was observed in the wild-type background and in Δ*derA*, but not in the Δ*atg1* and Δ*derA*Δ*atg1* background strains (Figure 5A,B), indicating that both wtGlaA::GFP and mtGlaA::GFP proteins are delivered to vacuoles upon starvation,

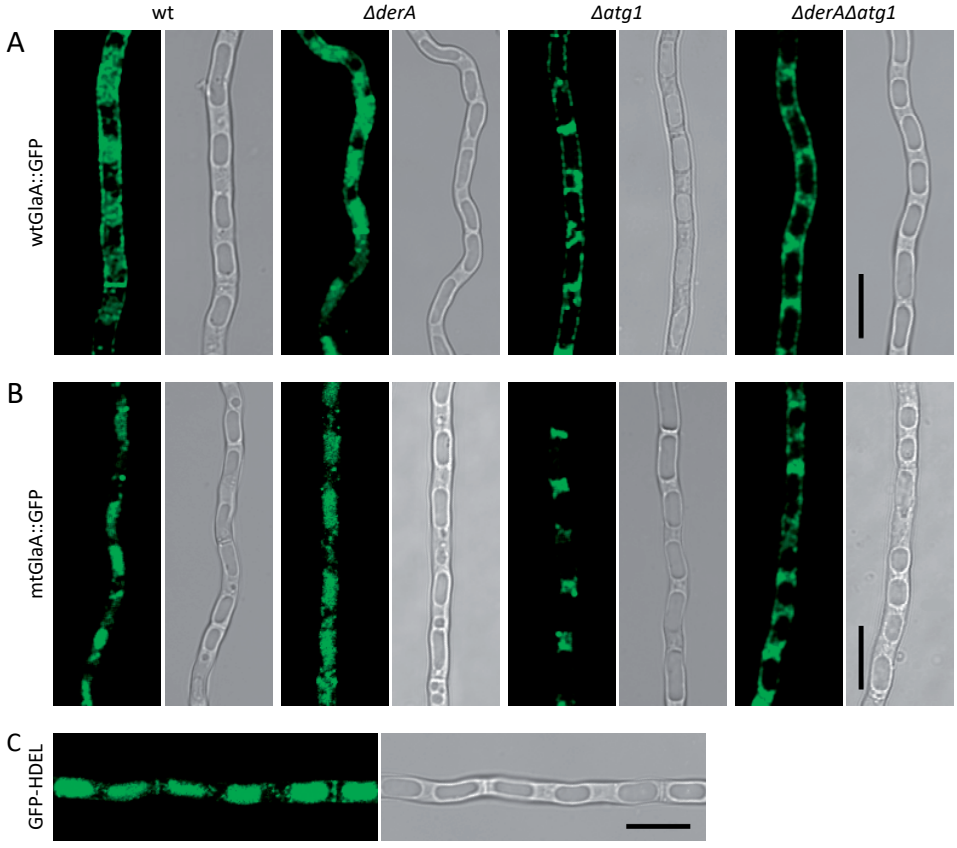

Figure 5 | Localization of wtGlaA::GFP, mtGlaA::GFP and GFP-HDEL during carbon starvation. The strains were pre-grown on coverslips in Petri dishes with MES-buffered MM (pH 6.0) for 7 hours at 30°C. Subsequently, the hyphae were washed and grown in MES-buffered MM (pH 6.0) without glucose for 40 hours at 30°C. Both wtGlaA::GFP (A) and mtGlaA::GFP (B) localized to vacuoles during starvation in wild-type (wt, MA169.4) and Δ*derA* backgrounds, but not in strains bearing the *atg1* deletion. GFP-HDEL also localized to the vacuoles (C). Scale bar: 10 µm.

which is mediated by autophagy. Subsequent examination of an *A. niger* strain expressing ER-targeted GFP under the same conditions showed localization of GFP in the vacuole (Figure 5C), suggesting that parts of the ER are delivered to vacuoles for degradation and recycling upon carbon starvation. Taken together, these results indicate that the transport of both wtGlaA::GFP and mtGlaA::GFP to the vacuole in response to carbon starvation is mediated via the autophagic pathway and is likely to occur as a consequence of bulk degradation of ER, and not specific for misfolded proteins in the ER.

Discussion

In this study, we investigated the effects of defective ERAD and autophagy on the degradation of misfolded secretory proteins in *A. niger*. The natural property of filamentous fungi to produce and secrete large amounts of proteins makes them attractive hosts for the industrial production of extracellular proteins. Approaches to further improve production yields have been successfully taken for the production of homologous proteins, but the secretion capacity for heterologous proteins is to date far lower (Nevalainen and Peterson, 2014). Besides the extracellular activity of efficiently produced secreted proteases (van den Hombergh *et al.*, 1997a; Punt *et al.*, 2008; Yoon *et al.*, 2009, 2011; Braaksma *et al.*, 2009), low product yields can also be the result of intracellular degradation of the protein, as a consequence of the strict quality control in the ER which eventually eliminates proteins that fail proper folding or processing (Jacobs *et al.*, 2009). Increased heterologous protein production has been reported upon the overexpression of genes encoding cellular foldases and chaperones in several *Aspergillus* species (reviewed by Heimel, 2015) and upon the deletion of some autophagy genes in *Aspergillus oryzae* (Yoon *et al.*, 2013). Deletion of ERAD components *derA* and *hrdC* in *A. niger* resulted in an increase in the intracellular accumulation of the heterologous Gla::Gus fusion protein, but had no severe effect on the growth phenotype (Carvalho *et al.*, 2011a). Since persistent accumulations of misfolded proteins are considered harmful to cells by causing severe ER stress, the modest phenotypic effect of compromising ERAD components in *A. niger* was surprising and suggests the presence of an alternative degradation mechanism.

A number of studies have pointed at the importance of autophagy in the degradation of misfolded proteins accumulating in the ER. Several disease-associated mutant proteins have been shown to be removed from the ER via autophagy. Accumulation of a mutant form of the human dysferlin transmembrane protein in the ER was increased after treatment with lysosome inhibitors and in atg5-deficient mice cells, whereas accumulation was inhibited upon induction of the autophagy pathway by rapamycin (Fujita *et al.*, 2007). Likewise, degradation of excess α1-Antitrypsin Z aggregates, which have been associated with the development of chronic liver disease, is dependent on a functional autophagy machinery in yeast and mice cells (Kamimoto *et al.*, 2006; Kruse *et al.*, 2006). Furthermore, cytosolic aggregates of expanded polyglutamine (polyQ) are degraded via ER stress-mediated autophagy (Kouroku *et al.*, 2007). Surprisingly, the misfolded protein constructed in this study (mtGlaA::GFP) showed increased ER-accumulation in the ERAD-defective strain (Δ*derA*), but not in the autophagy mutant (Δ*atg1*) (Figure 4). The double knockout mutant exhibited accumulation levels similar to Δ*derA*, indicating that ERAD is involved in the degradation of misfolded proteins but autophagy is not. Even in the absence of functional ERAD, autophagy is not alternatively taking over its functions in degrading misfolded proteins from the ER.

Gene expression levels of UPR target genes *bipA* and *pdiA* were higher in strains expressing the misfolded mtGlaA::GFP protein, compared to the N402 strain (Figure 2). This suggests the presence of ER stress and has also been demonstrated upon overexpression of the heterologous GlaA::Gus protein in *A. niger* (Carvalho *et al.*, 2011a) and upon expression of a mutant form of cellobiohydrolase I (CBHI) in *Trichoderma reesei* (Kautto *et al.*, 2013). Strains bearing a *derA* deletion showed higher induction than wild-type and Δ*atg1* single knockout strains, indicating a stronger UPR in ERAD-defective backgrounds. However, the observed UPR seemed rather mild and therefore it cannot be excluded that the ER stress caused by the accumulation of mtGlaA::GFP is not sufficient to induce the autophagy pathway. Such an effect has been observed for the α1-Antitrypsin Z protein, as degradation was dependent on autophagy only when this ERAD substrate was overexpressed (Kruse *et al.*, 2006). Interestingly, mutant procollagen trimers are degraded via autophagy, while misfolded procollagen monomers are eliminated through the ERAD pathway (Ishida *et al.*, 2009), suggesting that autophagy is induced as an ultimate strategy for cell survival to remove protein aggregates which cannot be degraded by the ERAD system. Further research is required to determine whether further elevated expression levels (e.g. by constructing multicopy strains) of mtGlaA::GFP are able to induce the process of autophagy in *A. niger.* However, previous experiments have shown that the presence of severe ER stress upon constitutive expression of *hacA* or after treatment with either DTT or tunicamycin did not result in transcriptional induction of the autophagy process in *A. niger* (Guillemette *et al.*, 2007; Carvalho *et al.*, 2012). In addition, our observation that the Δ*derA*Δ*atg1* double mutant expressing mtGlaA::GFP is equally sensitive to the ER stress inducing compound DTT, further supports the conclusion that autophagy is dispensable to overcome ER stress in *A. niger*.

Induction of the autophagy process upon starvation has been demonstrated in many organisms, including *A. niger* (Nitsche *et al.*, 2012). To elucidate whether misfolded proteins are targeted to vacuoles under autophagy-inducing conditions, wtGlaA::GFP and mtGlaA::GFP strains were microscopically examined after 40 hours of carbon starvation. The results show that both wtGlaA::GFP and mtGlaA::GFP proteins are transported to vacuoles in an autophagy-dependent manner (Figure 5A,B). Kimura *et al.* (2011) demonstrated that a misfolded form of the α-amylase protein was delivered to vacuoles via autophagy in late phase cultures of *A. oryzae*. However, in contrast to the observations reported in that study, we found wtGlaA::GFP also being targeted to the vacuoles dependent on autophagy, indicating that vacuolar targeting of proteins from the ER is not specific to misfolded proteins only. During starvation conditions, recycling of cytosolic contents and organelles via autophagy is important for the cell to survive. In this context, the ER can become a substrate of autophagic degradation, which is a specific type of autophagy known as ER-phagy or reticulophagy (Kraft *et al.*, 2009). Recently, Atg39 and Atg40 were identified as receptor proteins mediating this process in yeast and mammalian cells (Khaminets *et al.*, 2015; Mochida *et al.*, 2015). No homologs of Atg39 or Atg40 were identified in the *A. niger*

genome by BLASTp analysis, but in this study we provide evidence for the presence of ER-phagy (Figure 5C). Taken together, these data suggest that the autophagy-dependent delivery of proteins from the ER to the vacuoles upon starvation is likely the result of bulk degradation of ER and its contents.

In this study, a fluorescently labeled misfolded secretory protein was successfully expressed as a reporter in *A. niger*, which is a powerful tool to study the secretory pathway and the response to accumulations of improperly folded proteins in the ER. By expressing this mutant protein in different *A. niger* mutants, we were able to show that autophagy is not specifically involved in the degradation of misfolded proteins from the ER. Surprisingly, the ERAD/autophagy double knockout mutant expressing the misfolded protein was not impaired in growth, although it accumulated the mutant protein in the ER. Since intracellular protein accumulations are normally highly detrimental for cellular functions, it seems likely that the degradation of misfolded mtGlaA::GFP proteins is not completely impaired in Δ*derA* and Δ*derA*Δa*tg1* strains. Other mechanisms might be involved to limit protein accumulations to levels which *A. niger* is able to cope with. In this study we showed that autophagy does not play a role in this process, and we consider vacuolar targeting independent of the autophagic pathway as unlikely, as we did not observe transport of mtGlaA::GFP to vacuoles in *atg1* deletion strains. Yet another possibility is proteasomal degradation independent of DerA. In *A. fumigatus* it has been demonstrated that deletion of the ERAD gene *hrdA* in addition to the deletion of *derA* highly increased the susceptibility to ER stressing agents such as tunicamycin (Krishnan *et al.*, 2013), indicating that DerA and HrdA have synergistic functions. Further studies are required to explore the proteasomal pathway either by making double knockout mutants of the ERAD system or by applying proteasome inhibitors like MG132 (Kautto *et al.*, 2013) and the fluorescent mtGlaA::GFP protein could be used as a reporter to monitor protein accumulations under these conditions.

Acknowledgements

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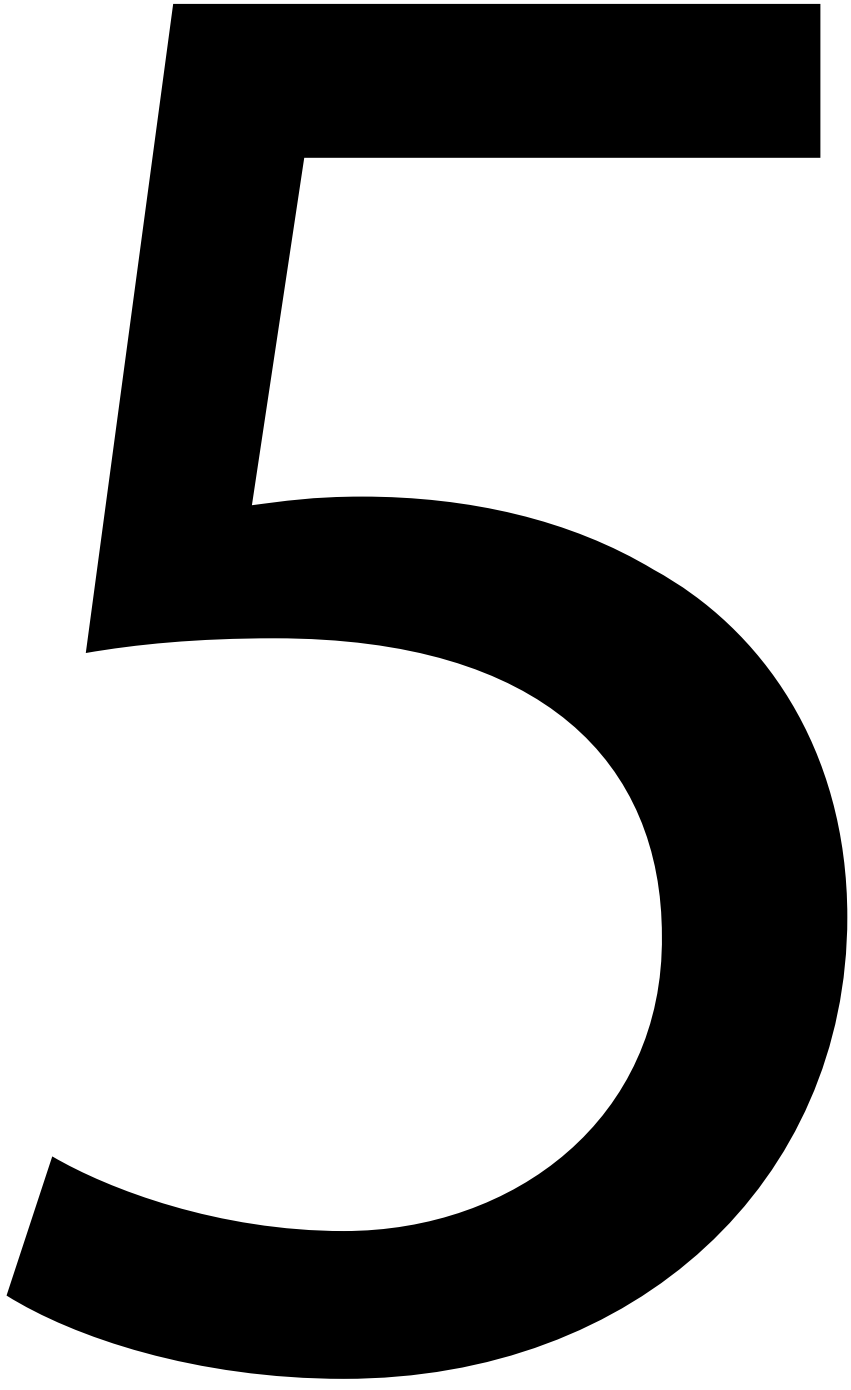

Chapter 5

The unconventional secretion of PepN is independent of a functional autophagy machinery in the filamentous fungus *Aspergillus niger*

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Abstract

During unconventional protein secretion (UPS), proteins do not pass through the classical endoplasmic reticulum (ER)-Golgi-dependent pathway, but are transported to the cell membrane via alternative routes. One type of UPS is dependent on several autophagy-related (Atg) proteins in yeast and mammalian cells, but mechanisms for unconventional secretion are largely unknown for filamentous fungi. In this study, we investigated whether the autophagy machinery is used for UPS in the filamentous fungus *Aspergillus niger*. An aspartic protease, which we called PepN, was identified as being likely to be secreted unconventionally, as this protein is highly abundant in culture filtrates during carbon starvation while it lacks a conventional N-terminal secretion sequence. We analyzed the presence of PepN in the culture filtrates of carbon starved wild-type, *atg1* and *atg8* deletion mutant strains by Western blot analysis and by secretome analysis using nanoLC-ESI-MS/MS (wild-type and *atg8* deletion mutant). Besides the presence of carbohydrate active enzymes and other types of proteases, PepN was abundantly found in culture filtrates of both wild-type and *atg* deletion strains, indicating that the secretion of PepN is independent of the autophagy machinery in *A. niger* and hence most likely occurs via a different mechanism.

Introduction

The vast majority of extracellular proteins is secreted via the classical secretory pathway, which involves vesicle-mediated transport from the endoplasmic reticulum (ER) through the Golgi apparatus to the cell membrane. An N-terminal signal peptide (leader sequence) is responsible for introducing these proteins into the secretion pathway. During their passage through the secretory pathway, secretory proteins are modified and processed to ensure that they are active and stable. These posttranslational modifications steps include the formation of disulphide bridges to ensure a proper tertiary structure of the protein, glycosylation on asparagine or serine and threonine residues, and further processing, e.g. by proteases.

During the last decades, several studies have reported on the presence of extracellular proteins lacking a typical signal peptide, being transported independently of the classical ER-Golgi route (Zhang and Schekman, 2013). This process is now generally known as unconventional protein secretion (UPS), which collectively describes several distinct vesicular and non-vesicular transport pathways. One form of unconventional secretion involving vesicles is associated with the autophagy pathway, requiring autophagy-related (Atg) proteins (Ponpuak *et al.*, 2015). Classically, autophagy is a constitutively active, catabolic process mediating the transport of cytoplasmic components within double membrane vesicles to vacuoles for degradation and recycling. It is strongly induced by starvation conditions as a mechanism to help cells cope with nutrient stress. Besides its degradative function, autophagy is also shown to be involved in unconventional protein secretion as autophagy-related proteins are present in compartments for UPS (CUPS) (Bruns *et al.*, 2011) and the unconventional secretion of different cargo proteins is dependent on several components of the autophagy machinery in yeast and mammalian cells (Duran *et al.*, 2010; Manjithaya *et al.*, 2010; Dupont *et al.*, 2011). Since genes encoding candidates for unconventionally secreted proteins such as PepN, ChiB and hemolysin are expressed during late exponential or stationary phase in *A. niger* (Braaksma *et al.*, 2010; Nitsche *et al.*, 2012; van Munster *et al.*, 2015b), which coincides with the induction of autophagy-related genes (Nitsche *et al.*, 2012), the autophagy process is a likely candidate to be involved in the unconventional secretion of these proteins. PepN has been previously described as PepAb (Wang *et al.*, 2008). As the gene name does not fulfill the gene nomenclature guidelines, we have renamed the gene *pepN*.

In this study we investigated whether the autophagy machinery is involved in the secretion of PepN in *A. niger*. The PepN protease was identified as an extracellular protein likely to be secreted via the unconventional secretion pathway and hence used as a target protein to show its presence in the secretome of Δ*atg1* and Δ*atg8 A. niger* mutants. Atg1 and Atg8 are the orthologs of the Atg1 and Atg8 proteins of *Saccharomyces cerevisiae* (Nitsche *et al.*,

2013). Proteome and Western analyses showed that PepN was abundantly present in culture filtrates of carbon starved cultures of both wild-type and *atg* deletion mutants, indicating that the secretion of PepN is not dependent on a functional autophagy machinery in *A. niger*.

Materials and Methods

Strains, culture conditions and molecular techniques

A. niger strains used in this study are listed in Table 1. Strains were cultivated in standard minimal medium (MM) or complete medium (CM) (Arentshorst *et al.*, 2012). For plate growth, the medium was solidified by the addition of 1.5% agar. Transformation plates were supplemented with 200 µg ml^{-1} hygromycin and 500 µg ml^{-1} caffeine and hygromycin-resistant transformants were subsequently purified on plates containing 100 µg ml^{-1} hygromycin. Transformation of *A. niger* and genomic DNA extraction were performed as described by Arentshorst *et al.* (2012). Southern blot analysis was conducted as described by Sambrook and Russell (2001), using [α-32P]dATP-labeled probes synthesized with the DecaLabel DNA labeling kit (Thermo Scientific).

Generation of an *A. niger pepN* deletion mutant

The *A. niger pepN* gene (An01g00370) was deleted by replacing its open reading frame (ORF) with the hygromycin resistance cassette using the split marker approach as was described in detail by Arentshorst *et al.* (2015b). Approximately 700 bp flanking regions of the *pepN* ORF were PCR amplified from genomic DNA of the N402 strain using primer pairs as listed in Table 2. The hygromycin cassette was PCR amplified from the plasmid pAN7.1 (Punt *et al.*, 1987) using primers hygP6for and hygP7rev (Table 2). Subsequently, 5' and 3' split marker fragments were obtained in two separate fusion PCR amplifications using the respective flank and the hygromycin cassette PCR products as a template and primer pairs according to Table 2. The split marker fragments were transformed to *A. niger* strain MA234.1 and homologous integration was confirmed by Southern analysis (Fig. S1). Strain MA234.1 (*ku70*$^{-}$) was obtained by transformation of strain MA169.4 (*ku70*$^{-}$, *pyrG*$^{-}$) with a 3.8 kb *Xba*I fragment containing the *A. niger pyrG* gene, resulting in the full restoration of the *pyrG* locus in strain MA234.1.

Proteolytic activity

Proteolytic activity was assayed on pre-acidified (pH 3.0) MM plates supplemented with 0.3% skim milk and solidified with 1.5% agarose. Halo sizes were compared after seven days of growth at 30°C. Fluorometric analysis was performed at pH 2.8 using the P-CHECK protease detection kit (Jena Bioscience) according to manufacturer's instructions.

Table 1 | A. niger strains used in this study

Strain	Genotype	Reference
N402	*cspA1* derivative of ATCC9029	Bos *et al.* (1988)
AB1.13	*cspA1*, *prtT*$^-$	Punt *et al.* (2008)
MA234.1	*kusA*::DR-*AmdS*-DR	This study
AW45.1	Δ*pepN*::*hyg* in MA234.1	This study

Table 2 | Primers used in this study

Primer	Sequence (5′ – 3′)	Target	Reference
PepN_P1F	AAGATGGAGT CAGAGCCACC C	5′ flank *pepN* and 5′ split marker fragment	This study
PepN_P2R	CAATTCCAGC AGCGGCTTGA TGGAGGATAA TTGATCAAAG	5′ flank *pepN*	This study
PepN_P3F	ACACGGCACA ATTATCCATC GACGAGTGGC TGAATCTATC ATG	3′ flank *pepN*	This study
PepN_P4R	AAGCCCGTCA GCTCCTTGA	3′ flank *pepN* and 3′ split marker fragment	This study
hygP6for	AAGCCGCTGCTGGAATTGGGCTCTGAGGTGCAGTGGAT	hygromycin cassette	Arentshorst *et al.* (2015b)
hygP7rev	CGATGGATAATTGTGCCGTGTTGGGTGTTACGGAGCATTCA	hygromycin cassette	Arentshorst *et al.* (2015b)
hygP9r	GGCGTCGGTTTCCACTATC	5′ split marker fragment	Arentshorst *et al.* (2015b)
hygP8f	AAAGTTCGACAGCGTCTCC	3′ split marker fragment	Arentshorst *et al.* (2015b)

Secretome analysis

For protein identification, 1 mL of culture filtrate was freeze dried and analyzed using nanoLC-ESI-MS/MS, which was performed by Proteome Factory (Berlin, Germany). Proteins were identified using MS/MS ion search of the Mascot search engine (Matrix Science, London, England) and the non-redundant protein database (National Center for Biotechnology Information, Bethesda, USA). Signal peptide predictions were taken from Braaksma *et al.* (2010) where available or by using the SignalP 4.0 algorithm (Petersen *et al.*, 2011).

Western blot analysis

Anti-PepN antibodies used for Western blot analysis were developed in rabbits by Davids Biotechnologie (Regensburg, Germany) against two different synthetic peptides (Fig. S2) and purified by affinity purification. Extracellular protein samples from bioreactor cultures were obtained by vacuum filtration of the culture broth through glass microfiber filter (Whatmann). Shake flask cultures were filtered through miracloth. Filtrate samples were

mixed with 4x SDS protein sample buffer (8% SDS, 40% glycerol, 20% beta-mercaptoethanol, 0.08% bromophenol blue and 240 mM Tris-HCl pH 6.8), heated for 5 minutes at 95°C and loaded on 9% SDS polyacrylamide gels. Proteins were either stained with the SYPRO® Ruby protein gel stain (Life Technologies) or blotted to a PVDF membrane through semi-dry electrophoretic transfer. The membranes were blocked for 1 hour with 5% non-fat dry milk in PBST (PBS, 0.05% (v/v) Tween-20), incubated with the anti-PepN antibody (1:50) for 1 hour and subsequently incubated with the goat anti-rabbit horseradish peroxidase secondary antibody conjugate (1:20000) (Bio-Rad). Immunoreactive bands were detected using peroxide and luminol/enhancer reagents (Bio-Rad).

Results

PepN is likely to be secreted via an unconventional secretion mechanism

The PepN protein was used as a target protein to study unconventional secretion of proteins in *A. niger.* PepN is a pepsin-like aspartic protease which is abundantly present in the culture filtrate of carbon starved cultures as determined by proteomics studies (Braaksma *et al.*, 2010; Nitsche *et al.*, 2012; Krijgsheld *et al.*, 2012). In contrast to other secreted proteases, it is predicted not to have a typical leader sequence (Wang *et al.*, 2008; Braaksma *et al.*, 2010), indicating that this protein might not pass through the classical secretory pathway, but is secreted by unconventional means. Comparison of the protein sequence of PepN with the secreted aspartic protease PepA in different *Aspergillus* species further showed that PepN misses the conserved pro-peptide which is present in PepA orthologs (Fig. S3). It should be noted that from the available proteome data no peptide sequences could be identified from the first 100 amino acids of PepN (Fig. S2). Although this might be explained by the distribution of arginine and lysine residues in this part of the sequence rendering two very large and several very small peptides upon digestion, it precludes formal proof of secretion of the predicted full length PepN protein. Considering the C-terminal end of the sequence, peptides could be identified resolving the correct annotation of the PepN protein out of two available sequences (NCBI accession: XP_001388485). The protein sequence of PepN contains four putative N-glycosylation sites, but none of these are conserved in other PepN orthologs (Fig. S3), suggesting that these sites in PepN are not glycosylated *in vivo* (Park and Zhang, 2011). This was supported by proteome analyses, which identified two putative glycosylation sites as non-glycosylated residues in the identified peptides (Fig. S2) and by the observed activity of a PepN antibody developed against an epitope containing one of the sites (see below).

The deletion of *pepN* in *A. niger* does not affect proteolytic activity

An *A. niger* Δ*pepN* mutant was generated by replacing the open reading frame with the hygromycin resistance cassette using the split marker approach (Arentshorst *et al.*,

2015b). Homologous integration was confirmed by Southern analysis (Fig. S1). Wild-type and mutant strains were subsequently tested for proteolytic activity by growing them on casein-containing medium for seven days. The size of the clear zone (halo) which is being formed upon degradation of casein correlates to the protease production in the respective strain (Sekine *et al.*, 1969). The results show that halo sizes were comparable between wild-type and Δ*pepN* strains, indicating that strains defective for PepN are not largely affected in total proteolytic activity (Fig. 1A), although Wang *et al.* (2008) showed that deletion of the *pepN* gene affected secreted levels of a heterologous laccase. In agreement with the plate assays, fluorometric analysis of total protease activities of culture filtrates harvested at different time points showed no difference between wild-type and Δ*pepN* mutants (Fig. 1B). To analyze whether expression of the *pepN* gene is controlled by the regulator PrtT (Punt *et al.*, 2008) which regulates the major secreted protease genes *pepA* and *pepB* (van den Hombergh *et al.*, 1997b), transcriptome data of the *prtT* mutant strain AB1.13 (Mattern *et al.*, 1992; Punt *et al.*, 2008) were analyzed for *pepN* expression. In contrast to the very low expression levels for *pepA* and *pepB*, *pepN* expression was still highly abundant (Fig. 1C), indicating that expression of *pepN* is not regulated by PrtT. Whereas PrtT-regulated proteases are responsible for the majority of the protease activity in *A. niger*, PepN activity could be involved in extracellular proteolysis of specific substrates.

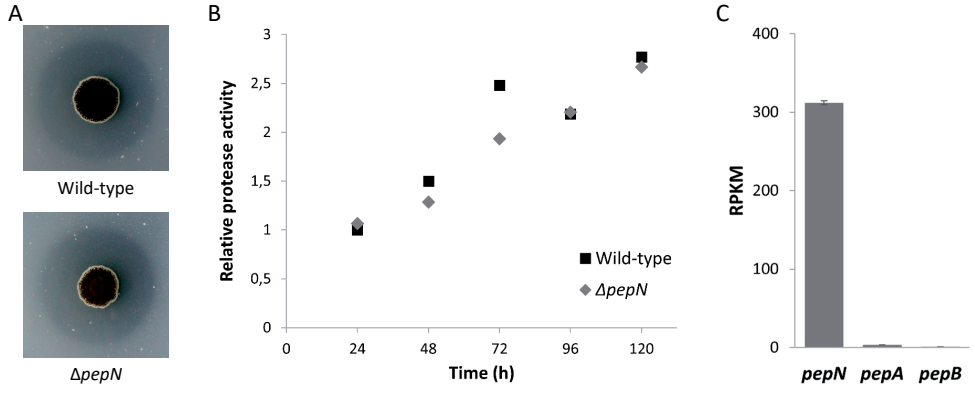

Figure 1 | Deletion of *pepN* in *A. niger* does not affect proteolytic activity. A) Spores from wild-type and Δ*pepN* strains were point inoculated on pre-acidified skim milk plates (pH 3.0) and grown for seven days at 30°C before halo sizes were compared. B) Wild-type and Δ*pepN* strains were grown in CM at 30°C and supernatants were collected at the indicated time points. Proteolytic activities were normalized using wild-type (24h) as a reference. C) Expression of genes encoding selected proteases (PepA, PepB and PepN) in the *prtT*- mutant strain AB1.13. RNA for RNAseq analysis was isolated from shake flask cultures grown for 48 hours in Medium 12 as described by Li *et al.* (2012). Only low RPKM (reads per kilobase per million) values were found for *pepA* and *pepB* protease-encoding genes, whereas higher expression was observed for *pepN*. Error bars indicate standard deviation from two biological replicates.

Proteome analysis of the *A. niger* Δ*atg8* mutant during carbon starvation shows the presence of PepN in culture filtrate

To investigate whether the secretion of PepN is dependent on a functional autophagy machinery, secretomes of wild-type and Δ*atg8* mutant strains were determined and analyzed. The bioreactor cultivations of the wild-type and Δ*atg8* mutant were performed and described previously (Nitsche *et al.*, 2013). For this study, proteins were precipitated from culture filtrates of wild-type and Δ*atg8* bioreactor cultures after 20 hours of carbon starvation, digested with trypsin, and analyzed by mass spectrometry, which revealed that PepN was abundantly present in both wild-type and Δ*atg8* secretomes (Table 3). In agreement with the observed increase in activity of extracellular hydrolases during carbon starvation (White *et al.*, 2002; Nitsche *et al.*, 2012), other proteins that were identified include several proteases and carbohydrate active enzymes, together with some cell wall proteins. The profile of identified proteins showed only minor differences between wild-type and Δ*atg8* secretomes, with some proteins being present in wild-type but not in Δ*atg8* and vice versa. Interestingly, PepN was the only identified protein lacking a predicted signal peptide.

Autophagy-independent secretion of PepN is confirmed by Western analysis of Δ*atg1* and Δ*atg8* culture filtrates.

The presence of PepN in culture filtrates of both wild-type and Δ*atg8* strains indicates that the secretion of PepN is not dependent on a functional Atg8 protein. In order to confirm this result and to test other mutants on their ability to secrete PepN into the culture medium, Western blots were performed using PepN specific antibodies. Two individual peptide antibodies were produced (Fig. S2), which were first tested for their specificity to PepN using culture filtrates of the wild-type and the Δ*pepN* mutant, collected from shake flask cultures during carbon starvation. Protein staining following gel electrophoresis showed a comparable protein profile between wild-type and Δ*pepN* samples (data not shown). Furthermore, both of the antibodies showed activity against the PepN protein (data not shown), and the antibody against the epitope closest to the C-terminal was chosen to be used for further experiments. It showed a clear band in wild-type samples between 40 kDa and 50 kDa, which corresponds to the expected size of the non-glycosylated protein (47 kDa) (Fig. 2A). As expected, this band was not observed in Δ*pepN* culture filtrates. It should be noted that the antibody showed cross-reactivity towards a slightly larger protein, but this cross reactivity was also found in proteins from the culture fluid of the *pepN* mutant (Fig. 2 and data not shown). The detection of this cross-reactive protein was variable between different experiments. The antibody was subsequently used to investigate whether PepN was also present in bioreactor culture filtrates of the autophagy mutants Δ*atg1* and Δ*atg8* (Nitsche *et al.*, 2013). In agreement with the results from the secretome analysis, PepN could be detected on Western blot in culture filtrates of the wild-type and the Δ*atg8* mutant after 20 hours of carbon starvation (day 1, Fig. 2B). Additionally, PepN was not present during the exponential growth phase, but was found on all other carbon starvation time points tested,

Table 3 | Secretome data

			Protein abundance[a]		
Identifier	**Gene**	**(Predicted) function**	**N402**	**Δ*atg8***	**SP[b]**
Proteases/peptidases					
An01g00370	*pepN*	Aspartic protease	+++	++++	No SP
An01g00530	*pepB*	Acidic protease	+	-	SP
An01g01750		Sedolisin protease	+	+[c]	SP
An02g04690		Serine-type carboxypeptidase	+	+[c]	SP
An03g05200	*protF*	Carboxypeptidase	+	+	SP
An06g00190		Sedolisin protease	+	+[c]	SP
An08g04490	*protA*	Lysosomal carboxypeptidase	++	-	SP
An08g04640	*protB*	Sedolisin protease	+	-	SP
An14g04710	*pepA*	Aspartic protease	++++	++++	SP
An18g01320		Pepsin protease	+[c]	+	SP
ATCC 53364		Aspartic protease	-	+	SP
Carbohydrate active enzymes					
An01g11010	*crhD*	Putative chitin-β-glucanosyltransferase	+	++	SP
An01g12450	*exsG*	Exo-1,3-β-glucanase	+	+++	SP
An01g12550	*msdS*	1,2-α-mannosidase	-	++	SP
An03g05290	*bgtB*	Glucan endo-1,3-β-glucosidase	++	+	SP
An03g06550	*glaA*	Glucan 1,4-α-glucosidase	++++	++++	SP
An09g00670	*gelD*	1,3-β-glucanosyltransferase	+	+[c]	SP
An11g03340	*aamA*	acid α-amylase	++	+++	SP
An15g02300	*abfB*	α-arabinofuranosidase	++	++	SP
An16g03330		GPI-anchored cell wall protein	-	+	SP
An16g06800	*eglB*	Endo-1,4-β-glucanase	+	+[c]	SP
ATCC 53033		1,3-β-glucanosyltransferase	+	++	SP
Cell wall proteins					
An04g01230	*ecmA*	Putative cell wall organization protein	+	++	SP
An06g00160		Hypothetical protein	++	+++	SP
An08g09420		Antigenic cell wall galactomannoprotein	-	+	SP
An14g01820	*phiA*	Cell wall protein	+	-	SP
An14g02100	*cwpA*	GPI-anchored cell wall mannoprotein	+	++	SP
Other					
An02g13750		Glutaminase A	+	++	SP
An07g08400		Strong similarity to rAsp f 4	-	+	SP
An16g01880	*lipanI*	Lysophospholipase	++	+[c]	SP
ATCC 55058		Hypothetical protein	+[c]	+	SP

[a] Protein abundance as roughly determined from biological duplicate experiments as described by Ishihama *et al.* (2005): (++++) > 10%; (+++) > 4%; (++) > 2%; (+) > 0%; (-) not detected.
[b] Signal peptide prediction (Braaksma *et al.*, 2010; Petersen *et al.*, 2011).
[c] Result based on detection in only one of the duplicates.

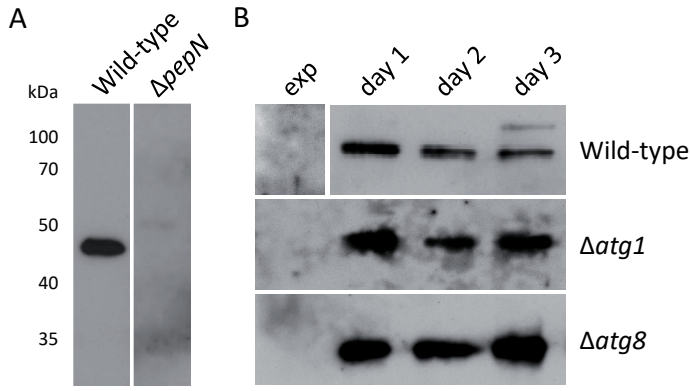

Figure 2 | Western blot analysis on the presence of the PepN protein. A) Culture filtrate samples were collected from wild-type and Δ*pepN* shake flask cultures after 120h of growth at 30°C in CM. The protein samples were separated by gel electrophoresis and immunodetected with an anti-PepN antibody. B) Culture filtrates were collected from wild-type, Δ*atg1* and Δ*atg8* bioreactor cultures (Nitsche *et al.*, 2013) during the exponential growth phase (exp) and during carbon starvation as indicated (day 1 – day 3). Equal amounts of culture fluid (15 µL) were loaded in each lane to be separated by gel electrophoresis and immunodetected with an anti-PepN antibody. In some cases, a second band was observed above the PepN band, which is probably the result of cross-reactivity of the antibody with another protein in the sample.

both in wild-type, Δ*atg1* and Δ*atg8* culture filtrates. This indicates that the secretion of PepN is induced by carbon starvation conditions, which has been reported before (Braaksma *et al.*, 2010; Nitsche *et al.*, 2012), but neither *atg1* nor *atg8* is required for the efficient secretion of PepN into the culture broth.

Discussion

Improving our understanding of the mechanisms for UPS in filamentous fungi is of great interest, because the use of these pathways for the production of heterologous proteins could result in highly improved production yields and activities as proteins exported via unconventional secretion most likely do not undergo unwanted posttranslational modifications. One of the major bottlenecks for the production of heterologous proteins in filamentous fungi arises with the strict protein quality control in the ER, which recognizes misfolded proteins and targets them for degradation via the ER-associated degradation pathway (Ruggiano *et al.*, 2014). Unwanted modification processes also include glycosylation and processing by Golgi-localized proteases, which might affect the activity of the secreted proteins (Schmidt, 2004; Nevalainen and Peterson, 2014). To circumvent the posttranslational modification of extracellular proteins during their passage through the classical secretory pathway, the use of UPS pathways is a promising alternative. This

principle has already been successfully applied in *Ustilago maydis*, in which heterologous proteins were secreted unconventionally by fusing them to the N-terminus of Cts1 (Stock *et al.*, 2012). However, in contrary to other eukaryotic organisms, the mechanisms by which proteins are exported via UPS pathways are largely unknown for filamentous fungi. To gain more insights into the process of UPS in *A. niger*, we investigated whether autophagy is an involved mechanism by analyzing secretomes of *atg* deletion mutants for the presence of the PepN protein.

An increasing number of proteins have been shown to be secreted in an unconventional manner. As such, we identified the PepN protein to be exported most likely without transiting through the conventional secretory pathway. An alternative explanation for the presence of PepN in the culture filtrate could be the occurrence of cell lysis. However, in previous studies (Braaksma *et al.*, 2010; Nitsche *et al.*, 2012), and in this present study, PepN was found to be abundantly present in the culture fluid while the contribution of cytosolic proteins in these studies was found to be very limited. Currently, we can formally not exclude that PepN is a cytosolic protein that is present in the culture filtrate after cell lysis and that detection of PepN is possible because it is resistant against proteolytic degradation while other cytosolic proteins are quickly degraded. However, we find this scenario less probable also given the fact that unconventional secretion of other proteins, e.g. Cts1 in *Ustilago maydis* has been shown to take place (Stock *et al.*, 2012). The structure of the PepN protein sequence is different from that of the secreted protease PepA, as it lacks the conserved pro-peptide sequence and a typical secretion peptide (Fig. S3). BLASTp analysis showed that PepN orthologs are found in other *Aspergillus* species, as well as in *Trichoderma* and *Penicillium* species, also lack a predicted signal peptide sequence (see Fig. S3). The absence of a predicted signal peptide provides evidence for the PepN protein to be secreted via an unconventional pathway. Furthermore, no indications were found for posttranslational processing events on PepN, as putative glycosylation sites were determined to be non-glycosylated *in vivo* (Fig. S2), while the size of the protein on Western blot corresponded well to the expected full-length protein size (Fig. 2A).

The deletion of *pepN* in a heterologous laccase producing Δ*pepA* background increased the total extracellular laccase activity compared to the parental strain (Wang *et al.*, 2008), indicating that eliminating the proteolytic activity deduced by PepN could improve production yields. Proteolytic activity was also decreased upon the deletion of *pepA* or *pepB* in *A. niger* (Mattern *et al.*, 1992; van den Hombergh *et al.*, 1997b). However, the single deletion of *pepN* performed in this present study did not have an effect on total proteolytic activity compared to wild-type (Fig. 1), which might indicate that its activity is highly substrate specific as was observed for other aspartic proteases such as chymosin (Kageyama, 2002) or could only be observed in combination with the deletion of other highly expressed protease genes like *pepA* or *pepB*, or under specific growth conditions.

Autophagy has been associated to UPS based on studies in yeast and mammalian cells showing that the unconventional secretion of several proteins is dependent on elements of the autophagy machinery (Ponpuak *et al.*, 2015). The unconventional secretion of Acyl-CoA binding protein (Acb1) in yeast requires several autophagy-related genes, including *atg1* and *atg8* (Duran *et al.*, 2010; Manjithaya *et al.*, 2010). Similarly, in mammalian cells it was shown that the unconventional secretion of the cytokine IL-1β is promoted by the induction of autophagy in an Atg5-dependent manner (Dupont *et al.*, 2011). By using both proteome (mass spectrometrical) and immunoblotting analyses, we were able to show that PepN was present in secretomes of wild-type, Δ*atg1* and Δ*atg8* mutants (Table 3; Fig. 2B), indicating that both Atg1 and Atg8 are not required for the secretion of PepN in *A. niger*. Since these autophagy proteins are considered to have important roles in the initiation of autophagy and the formation of autophagosomal vesicles, respectively (Inoue and Klionsky, 2010; Bartoszewska and Kiel, 2011), and were shown to be essential for autophagy in *A. niger* (Nitsche *et al.*, 2013), it was concluded that the secretion of PepN is independent of the autophagy machinery. However, it cannot be excluded that autophagy is involved in the unconventional secretion of proteins other than PepN, although this might be dependent on specific culture conditions as PepN was the only protein identified during this study lacking a predicted signal sequence. An obvious candidate gene with a possible function in non-classical secretion in *A. niger* is the GRASP (Golgi-reassembly and stocking protein) homolog. GRASP has been shown to be required for the unconventional secretion of Acb1 in *Dictyostelium discoideum* and *Pichia pastoris* (Kinseth *et al.*, 2007; Manjithaya *et al.*, 2010). A BLASTp search identified a candidate GRASP protein in *A. niger* (An11g05650) and its possible role in PepN secretion could be addressed in future experiments. It should be noticed however that subsequent studies also showed that Acb1 secretion required, in addition to GRASP, autophagy proteins (Duran *et al.*, 2010; Manjithaya *et al.*, 2010). Although our study does not exclude the possibility of GRASP involvement, the mechanism of unconventional secretion of PepN in *A. niger* is likely to be different from that of the Acb1 protein as autophagy is dispensable for PepN secretion. Further studies are required in order to increase our understanding of mechanisms for UPS as well as to identify genes that are involved in these processes in *A. niger.*

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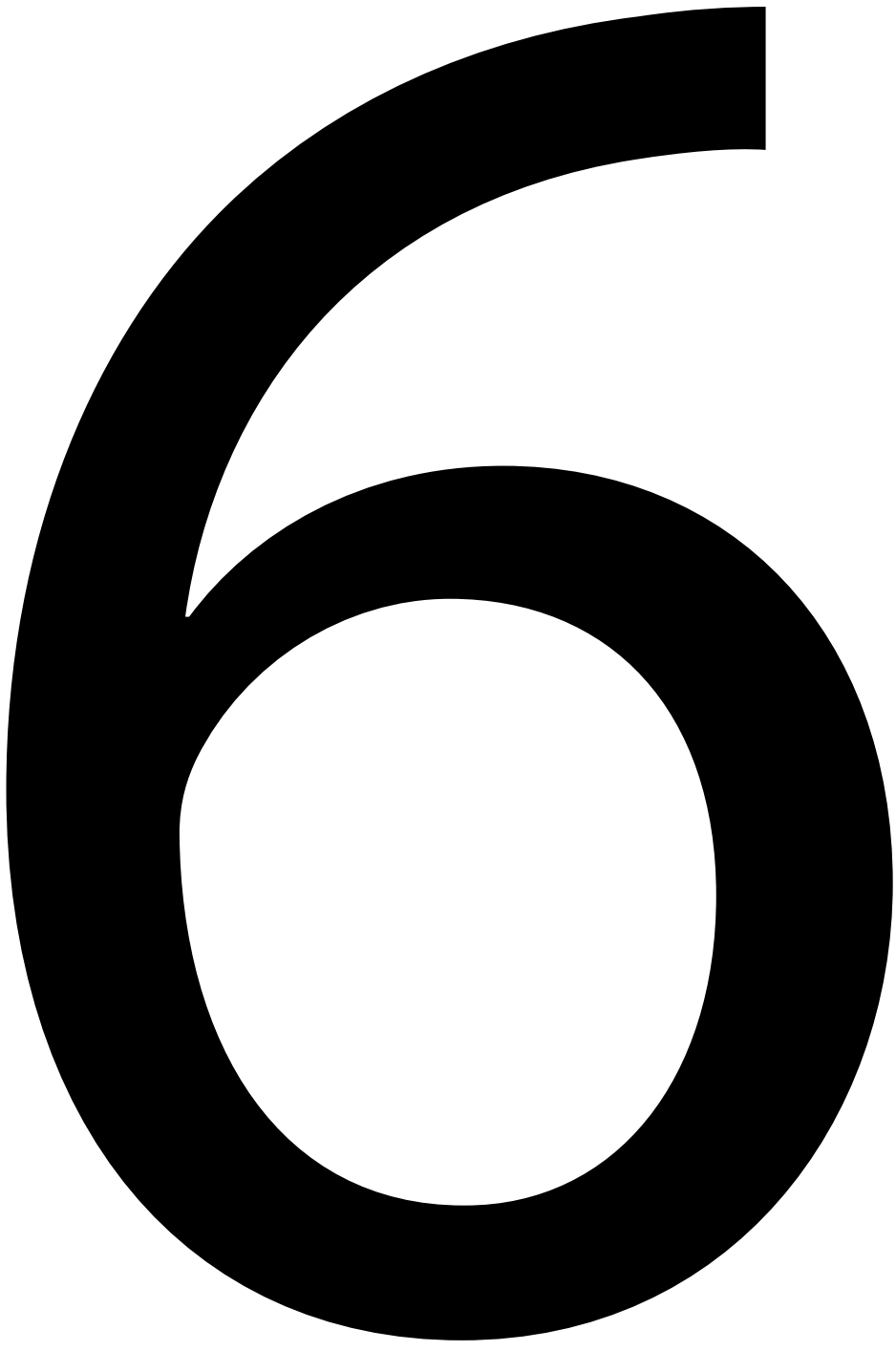

Chapter 6

Summarizing discussion

The carbon starvation response – induction of autophagy

Aspergillus niger is a filamentous fungus with a saprophytic lifestyle, proliferating on organic materials originating from plants. Sensing the available nutrients in the growth environment and efficient utilization is important in order to promote fungal growth and development. As such, *A. niger* features a large variety of enzymes and a naturally high secretion capacity which help the fungus to appropriately deal with the available nutrient sources. However, as nutrients are not always abundantly present and limitations are common, fungi possess specific response mechanisms serving to adapt to such environmental stress conditions and enabling cell survival. From the different nutrient sources, the response to limitation of carbon has been most well-studied, both in filamentous fungi in general and in *A. niger.* The carbon starvation response (reviewed in chapter 1) is rather complex and aims at maintaining biomass and scouting for new carbon sources at the one hand and the formation of asexual reproductive structures on the other hand. For both ways, liberating of energy (ATP) and building blocks through endogenous recycling or extracellular hydrolysis is essential in order to fuel outgrowth of hyphae and conidiation. In this respect, autophagy is an important process during starvation, as was supported by transcriptomics studies showing that autophagy is one of the most dominantly induced processes upon starvation conditions in *Aspergilli* (Nitsche *et al.*, 2012; Krohn *et al.*, 2014). Besides its importance in nutrient recycling during starvation, other studies have shown that autophagy might also be involved in e.g. protein secretion, pathogenicity and degradation of damaged proteins and organelles. In this thesis, different roles of autophagy (both in nutrient-rich and during starvation conditions) in *A. niger* were studied. *A. niger* mutants defective for autophagy were constructed and phenotypically analyzed both in surface and in submerged growth (chapter 2). Subsequently, whole transcriptome analysis comparing the autophagy mutant with the wild-type during submerged cultivation was performed (chapter 3) and the specific role of autophagy in the degradation of misfolded proteins (chapter 4) and in unconventional protein secretion (chapter 5) was assessed.

Phenotypic, morphologic and transcriptomic analysis of autophagy mutants during starvation

Genome-wide transcriptional profiling has shown that the majority of the autophagy orthologs in *A. niger* is transcriptionally induced during carbon starvation (Nitsche *et al.*, 2012), suggesting an important role for autophagy in nutrient-limited conditions. To investigate the functions of autophagy during starvation in more detail, gene deletion mutants were constructed after which Δ*atg1* and Δ*atg8* strains were found to be severely impaired in autophagy (chapter 2). During submerged carbon starvation, these mutants showed accelerated cell death of older hyphal compartments accompanied by a faster emergence of thin non-branching hyphae compared to the wild-type. Furthermore, transport of mitochondria to the vacuoles was severely impaired. From transcriptome data comparing the Δ*atg1* mutant with the wild-type, it was shown that ribosomal genes

were lower expressed whereas genes related to DNA repair and cell cycle showed higher expression (chapter 3). Taken together, the results suggest that autophagy is important for the adaptation to nutrient-limited conditions by influencing multiple cellular processes. However, these responses are rather complex and far from understood. For example, autophagy has been shown to be related to programmed cell death both in yeast and mammalian cells, but depending on environmental factors it can either contribute to or prevent it (Nikoletopoulou *et al.*, 2013; Liu and Levine, 2015; Falcone and Mazzoni, 2016). Furthermore, it has been suggested that the antifungal protein AFP contributes to survival of *A. niger* during carbon starvation through interaction with the autophagy machinery (Paege *et al.*, 2016). The *afp* gene was found to be co-expressed with *atg4* and *atg8* during carbon starvation and induction of the *afp* promoter was specifically observed in highly vacuolated compartments. So far, studies on autophagy during starvation mainly focus on carbon starvation, hence the role of autophagy during nitrogen starvation is limited studied. Preliminary results from transcriptome analysis on prolonged nitrogen-starved *A. niger* batch cultures confirmed that the expression of autophagy genes is also significantly increased under limitations of nitrogen (unpublished data). However, as biomass growth and the formation of hyphal ghosts behaved differently as compared to carbon starvation conditions, studying the role of autophagy during nitrogen starvation would be of interest for future research.

Analysis of autophagy mutants during normal growth conditions

Autophagy is a constitutively active process, hence it is suggested to be also involved in cellular functions during normal growth conditions. Somewhat surprisingly however, the effects of deleting genes essential for autophagy in *A. niger* were only mild in comparison with other filamentous fungus species e.g. *Podospora anserina* and *A. oryzae* (Pinan-Lucarré *et al.*, 2005; Kikuma *et al.*, 2006), which were severely affected in the formation of aerial hyphae and conidia. In *A. niger*, the deletion of the autophagy-essential genes *atg1* or *atg8* in *A. niger* rendered viable mutants, which showed only a mild reduction in growth rate both in surface and in submerged growth during the exponential phase (chapter 2). Accordingly, only a small minority of genes were differentially expressed in the Δ*atg1* mutant compared to the wild-type during exponential growth (chapter 3). Despite that autophagy is supposed to play an important role in cellular maintenance during normal growth (Papáčková and Cahová, 2014), *A. niger* mutants defective for autophagy are not severely impaired in cellular functions.

Autophagy and ER stress conditions

The autophagic pathway aims at the degradation of cellular components in order to free energy and reuse building blocks. Remarkably, not only small cytosolic proteins are recycled via autophagy, but also larger cellular components, like organelles, are transported to vacuoles in autophagic vesicles (Kanki *et al.*, 2015; Kikuma *et al.*, 2017a). We have

demonstrated the autophagy-dependent vacuolization of mitochondria and endoplasmic reticulum (ER) in *A. niger* (chapter 2, chapter 4) and this has also been shown in other *Aspergilli* species. In addition, whole nuclei and peroxisomes can also become the subject of autophagic degradation (Amor *et al.*, 2000; Kikuma *et al.*, 2017b). The uptake of such a variety of cellular constituents by autophagy raises the question whether there might be specificity for degrading components that are harmful for the cell, e.g. reducing cellular ROS levels by specific turnover of (damaged) mitochondria or removing intra-ER accumulations of misfolded proteins by degrading specific parts of the ER. In fact, a number of studies have shown that several disease-associated mutant proteins are being removed from the ER via autophagy both in yeast and mammalian cells (reviewed in: Ciechanover and Kwon, 2017). Also in *A. oryzae* and *A. nidulans* misfolded mutant proteins were transported to vacuoles using the autophagic pathway (Kimura *et al.*, 2011; Evangelinos *et al.*, 2016), although this was observed only after subjecting the fungi to starvation conditions. To study whether autophagy is involved in overcoming ER stress caused by accumulations of misfolded proteins in *A. niger*, we induced ER stress by expressing a mutant form of the secretory protein glucoamylase, in which disulfide bonds were mutated supposedly resulting in misfolding of the protein (chapter 4). Fusion of the protein to GFP enabled the visualization of its cellular localization during different environmental conditions. As expected, the degradation of the mutant GlaA::GFP was dependent on a functional proteasomal pathway as the mutant protein accumulated in the ER in strains defective for ER-associated degradation (ERAD). The mutant protein was not degraded via the autophagic pathway, not even in the absence of functional ERAD. Furthermore, the ERAD autophagy double knockout mutant did not show increased sensitivity to the ER-stress inducing agent dithiothreitol, indicating that autophagy is dispensable even under severe ER stress conditions.

Autophagy and unconventional secretion of PepN

Autophagy generally is considered a catabolic process, transporting cytoplasmic components within double membrane vesicles to vacuoles for degradation and recycling. However, increasing evidence shows that autophagic vesicles can also be used for the vesicle-mediated secretion of extracellular proteins in a non-classical manner (Ponpuak *et al.*, 2015). This unconventional protein secretion (UPS) comprises several distinct vesicular and non-vesicular pathways and mediates the transport of proteins lacking a typical signal peptide independently of the classical ER-Golgi route. Based on studies in yeast and mammalian cells, several proteins have been identified that are being unconventionally secreted dependent on elements of the autophagy machinery e.g. IL-1β in mammalian cells and Acb1 in yeast (Ponpuak *et al.*, 2015). By using the model protein PepN, which is likely being secreted in an unconventional manner, we investigated whether the autophagy machinery is used in UPS in *A. niger*. The results showed that PepN was being secreted independent of the availability of functional autophagy components (chapter

5). In accordance with our results, a more recent study in *A. oryzae* demonstrated that the autophagy-related protein Atg1 was not required for the unconventional secretion of another non-classical protein, namely the Acb1 ortholog Acb2 (Kwon *et al.*, 2017). This data suggest that unconventional secretion, unlike UPS in *S. cerevisiae*, is independent of the autophagy machinery in *Aspergillus* species. However, further candidate proteins should be identified and studied to further confirm this conclusion. At the other hand, unraveling which mechanism is being used to secrete non-classical proteins such as PepN would be of much interest.

Concluding remarks and future outlook

The high secretion capacity of *A. niger* is successfully exploited for the large-scale industrial production of a wide range of organic acids, enzymes and proteins. A better understanding of (stress-related) processes that hamper the production process and lower production yields might contribute to the improvement of *A. niger* as a cell factory. In this respect, autophagy was an obvious process to study, since this pathway is highly induced during nutrient limitation conditions. It was anticipated that autophagy might be related to UPR/ERAD and unconventional protein secretion. However, no evidence was found that autophagy is involved in these processes and hence the role of autophagy is smaller than expected.

In conclusion, this thesis shows that autophagy is an important process in the filamentous fungus *A. niger*, as corresponding gene sets were constitutively active under normal growth conditions and even highly upregulated during starvation conditions. However, it has been proven difficult to specifically identify how autophagy is involved in cellular biology, especially during normal growth circumstances. Whereas the deletion of genes that are essential for the process of autophagy in *A. niger* clearly affected growth and development during carbon starvation, this was not the case during normal growth conditions. Autophagy deletion mutants were neither hampered in the degradation of a misfolded mutant protein mtGlaA::GFP nor in the secretion of unconventionally secreted proteins such as PepN and Acb2. Further research is required to further unravel the functions of autophagy and discover its interactions with other processes enabling the maintenance of cellular homeostasis. This is in particular of interest since in unicellular fungi and mammalian cells deletion of autophagy-related genes has a much more severe effect on growth characteristics. Possibly, the mycelial growth phenotype of filamentous fungi such as *A. niger*, together with the ability to produce spores may allow escape from these effects, explaining the proliferous growth of filamentous fungi also under extremely stressful growth conditions.

Samenvatting

Voor filamenteuze schimmels die leven op de restanten van dood plantaardig materiaal, zoals *Aspergillus niger*, is het niet ongewoon dat er tekorten optreden in de beschikbare koolstofbronnen die uit dat plantaardige materiaal kunnen worden vrijgemaakt. Om zulke omstandigheden te kunnen overleven, worden specifieke overlevingsstrategieën geïnduceerd. Een van de processen die tijdens koolstoflimitatie wordt geïnduceerd, is autofagie.

Autofagie is een intracellulair afbraakmechanisme waarbij cytosolische componenten worden afgebroken en gerecycled zodat de bouwstenen kunnen worden hergebruikt door de cel. Dit proces komt in veel organismen voor, ook in schimmels, waaronder in *A. niger*. Gedurende autofagie in *A. niger* worden cytosolische eiwitten en organellen opgenomen in blaasjes omgeven door een dubbele membraan om zo getransporteerd te kunnen worden naar de vacuole. Deze blaasjes worden autofagosomen genoemd. De buitenste membraan van het autofagosoom fuseert met de membraan van de vacuole, waarna een blaasje met een enkele membraan uitkomt binnenin de vacuole. Dit blaasje en de inhoud daarvan worden dan in de vacuole afgebroken en de afbraakproducten worden terug getransporteerd naar het cytosol, zodat ze kunnen worden hergebruikt. De eiwitten die betrokken zijn bij zowel de regulatie van autofagie als bij het proces zelf, worden autofagie-gerelateerde (Atg) eiwitten genoemd. Deze Atg eiwitten worden gecodeerd door *atg* genen. Het proces van autofagie en de genen die coderen voor Atg eiwitten worden sterk geïnduceerd door een gebrek aan koolstofbronnen in de omgeving, een omstandigheid waarin het recyclen van nutriënten van groot belang is voor het onderhouden van essentiële cellulaire functies, sporevorming en differentiatie. Naast autofagie zijn er verschillende andere mechanismes die bijdragen aan het overleven in condities van tekorten aan koolstofbronnen. Een overzicht van de complexe reacties op koolstofbrontekorten in *Aspergillus* soorten wordt gegeven in hoofdstuk 1 van dit proefschrift.

Naast dat autofagie belangrijk is voor het recyclen van nutriënten tijdens (koolstof) hongering, wordt verondersteld dat het betrokken is bij eiwitsecretie, pathogeniteit en afbraak van beschadigde eiwitten en organellen. In dit proefschrift zijn verschillende rollen van autofagie bestudeerd, zowel in nutriëntrijke als in nutriëntarme omstandigheden. Hiervoor zijn deletiemutanten gemaakt van een aantal autofagie-gerelateerde genen. In hoofdstuk 2 worden de effecten van deze deleties beschreven op zowel groei op plaat als in vloeibare culturen. Microscopisch onderzoek van reporterstammen heeft aangetoond dat de genen *atg1* en *atg8* essentieel zijn voor autofagie in *A. niger*, terwijl *atg17* dat niet is. Het uitschakelen van *atg1* of *atg8* in *A. niger* leidde tot effecten op het fenotype. Op plaat

werd een verminderde sporeproductie gezien voor deze mutanten en in bioreactorculturen was de maximale specifieke groeisnelheid voor deze stammen lager dan in de wild-type stam. Daarnaast werd de vorming van dunne, onvertakte hyfen en het daarbij behorende ontstaan van lege celcompartimenten in de autofagiemutanten versneld.

Transcriptoomanalyse op verschillende tijdspunten tijdens koolstofhongering heeft aangetoond dat er in *A. niger* wild-type onderscheid te maken is tussen de vroege en de late respons op koolstofhongering (zie hoofdstuk 1). Dit onderscheid werd ook teruggezien in de vergelijking tussen de Δ*atg1* autofagie mutant en het wild-type (hoofdstuk 3). Vroeg in de post-exponentiële fase werden namelijk andere groepen van genen met een verschillend expressie niveau geïdentificeerd dan laat in de post-exponentiële fase. Een dag nadat de koolstofbron was uitgeput, kwamen genen gerelateerd aan DNA herstel en celdeling in de autofagiemutant hoger tot expressie dan in het wild-type. Nog later in de tijd (zes dagen nadat de koolstofbron uitgeput was) kwamen ribosomale genen en metabolische processen hoger tot expressie.

De rol van autofagie bij verschillende cellulaire processen werd verder onderzocht door te kijken naar de mogelijke betrokkenheid van autofagie bij het afbreken van misgevouwen eiwitten in het endoplasmatisch reticulum (ER) (hoofdstuk 4). Ongevouwen en misgevouwen eiwitten worden normaal gesproken afgebroken via de ER-associated degradation (ERAD) pathway, maar *A. niger* ERAD deletiemutanten zijn nauwelijks belemmerd in groei, zelfs niet als ER stress wordt geïnduceerd. Om te bestuderen of autofagie de rol van ERAD overneemt, werden mutanten gemaakt die zowel een deletie van een ERAD gen als van een autofagie gen hadden. Deze dubbelmutanten werden vergeleken met de enkele ERAD mutant. Er werden geen verschillen gevonden in groei en in de dubbelmutant was er geen sprake van meer ophoping van misgevouwen heterologe eiwitten in het ER. Daaruit blijkt dat autofagie in *A. niger* geen rol speelt als alternatief in de afbraak van misgevouwen eiwitten uit het ER bij afwezigheid van ERAD.

In hoofdstuk 5 van dit proefschrift is onderzocht of autofagie betrokken is bij onconventionele eiwitsecretie (UPS, unconventional protein secretion) in *A. niger*. Tijdens UPS worden secretoire eiwitten onafhankelijk van de klassieke ER-Golgi route naar de celmembraan getransporteerd. De protease PepN is in *A. niger* geïdentificeerd als een eiwit dat tijdens koolstofhongering gesecreteerd wordt via de niet-klassieke secretieroute. Onderzoek aan filtraten van *A. niger* autofagiemutanten toonde aan dat PepN ook aanwezig was in het secretoom van stammen waarin autofagie niet functioneert. Dit toont aan dat de secretie van PepN niet afhankelijk is van een werkend autofagieproces in *A. niger*.

Het onderzoek beschreven in dit proefschrift heeft nieuwe inzichten gegeven in de functies van autofagie in de schimmelbiologie. De rol van autofagie in *A. niger* blijkt complexer

dan verwacht en verschillend van de rol die het speelt in andere schimmelsoorten. Het uitschakelen van genen die essentieel zijn voor het autofagieproces in *A. niger* leidde tot duidelijke effecten op de groei en de ontwikkeling van dunne hyfen tijdens koolstofhongering, maar deze effecten waren gering in vergelijking met de resultaten zoals gevonden in unicellulaire schimmels en zoogdiercellen. Daarnaast toont ons onderzoek aan dat in *A. niger* autofagie en ERAD niet functioneel redundant zijn als het gaat om het afbreken van misgevouwen eiwitten en dat autofagie niet betrokken is bij de onconventionele secretie van PepN. Verder onderzoek is nodig om de functies van autofagie in *A. niger* te identificeren en interacties met andere processen te ontdekken.

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Supplementary material

Chapter 2

Chapter 3

Chapter 4

Chapter 5

List of publications

Jørgensen, T.R., **Burggraaf, A.-M.**, Arentshorst, M., Schutze, T., Lamers, G., Niu, J., Kwon, M.J., Park, J., Frisvad, J.C., Nielsen, K.F. Meyer, V., Hondel, C.A.M.J.J. van den, Dyer, P.S., and Ram, A.F.J. (2020) Identification of SclB, a Zn(II)2Cys6 transcription factor involved in sclerotium formation in *Aspergillus niger*. *Fungal Genet Biol* **139**: 103377.

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Curriculum vitae

Anne-Marie Burggraaf werd geboren op 24 februari 1988 te Alphen aan den Rijn en groeide op in Stolwijk. Na het behalen van haar VWO diploma in 2006, studeerde ze Biologie aan de Universiteit Leiden. Tijdens haar master Molecular and Cellular Biosciences deed zij een onderzoeksproject bij de afdeling Molecular Microbiology and Biotechnology. In 2011 studeerde ze cum laude af en begon in dezelfde onderzoeksgroep aan het promotieonderzoek wat resulteerde in dit proefschrift. Anne-Marie werkt momenteel als data engineer bij Eneco.