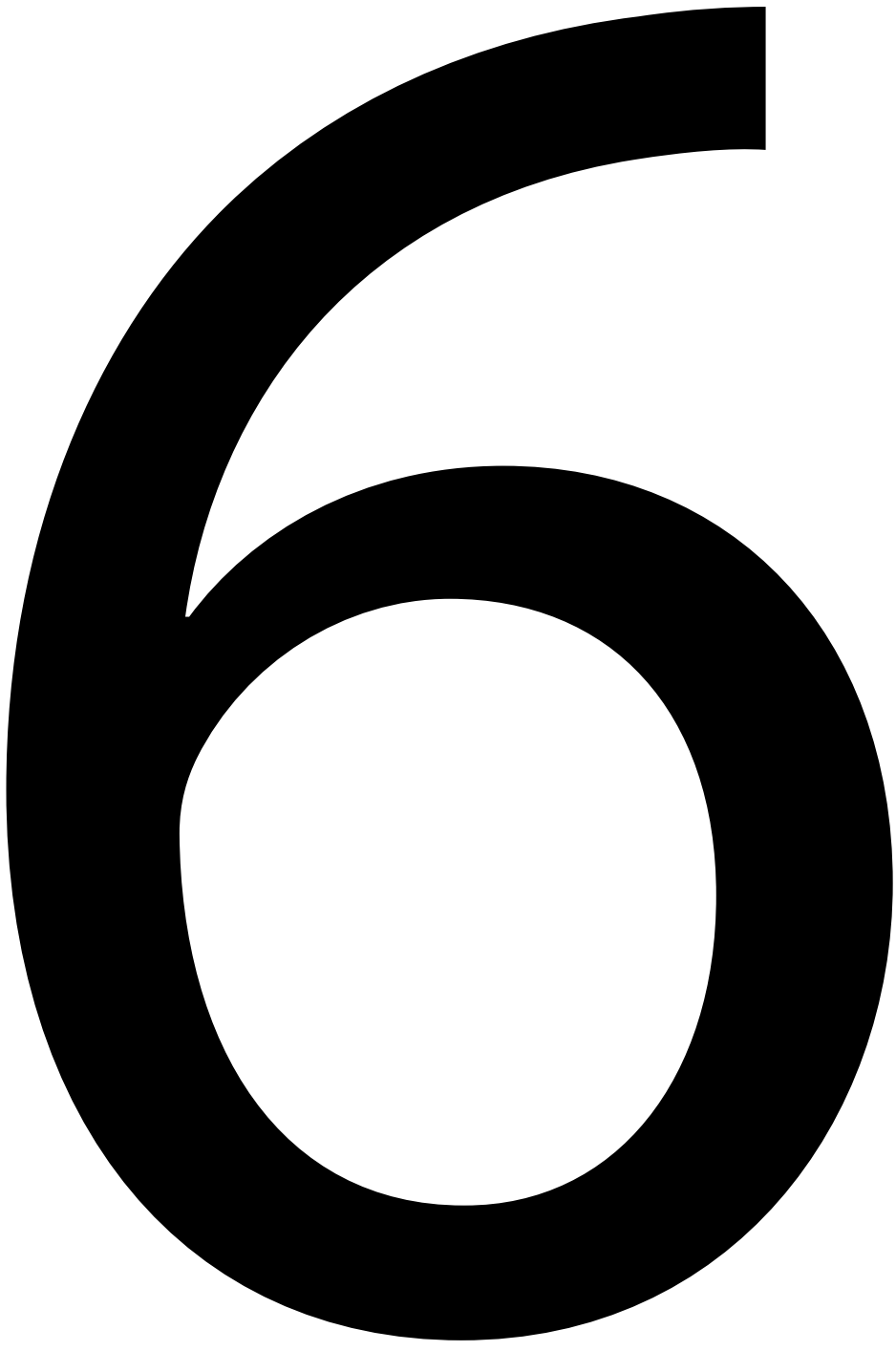
The role of autophagy during carbon starvation in Aspergillus niger

Burggraaf, M.A.



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.