Chapter 6

General Discussion

The ability of *Aspergillus niger* to secrete a high quantity and wide range of enzymes and organic acids to the extracellular environment makes this fungus a versatile cell factory (Pel et al. 2007). Due to its well annotated genome sequence, the rapid development of omics technologies (mainly transcriptomics and proteomics), and genetic tools such as newly established gene transfer systems, *A. niger* has become a model fungus for industrially exploited filamentous fungi (Adav et al. 2010; Carvalho et al. 2010; Fleissner and Dersch 2010; Jacobs et al. 2009; Meyer et al. 2011b; Pel et al. 2007). Like other filamentous fungi, a key feature of *A. niger* is the highly polarized growth of its hyphae by apical extension at the hyphal tip. After spore germination, the axis of growth remains fixed in the primary germ tube while the formation of secondary germ tubes and subapical lateral branches establish new growth axes (Momany 2002). This polar or asymmetric cell growth is found not only in filamentous fungi but also ubiquitously throughout other phyla e.g. filamentous bacteria (actinomycetes), pollen tubes, plant root hairs and developing neuronal cells (Horio 2007). Because of the relatively simple cytoskeletal organization and similarity to higher eukaryotic systems, the morphology of filamentous fungi including *A. niger* are important model systems to study polar cell growth (Horio 2007). In addition, fungal morphology is an important factor determining the efficiency of product formation in terms of yield, mixing or mass/oxygen transfer in industrial fermentations (Grimm et al. 2005; McIntyre et al. 2001; Papagianni 2004). Better understanding of the process determining morphology could lead to the improvement of fungal cells for the production of enzymes or acids.

Polarized growth and secretion involve many factors including transport of secretory vesicles containing different cargos to the tip of the cell. Direction of growth is determined by a vesicle-rich region located in the hyphal tip called the Spitzenkörper (Steinberg 2007; Virag and Harris 2006b). Moving the Spitzenkörper from its apical position by optical tweezers has substantial effects on the cell shape, which indicates that the position of the Spitzenkörper is crucial and directs polar growth (Bartnicki-Garcia et al. 1995). Secretory vesicles are transported along the hyphae to the Spitzenkörper via microtubules (MT) powered by kinesin motor proteins while short distance transport from the Spitzenkörper to the plasma membrane is mediated via the actin cables by myosin motor proteins (Fig. 1) (Saloheimo and Pakula 2012; Steinberg 2007; Taheri-Talesh et al. 2008; Taheri-Talesh et al. 2012). Thus the polarity of the cytoskeleton (tubulin and actin filaments) is a crucial factor in establishing and maintaining polar growth and secretion. Rho GTPases present in many systems including mammalian cells are well known to regulate morphology by

organizing the dynamics of the actin cytoskeleton (Bosco et al. 2009; Heasman and Ridley 2008; Park and Bi 2007; Ridley 2006). We systematically investigated the function of all members of the Rho family GTPases present in *A. niger* (Chapter 2). Based on loss-of-function studies, we showed that six Rho GTPases (RacA, CftA, RhoA, RhoB, RhoC, RhoD) exert distinct and overlapping functions during the life cycle of *A. niger*. Additionally the localization of RacA protein was observed as a crescent shape at the actively growing hyphal tips, which is incorporated into Fig. 1.

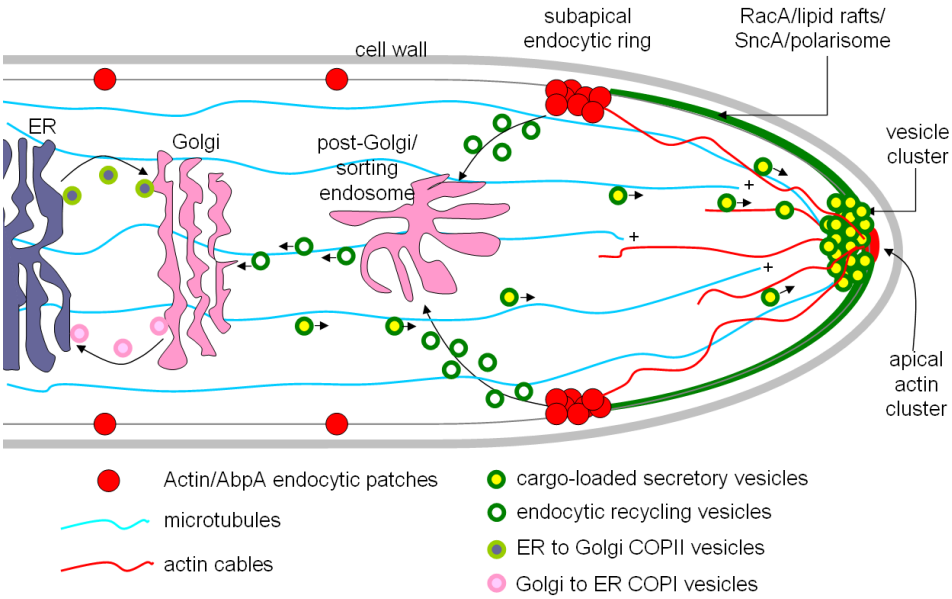

Fig. 1. A schematic model for polarized growth and secretion in *A. niger*. Secretory vesicles move to the Spitzenkörper (vesicle clusters) via the microtubules powered by kinesin motor proteins while short distance transport from the Spitzenkörper to the plasma membrane (PM) acts via the actin cables by myosin motor proteins (Steinberg 2007; Taheri-Talesh et al. 2008; Taheri-Talesh et al. 2012). The transport of the secretory vesicle cargo from the ER to the Golgi is mediated by the COPII carrier while retrieval of escaped luminal proteins as well as other machinery required for optimal anterograde (ER to Golgi) transport is mediated by the COPI carrier. Golgi derived secretory vesicles fuse with the PM, releasing their contents and the components of the membranes of the secretory vesicles. As the tip grows, the ring of actin/AbpA endocytic patches moves forward, removing SncA and other vesicle membrane components from the PM and incorporating them into endocytic vesicles for recycling. Adapted from (Taheri-Talesh et al. 2008).

Interestingly, a gene encoding the predicted acetyl-coenzyme A (CoA) transporter (An02g13410) was consistently up-regulated in all the compared protein overexpression and ER stress conditions transcripomes (Chapter 4). The putative acetyl-CoA transporter (An0213410) is predicted to contain a transmembrane spanning domain like the acetyl-CoA transporter AT-1 in humans (based on TMHMM) and shares 38% amino acids identity (Pehar and Puglielli 2013). In higher eukaryotes, this protein is shown to be involved in translocation of acetyl-CoA from the cytosol into the ER. The ER localized acetyl-CoA is subsequently used for acetylation of ER-transiting proteins including membrane proteins and possibly secretory proteins, thereby improving the folding efficiency (Fig. 2) (Jonas et al. 2010; Pehar and Puglielli 2013). Only acetylated nascent proteins can leave the ER and enter the Golgi where they are deacetylated (Fig. 2). Interestingly, also in mammalian

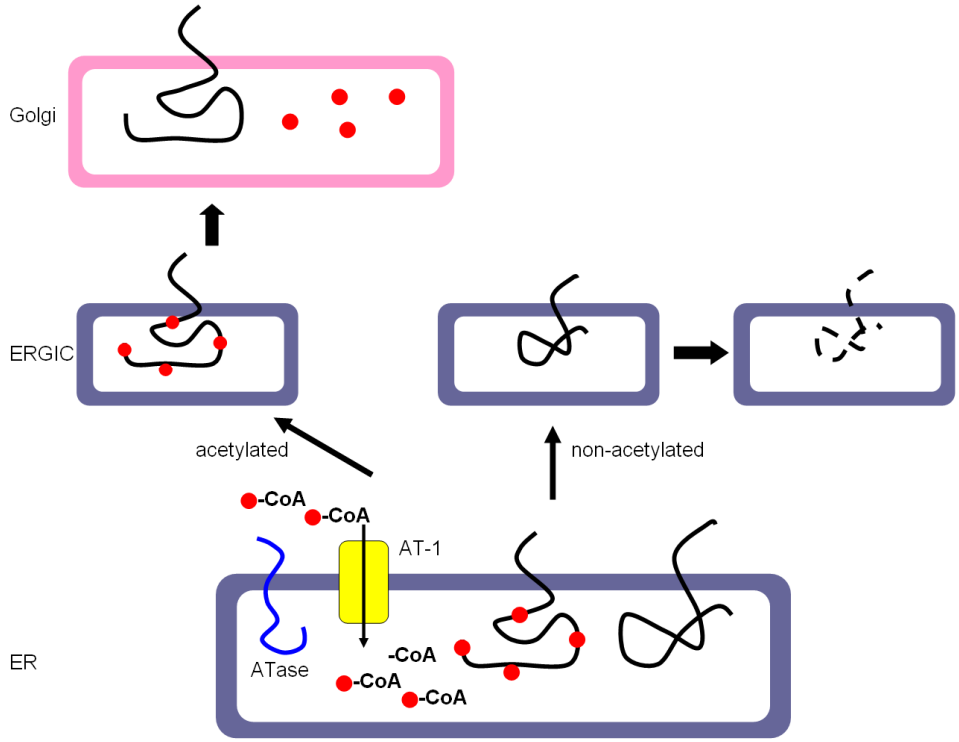

Fig. 2. A schematic view of acetylation in the ER. Acetyl- CoA is translocated from the cytosol into the lumen of the ER by an acetyl-CoA transporter (AT-1) and subsequently used for acetylation of nascent ER-transiting proteins including membrane proteins and possibly secretory proteins by an acetyltransferase (ATase). If acetylated, the nascent proteins can reach the Golgi where they are deacetylated and complete maturation while non-acetylated proteins are retained and degraded in the ER Golgi intermediate compartment (ERGIC). The acetyl group is shown as a red circle. Adapted from (Pehar and Puglielli 2013).

cells the gene encoding the acetyl-CoA transporter (AT-1) is up-regulated in response to ER stress (Shaffer et al. 2004). This suggests conservation of the possible role of acetylation in the ER as a part of the unfolded protein response (UPR) in eukaryotic cells including filamentous fungi.

Growth and secretion in fungi are considered to be tightly linked processes. However, with previous results from our group (Jørgensen et al. 2009) and our current results (Chapter 4), we observed a growth rate independent increase in protein secretion (Kwon et al. 2013b). The mechanism(s) which enables *A. niger* to secrete more proteins although cultivated at the same growth rate is not well known. Some possible mechanisms have been addressed in the general introduction part of this thesis (see Chapter 1). One of the important features in the further research will be based on the dynamics of processes related to the secretion. In the last decade, many proteins involved in hyphal tip growth and the polarized secretory pathway have been identified, analyzed and visualized using fluorescent proteins (FPs) (Sudbery 2011). In addition, some important proteins have been labeled in this thesis and our group: GFP-SncA (secretory vesicles), SlaB-YFP, AbpA-CFP (endocytic actin), CFP-TubA (tubulin), GFP-RacA (RacA) from this thesis and GmtA-GFP (Golgi, (Carvalho et al. 2011b)), SpaA-CFP (polarisome, (Meyer et al. 2008)), GlaA-GFP-HDEL, H2B-GFP, MTS-GFP (ER, nuclei, mitochondria respectively, unpublished data). Actin was visualized indirectly using immunostaining (Chapter 2), as approaches to fuse GFP to actin to perform life imaging of actin dynamics were not successful despite several attempts to fuse GFP either to the N- or C-terminus of actin (data not shown). We showed that the organization of the hyphal tip apparatus (or at least endocytic/exocytic events) is similar to what is observed in *A. nidulans*. To understand the high secretion capacity of *A. niger* or to understand mechanisms to explain different production rates at the same growth rates, the dynamics of the secretion machinery e.g. endo/exocytosis or COPII/COPI turn-over rates need to be studied in more detail in the future.

Two major findings have been achieved from this thesis, which will bring further follow-up studies. The first important finding is the acetylation of ER proteins (Chapter 4). The acetylation takes place in the ER involving the protein secretory pathway. This is quite a new finding in filamentous fungi. Since we only have transcriptomic data for this so far, it has to be proven whether the predicted *A. niger* acetyl-CoA transporter fulfills the same function. If acetylation takes place in *A. niger*, it has to be studied whether the acetylation of proteins in *A. niger* is part of the quality control mechanism, and other proteins involved in this process like acetyltransferases or deacetylases need to be identified in future studies.

Possibly this will open a new opportunity to understand and thus improve (heterologous) protein production in *A. niger*.

The second major finding from this thesis is the possible importance of lipid signaling networks and lipid remodeling that are involved in polar tip growth (Chapter 3). The synthesis of important phospho- and sphingolipid molecules functioning as secondary messengers in eukaryotes (DAG, IP, PA, PIP2, S1P) and as components of the plasma membrane (e.g. ergosterol, sphingolipids, glycerophosphocholine) were modulated during apical branching (Δ*racA*, *ramosa-1*) and apolar growth (P*glaA-racA*^{G18V}). In *S. cerevisiae* and filamentous fungi, sphingolipids and ergosterol concentrate to form lipid rafts in the plasma membrane which organize and regulate signaling cascades involved in polar growth control (Takeshita et al. 2012; Wachtler and Balasubramanian 2006). It is also known that the membrane lipid modification is important for Rho GTPase activity in addition to the GTP/GDP cycle (Casey 1994; Dransart et al. 2005; Ridley 2006), and as a consequence of this, localization of Rho GTPases is also influenced by the lipid composition of the membrane. In this context, it is worth to highlight that RacA localized mainly at the hyphal apex displaying a crescent-like form (Chapter 2, Fig. 5). Since RacA is post-translationally prenylated, RacA seems to be localized at the site with specific lipid compositions. An interesting question for future research is to determine factors to ensure the presence of RacA both at the right place and at the right time and to determine to which extend the lipid composition of the membrane affects RacA localization.