Title: Endocytosis and Exocytosis in Hyphal Growth

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10 Abstract

Two ancient processes, endocytosis and exocytosis, are employed by eukaryotic cells to shape their plasma membrane and interact with their environment. Filamentous fungi have adapted them to roles compatible with their unique ecological niche and morphology. These organisms are optimal systems in which to address questions such as how endocytosis is localized, how endocytosis and exocytosis interact, and how large molecules traverse eukaryotic cell walls. In the tips of filamentous (hyphal) cells, a ring of endocytosis encircles an apical crescent of exocytosis, suggesting that this area is able to support an endocytic recycling route, although both processes can occur in subapical regions as well. Endocytosis and exocytosis underlie growth, but also facilitate disease progression and secretion of industrially relevant compounds in these organisms. Here we highlight recent work on endocytosis and exocytosis in filamentous fungi.

Keywords: Hyphae, Cell Polarity, Vesicle Trafficking, Spitzenkörper, Polarized Growth

Abbreviations: Golgi Equivalent (GE), Plasma Membrane (PM), Filamentous fungi (FF)

1. Introduction

Filamentous fungi (FF) are diverse organisms defined by the remarkably polarized growth of their characteristic cell type, the hypha. Underlying this lifestyle is an exquisite spatial control over two ubiquitous cellular processes: endocytosis and exocytosis (Araujo-Bazán et al., 2008; Caballero-Lima et al., 2013; Shaw et al., 2011; Taheri-Talesh et al., 2008; Upadhyay and Shaw, 2008). Endocytosis, or membrane internalization, and exocytosis, or secretion, govern a large portion of the interactions of cells with their environment. Both are highly regulated and complex, with endocytosis in budding yeast involving more than 60 proteins (Brach et al., 2014; Weinberg and Drubin, 2012; Weinberg and Drubin, 2014) and exocytosis employing conserved tethering complexes, lipids, and the cytoskeleton to overcome the energetic barrier for membrane fusion (**Figure 1A, B**) (Finger and Novick, 1998; He and Guo, 2009; Jahn and Südhof, 1999). Exocytosis has long been implicated in membrane expansion of vegetative cells in FF, but a role for endocytosis in maintaining hyphal shape has only recently been appreciated (Caballero-Lima et al., 2013; Hervás-Aguilar and Peñalva, 2010; Lee et al., 2008; Read and Kalkman, 2003; Upadhyay and Shaw, 2008). Much of what is known about these processes comes from studies in the budding yeast *Saccharomyces cerevisiae*, and a large portion of the machinery involved is conserved among eukaryotes. However, FF have some distinctions from yeast. For example, hyphae exhibit an enormous rate of exocytosis (Bartnicki-Garcia et al., 1989; Howard, 1981). Moreover, filamentous ascomycetes and basidiomycetes possess a Spitzenkörper, an organelle that regulates secretion at the tips of growing hyphae (Brunswick, 1924; Dijksterhuis and Molenaar, 2013; Girbardt, 1957; Jones and Sudbery, 2010; Riquelme and Sánchez-León, 2014). Additionally, endocytosis and exocytosis are clearly separated in growing hyphae (Sudbery, 2011; Taheri-Talesh et al., 2008). Here we summarize the most recent work done on endocytosis and exocytosis in FF, with a focus on growth and their importance in microbe-host interactions.

2. Exocytosis: vesicle tethering, secretion, and the Spitzenkörper

2.1 The Exocytic Machinery

Exocytosis is the process by which vesicles associate with the plasma membrane (PM) and empty their contents into the extracellular space (Finger and Novick, 1998; He and Guo,

2009; Jahn and Südhof, 1999). Membrane fusion in living cells involves donor membranes (e.g. secretory vesicles) passing multiple tests for specificity with target membranes. These include at least three components for exocytosis in yeast (and, presumably, FF). The Rab GTPase Sec4p associates with secretory vesicles in its active GTP-bound form and interacts with the membrane fusion machinery (Guo et al., 1999b). Next, a pair of Soluble N-ethylmaleimide-sensitive factor Attachment REceptor (SNARE) proteins, one on the vesicle (v-SNARE) and one on the target membrane (t-SNARE), are required to complete membrane fusion (Ferro-Novick and Jahn, 1994; Söllner et al., 1993). Finally, exocytosis involves an octameric tethering complex, the exocyst, and associated Sec1/Munc18 (SM) proteins (Carr et al., 1999; Novick et al., 1981). Strains with a disrupted exocyst accumulate secretory vesicles in the cytoplasm and are severely compromised in polarized growth, highlighting the importance of this complex in exocytosis and membrane expansion (Guo et al., 1999a). In fungi, the exocyst is composed of eight proteins, corresponding to *S. cerevisiae* Exo70p, Exo84p, Sec3p, Sec5p, Sec6p, Sec8p, Sec10p, and Sec15p (TerBush et al., 1996, Guo et al., 1999). Of these proteins, only Sec3 is dispensable in yeast (Haarer et al., 1996), which also holds true for *Aspergillus niger* and *Candida albicans* (Kwon et al., 2014; Li et al., 2007). In *N. crassa*, however, Sec-3 was essential and Sec-5 was not (Riquelme et al., 2014), and both a *Magnaporthe oryzae* *exo70* and a *sec5* deletion were viable (Giraldo et al., 2013). These findings indicate that more work needs to be done to understand the assembly and structure of the exocyst in fungi.

Tethering complexes are important throughout eukaryotes for vesicle fusion with various cell destinations such as the PM, the Golgi, and the vacuole. The yeast homotypic fusion and vacuole protein sorting (HOPS) complex can increase luminal mixing of SNARE-bearing proteoliposomes by 100-fold (Zick and Wickner, 2014). The role that the exocyst plays in fusion is not completely clear, but it appears to be able to associate with the Rab proteins on secretory vesicles, as well as promote binding between SNARE proteins on vesicles and the PM (Guo et al., 1999b; He and Guo, 2009; TerBush et al., 1996). Sso1/2p and Snc1/2p are the exocytic t- and v-SNARE proteins in *S. cerevisiae*, respectively (Valdez-Taubas and Pelham, 2003). Intriguingly, the Sso1p homologue is localized throughout the entire PM in nearly all eukaryotes, including FF (Guo et al., 2000; Taheri-Talesh et al., 2008; Treitschke et al., 2010; Valkonen et al., 2007). In general, however, the Snc1/2p homologues are only found at areas of growth (Hervás-Aguilar and Peñalva, 2010; Taheri-Talesh et al., 2008). Many t-SNAREs can interact with multiple v-SNAREs (Banfield et al., 1995; Götte and Fischer von Mollard, 1998), but whether this occurs for Sso1/2p is not currently known. Currently, therefore, exocytosis in hyphae can be thought to be delimited by the location of the exocyst.

The earliest study of exocyst localization in hyphae was in *Aspergillus nidulans*, where SecC/Sec3p was localized to a small cap at hyphal tips (Taheri-Talesh et al., 2008). Next, *Ashbya gossypii* Exo70p, Sec3p, and Sec5p were observed, depending on growth rate, to a crescent at the apex (slow growth), or the Spitzenkörper (fast growth) (Köhli et al., 2008). The exocyst of *C. albicans* hyphae, on the contrary, forms a stable apical crescent even when the cytoskeleton is disrupted (Jones and Sudbery, 2010). More recently, Riquelme et al. (2014) observed that EXO-70 localizes to the periphery of the Spitzenkörper in an actin and microtubule-dependent manner in *N. crassa*, and these subunits may be responsible for tethering this organelle to the rest of the complex. This would be opposed to the situation in *S. cerevisiae* (He and Guo, 2009) and *Schizosaccharomyces pombe* (Bendezu et al., 2012) where Exo70p and Sec3p bind to the PM and recruits the rest of the complex. It would, however, generally agree with the notion that the exocyst exists as two multimeric subunits, one of which travels on

vesicles, that assemble at the PM in a reaction that promotes vesicle tethering (Guo et al., 1999a; Guo et al., 1999b; He et al., 2007).

2.2 The Spitzenkörper and Exocytosis

The Spitzenkörper is commonly viewed as a “vesicle supply center” that provides the membrane for tip growth in some fungi (Gierz and Bartnicki-Garcia, 2001; Riquelme and Sánchez-León, 2014). Exocytic traffic from fungal Golgi equivalents (GEs) awaiting fusion with the PM provides a large part of the membrane for this organelle (Pantazopoulou et al., 2014), which suggests that membrane fusion in the tip is limiting. This limitation may also influence the structure and size of the Spitzenkörper. Having exocyst proteins delivered to the tip on secretory vesicles could provide some of this regulation, if the fusion of subunits on vesicles with subunits on the PM was required for vesicle tethering, and PM-localized subunits were limited in quantity, for example. However, vesicles in the Spitzenkörper are also organized into different layers, with large macrovesicles in the outer layer (termed by the authors the “Spitzenring”) and smaller, microvesicles in the “core” (Howard, 1981; Riquelme and Sánchez-León, 2014; Verdín et al., 2009). In *N. crassa* these different layers are known to have different cargo, with the 1,3 β -glucan synthase GS-1 located in the outer layer and the chitin synthase Chs-1 present in the core (Verdín et al., 2009). *S. cerevisiae* also has distinct populations of high and low density vesicles that each have cargo destined for different locations (Harsay and Bretscher, 1995). It is possible that behavior among the different groups of vesicles could be different. Additionally, no studies to date in fungi have differentiated between exocytosis that results in full membrane fusion and the transient “kiss-and-run” exocytosis seen in animal and plant cells (Aravanis et al., 2003; Weise et al., 2000). Analyzing these two populations of vesicles, as well as how they interact with the plasma membrane, will be key to gaining more insight into the nature of the Spitzenkörper and hyphal growth.

2.2 The Cell Wall and Exocytosis

Along with membrane-building materials, secretory vesicles in fungi include proteins that are destined for the cell wall or the extracellular space. In yeast, this is represented by the subset of 100 nm “periplasmic” vesicles that include secreted proteins, such as the major exoglucanase Exg1p as cargo (Harsay and Bretscher, 1995). The cell wall is a formidable barrier to macromolecules, viruses, and bacteria (Moebius et al., 2014), but fungi are clearly able to surpass this, as demonstrated by their prodigious production of secreted proteins, during nutrient acquisition, disease progression in the host, secreted secondary metabolites, and a variety of antibiotics. Recently, more data has accumulated regarding trans-cell wall secretion. The presence of immunologically active vesicles in the culture supernatant of the basidiomycete pathogen *Cryptococcus neoformans*, as well as the ascomycetes *Histoplasma capsulatum* and *C. albicans*, are strong evidence for exocytosis being the preferred method of transport through walls (Albuquerque et al., 2008; Casadevall et al., 2009; Rodrigues et al., 2007). This mechanism involves the fusion of organelles such as multivesicular bodies (MVBs) delivering cargo-loaded vesicles into the extracellular space. Aflatoxin B1, a carcinogenic secondary metabolite synthesized by some *Aspergillus* spp. is produced in vesicles termed “aflatoxisomes” trafficking to the PM. This may be a tactic that is conserved in many fungi to deploy a variety of molecules (Chanda et al., 2009; Chanda et al., 2010).

3. Endocytosis: The Endocytic Collar and Hyphal Morphogenesis

3.1 The Endocytic Machinery

Electron micrographs of budding yeast cells regularly reveal small (<200nm) invaginations of the PM associated with endocytic proteins. Surprisingly, these are rarely seen in similarly prepared images of hyphae, although the importance of endocytosis in fungi is well-established (Araujo-Bazán et al., 2008; Fischer-Parton et al., 2000; Upadhyay and Shaw, 2008). Endocytosis in yeast involves two phases. The earliest phase is variable (60-180 seconds) during which cargo congregates at nascent sites and the clathrin coat is established around the invagination. The late phase is shorter and more ordered (~35 seconds) and in this step invagination is driven by actin nucleation, culminating in scission and movement away from the PM (Kukulski et al., 2012; Kukulski et al., 2011; Weinberg and Drubin, 2012). FF possess most of the endocytic proteins also present in yeast and mammals (**Figure 1B**) (Read and Kalkman, 2003) and uptake endocytic markers regularly (Fischer-Parton et al., 2000), but the conservation of these phases may have diverged considerably, as seen for the scission step (Conibear, 2010).

3.2 Endocytic Scission

Animals use the GTPase dynamin and the actin cytoskeleton for membrane bending and scission (van der Blik and Meyerowitz, 1991). In the absence of the yeast dynamin-1 homologue Vps1p, however, endocytosis proceeds normally. Vps1p also does not have an obvious PM localization (Peters et al., 2004), which has led some to conclude that it does not participate in endocytosis. The *A. nidulans* Vps1p homologue, VpsA, was functionally characterized, and although endocytosis was not addressed, the deletion appeared to result in a pleiotropic effect as it does in *S. cerevisiae* (Tarutani et al., 2001). These results may have obfuscated its importance in fungal endocytosis, however, as recent studies incorporating electron microscopy have revealed defects in membrane invagination in Vps1p mutants (Smaczynska-de Rooij et al., 2010). These phenotypes have also been linked to another set of proteins, the amphiphysins Rvs161p and Rvs167p, which are known to act in scission in yeast (Wang et al., 2011). In yeast, amphiphysins arrive at endocytic sites just before scission, and may bind to and stabilize the invaginating endocytic bud through their F-BAR domains, which recognize membrane curvature (Meinecke et al., 2013). In the absence of Rvs161p or Rvs167p ~20% of invaginations retract to the membrane without undergoing scission, and endocytosis is greatly reduced (Kishimoto et al., 2011; Youn et al., 2010). Interestingly, some FF appear to have more amphiphysin-like proteins than in yeast (e.g., *Aspergillus nidulans* and *N. crassa* each possesses two proteins similar to the amphiphysin Rvs167 in yeast (Arnaud et al., 2012), and from data in *N. crassa* at least some appear to be dispensable for hyphal growth (**Figure 1B**) (Muñoz et al., 2012). Alternatively, the unconventional Myosin Type I, MyoA, localizes to sites of endocytosis in *A. nidulans* (Yamashita et al., 2000) and interacts with Abp1p (Matsuo et al., 2013). Additionally, a constitutively active allele of MyoA increased endocytosis at hyphal tips (Yamashita and May, 1998). The *S. cerevisiae* myosins Type I, Myo3p and Myo5p, have also been implicated in endocytosis (Geli and Riezman, 1996), but direct evidence is still lacking for a role in scission. More likely, their chief role is in actin patch regulation.

The lipid composition of endocytic invaginations also appears to play a role in vesicle scission through the activity of the synaptojanins. Several endocytic adaptor proteins have been shown to attach to Phosphatidylinositol (4,5) bisphosphate (PIP₂) on invaginating buds through Pleckstrin-Homology (PH) domains. Synaptojanins hydrolyze PIP₂ and deplete it at these sites. The differential lipid composition between the bud ends and the neck can then provide an interfacial force that may promote scission, as well as uncoating of PH-domain proteins (Sun et

al., 2007). Most ascomycetes contain several, possibly redundant synaptojanin homologues, but as they regulate the intracellular PIP₂ level (Sun et al., 2007), which in turn is important for hyphal growth (Mähls et al., 2012; Sun and Drubin, 2012), their examination is warranted in future studies on endocytosis in filamentous fungi.

3.4 Alternate Endocytic Routes

Clathrin-mediated endocytosis is the best characterized internalization pathway in eukaryotes, but other types of endocytosis (e.g. phagocytosis) have been adapted in different organisms, collectively referred to as clathrin-independent endocytosis. In budding yeast, a clathrin-independent route for the uptake of FM4-64 was recently discovered (Prosser et al., 2011). Surprisingly, it was independent of the Arp2/3p complex, which is critical for forming the actin patches that are commonly viewed as sites of endocytosis. Rather, it depended on the formin Bni1p, which nucleates actin filaments, and the GTPase Rho1p, which are generally associated with exocytosis. Indeed SepA, the only formin in *A. nidulans*, and the ortholog Bni-1 in *N. crassa*, are located in the Spitzenkörper and the apical dome and are thought to be mainly involved in producing actin filaments along which vesicle traffic is sent to the apex (Lichius et al., 2012; Sharpless and Harris, 2002). Rho1p, additionally, interacts with the exocyst (Guo et al., 2001), and in *A. nidulans* its homologue RhoA is involved in cell wall deposition (Guest et al., 2004). An endocytic internalization route occurring at the apex of hyphae would not be an unprecedented proposition, however, as multiple endocytic routes are present in tip-growing plant cells (Bove et al., 2008; Ketelaar et al., 2008; Onelli and Moscatelli, 2013; Ovečka et al., 2012). Intriguingly, in *C. albicans* clathrin and the arp2/3 complex, which assists in the formation of the actin patches that are thought to provide force for PM invagination, are not required for endocytosis (Epp et al., 2013). These mutants could not form hyphae, however, adding support for the importance of endocytosis in hyphal growth. Similar studies have shown endocytosis to concentrate in a region just behind the apex, and to be essential for normal growth in *A. oryzae*, *N. crassa*, *A. nidulans*, *A. gossypii*, and *Fusarium graminearum*. Disruption of the actin crosslinking protein Fimbrin in each of these organisms resulted in an almost complete block of endocytosis and compromised hyphae (Echauri-Espinosa et al., 2012; Higuchi et al., 2009; Jorde et al., 2011; Upadhyay and Shaw, 2008; Zheng et al., 2014).

The identification of clathrin-independent endocytic routes in FF could, therefore, be important for understanding the importance of endocytosis in hyphal growth. Recent studies indicate that such processes do exist, although their role in morphogenesis is not as clear as in *C. albicans*. First, the protein flotillin promotes clathrin-independent endocytosis in animal cells, and it localizes throughout the PM in *A. nidulans*, but is excluded from apical regions (Takeshita et al., 2012). Another study examined proteins that interacted with actin binding protein A (AbpA) in *Aspergillus oryzae* and identified the membrane compartment of CanA/eisosomes in subapical regions of hyphae (Matsuo et al., 2013). Deleting components of this compartment did not have major effects on growth or FM4-64 uptake, but the internalization of AoCanA was disrupted. Eisosome disruption, moreover, did not obviously affect endocytic uptake in *A. gossypii* or *A. nidulans*, and their role in specialized endocytosis is still being explored in FF (Athanasopoulos et al., 2013; Seger et al., 2011). In summary, endocytosis is conserved in filamentous fungi, but specific roles of endocytic proteins may be unique in these organisms. Investigating different endocytic routes in hyphae is a promising path for fungal cell biologists.

4. Endocytosis and Exocytosis in Polarized Growth

4.1 Localization of Exocytosis and Endocytosis

Fungi are able to rearrange their growth axes in response to environmental cues (Brand et al., 2014; Hoch et al., 1987; Stephenson et al., 2014), and this involves a rapid dissolution and reorganization of the endocytic and exocytic machinery. How these processes become polarized and coupled, however, is unclear. In *S. cerevisiae*, endocytosis may polarize exocytosis by a dynamic “corralling” mechanism (Jose et al., 2013), but in germling hyphae, these two processes do not appear to be segregated (Sharpless and Harris, 2002; Taheri-Talesh et al., 2008; Upadhyay and Shaw, 2008). Additionally, in rapidly growing cells, the localization of many polarized proteins is growth rate dependent, and many components of hyphal tips rapidly mislocalize when cells stop growing, so any extremely deleterious mutation could cause the mislocalization of any tip protein. For example, although in budding yeast Cdc42p was implicated in directional traffic of the exocyst component Sec6p (Adamo et al., 2001), it is unclear from that study whether the cells were growing when imaged. This is an important issue that should be addressed when assessing how any process is localized in hyphae.

Membrane composition can determine the location of exocytosis. In budding yeast, Exo70p and Sec3p bind PIP₂, which is polarized in the PM at sites of growth (He and Guo, 2009; He et al., 2007). In *N. crassa*, however, Exo-70 is associated with secretory vesicles and requires the actin cytoskeleton for its localization (Riquelme et al., 2014), indicating a divergence of function between these proteins in filamentous fungi. PIP₂ concentration, as assessed by a fluorescent reporter, is somewhat polarized in hyphae, but forms a gradient that is much shallower than most tip components (Pantazopoulou and Peñalva, 2009; Vernay et al., 2012), although the PIP₂ synthase was required for *N. crassa* growth (Mähs et al., 2012). Exo84p was shown to be associated with Phosphatidylserine in *C. albicans*, and when it was phosphorylated it dissociated with this phospholipid and was released from the plasma membrane, which potentially allows it to be recycled back to the PM (Caballero-Lima and Sudbery, 2014). Filipin is a fluorescent marker for lipid rafts, and though staining with filipin is not accurate in live cells (Valdez-Taubas and Pelham, 2003), nearly all data to date indicate that these PM components are enriched at hyphal tips. It is possible that, as *S. cerevisiae* Exo70p recognizes PIP₂, some proteins may preferentially bind to lipid rafts. In animals, lipid rafts can mark sites of exocytosis (Chamberlain et al., 2001; Salaün et al., 2004), and the presence of these domains can influence the integrity of the cytoskeleton in hyphae (Pearson et al., 2004), but more work needs to be done to understand PM protein-lipid interactions in filamentous fungi.

4.2 Signaling the beginning of endocytosis

Endocytosis involves several dozen proteins and occurs in a sequential order, and in FF it is precisely localized. What directs this process to a specific space in the cell? The identity of a proteinaceous signal has been elusive, at least in fungi. Recently, the first seven proteins known to be involved in endocytosis were shown to be dispensable for endocytosis to localize and successfully complete in budding yeast (Brach et al., 2014). One possibility for the exclusion of endocytosis from the apical dome is that the high rate of exocytosis at the tip disallows buildup of the endocytic machinery. The process of endocytosis can take up to four minutes (Kukulski et al., 2011) which is unstable in a region with high membrane flux, such as the apex of hyphal tips. Supporting this is the observation that germlings, which experience much slower growth than mature hyphae, and thus less vesicle fusion at the tip, do not exhibit a separation of the exocytosis and endocytosis at the apex (Berepiki et al., 2010). However, this does not explain why endocytosis is highly concentrated at the collar, nor does it explain the relative stability of

actin patches at the collar, both of which indicate that this region of the hypha is “marked” for endocytosis. The membrane composition at the collar is clearly important, but there are many other components that may play a role.

The lipid composition of the PM could determine the location of the collar. For example, in yeast, a mutant that lacked phosphatidylserine also lost the ability to polarize endocytosis, whereas the loss of PIP₂ had an effect on scission but not initiation of endocytosis (Sun and Drubin, 2012). Phosphatidylserine promotes polarization of Cdc42p (Fairn et al., 2011), but this has not extended directly to the recruitment of endocytic proteins. Additionally, lipid rafts have been hypothesized to be involved in endocytosis (Parton and Richards, 2003), although they are generally present at the apex and taper off near the endocytic collar (Takeshita et al., 2008). It is possible that a precise composition of lipid rafts or phospholipids is optimal for the attraction of endocytic proteins. One last hypothesis comes from two recent studies in budding yeast and *A. nidulans*, respectively, that looked at the role of the endoplasmic reticulum (ER) at the plasma membrane. In yeast it was observed that endocytosis occurred in areas that were devoid of a cortical-ER connection, so that the PM was segmented into domains that were either endocytic, carried lipid rafts, or were attached to the ER (Stradalova et al., 2012). Interestingly, the *A. nidulans* peripheral ER localizes near the endocytic collar, and it was hypothesized that the actin cytoskeleton associated with the endocytic collar may act to scaffold the peripheral ER in place to provide growing hyphal tips with secretory ER (Markina-Iñarrairaegui et al., 2013).

4.4 Endocytic recycling in hyphae

The location of the endocytic collar suggests the presence of a recycling route near the hyphal apex. Endocytic recycling generally includes three phases. First, membrane proteins are recognized at sites of endocytosis by cargo adaptor proteins and internalized. Next, they are sent to early endosomes, which tubulate and send traffic to late GEs. From there, they are able to be sorted back into the secretory pathway for exocytosis. Several proteins, including Snc1p (Valdez-Taubas and Pelham, 2003) and Wsc1p (Piao et al., 2007) follow this pathway. The situation is different for filamentous fungi, however, as the Snc1p homologue SynA was still polarized in *A. nidulans* in a *vpt6/rabC* deletion, as well as an *rcyA* deletion, which presumably abolish most recycling traffic to late GEs. In mammals, recycling traffic travels through recycling endosomes, the Trans-Golgi Network (TGN), or is sent directly back to the PM through early endosomes in a so-called “quick recycling route” that is regulated by Rab4 (Grant and Donaldson, 2009), and it is likely that, if endocytic recycling does assist in hyphal growth, FF have adapted a more direct recycling route accommodate the rapid turnover that they likely require for some polarized proteins, although details of this route are unknown.

Some evidence has accumulated to support the presence of endocytic recycling in hyphal tips. Fischer-Parton et al. (2000) found that the endocytic marker FM4-64 localized to the Spitzenkörper soon after internalization in many fungi. Endocytic protein AoEnd4 was discovered to be important for polarized growth as well as recycling of the v-SNARE AoSnc1 in *A. oryzae* by Higuchi et al. (2008). Peñalva et al., additionally, showed that SynA was mislocalized to the PM when its endocytosis was blocked in *A. nidulans* (Hervás-Aguilar and Peñalva, 2010; Pantazopoulou and Peñalva, 2011). Most recently, Schultzhaus et al. showed that a plasma membrane flippase DnfA was recycled through the late Golgi and endocytosed through an NPFxD motif in *A. nidulans* (Schultzhaus et al., 2015). Fluorescence Recovery After Photobleaching (FRAP) on tip components in *C. albicans* demonstrated that proteins that localize to the Spitzenkörper such as myosin light chain are more transient than proteins that

form an apical crescent, such as the exocyst (Jones and Sudbery, 2010). These findings are in agreement with studies in yeast (Howell et al., 2012) that suggest that proteins that arrive at hyphal tips through vesicle trafficking, such as those trafficking via endocytic recycling, should accumulate in an area behind the membrane. Finally, Craven et al. performed an excellent description of the spatial orientation of the tip growth machinery in *C. albicans*, and determined that exocytosis and endocytosis were situated in a way that corresponded well with hyphal shape, although the specific identity and destination of the cargo of the endocytic collar has not been directly assessed (Caballero-Lima et al., 2013).

Recent studies suggest that, unlike the strict separation that occurs in hyphae, pollen tube tips uphold a complex current of vesicle traffic made of different endocytic and exocytic pathways, which are important for cell growth and shape (Bove et al., 2008; Helling et al., 2006; Onelli and Moscatelli, 2013). This model is built from microscopic observations that suggested that the greatest area of cell expansion is not the extreme apex, but rather in an annular region around the very pole (Geitmann and Dumais, 2009). Additionally, clathrin-dependent and independent endocytosis occurs at different locations throughout the apical compartment of pollen tubes, and the route taken by cargo of these routes can be complex (Onelli and Moscatelli, 2013). The rate of endocytosis has been quantified in *Arabidopsis* pollen tubes. This revealed that an excess of vesicles large enough to accommodate almost a minute of growth is present in the apex of these cells (Ketelaar et al., 2008; Ovečka et al., 2012), but how much membrane was recycled was unclear. Such studies would be valuable for understanding growth in filamentous fungi.

4.5 Subapical Endocytosis and Exocytosis

In agreement with the idea that fungi regulate endocytosis and exocytosis to accomplish growth, the machinery for both processes is found at growing septa (**Figure 1C**), as well as in new sites of branching. Septa represent subapical sites of polarization distinct from hyphal tip growth, and while the endocytic machinery can be seen intermittently throughout hyphae (likely for the internalization and recycling of a variety of transporters (Vlanti and Diallinas, 2008)) and secretion and growth (intercalary growth) have been observed occasionally in subapical compartments (Read, 2011), most studies focusing on the molecular machinery of endocytosis and exocytosis in these regions have centered around what occurs at septa (Delgado-Álvarez et al., 2014; Hayakawa et al., 2011; Mouriño-Pérez, 2013). As reviewed in Mouriño-Pérez (2013) the formation of a septum is a process that coordinates many influences, including cell wall growth, the cytoskeleton, and mitosis. Endocytosis and exocytosis at these locations are subject to different constraints than the hyphal tip. First, from studies on the septation machinery, proteins begin at the cell wall and move inward as the septum is made, following a structure called the contractile actin ring (Delgado-Álvarez et al., 2014). The exocytic machinery will therefore overlap or be removed as the septum contracts, and the curvature of the membrane at septa will be greater. Additionally, there is probably less microtubule traffic to the septa, as they are anchored at the tip, making actin more important for exocytosis at septa. This could explain why the well-studied formin *sepA*, when deleted in *A. nidulans*, completely blocks septum formation but not tip growth, although in *N. crassa*, the formin Bni-1 arrives after actin cables at septa (Mouriño-Pérez, 2013).

5. Endocytosis and Exocytosis and Pathogenesis

5.1 Effectors and the interfacial layer between pathogen and host

An important tool in the fungal pathogen repertoire is an effector, which is a secreted molecule that contributes to disease progression. These are so common that some fungal pathogens such as *Ustilago maydis* produce more than 150 potential effectors, all of which may have different targets in the host (Mueller et al., 2008). Effectors are secreted into their host, which is important as the molecules may have distant targets and even may be toxic to the pathogen. Interestingly, *M. oryzae*, the destructive rice blast pathogen, is able to deliver proteins to distinct extracellular regions, either into the host-pathogen interface or into the host cell, using two distinct mechanisms that require either the conventional Golgi-directed secretory machinery, or one that is Brefeldin A-insensitive and requires some components of the exocyst (Giraldo et al., 2013). Additionally, hyphae that grow inside plants are usually bulbous and misshapen in comparison to hyphae growing on media. Some changes in shape could be the result of the physical barriers encountered when growing inside a host, but a concomitant reorganization of exocytosis and endocytosis could also play a role in this change. Endocytosis, for example, is important for morphology as well as virulence in such diverse pathogens as *U. maydis* (Fuchs et al., 2006), *F. gramineicola* (Zheng et al., 2014), and the mammalian pathogen *C. albicans* (Douglas et al., 2009).

5.2 Endocytosis and drug resistance and mating

Endocytosis could also be a common route into cells for signaling molecules and antifungal drugs. Interestingly, amphiphysins were recently discovered in two independent screens to identify genes controlling Conidial Anastomosis Tube formation (Fu et al., 2011), as well as resistance to antifungal peptides (Muñoz et al., 2012). Amphiphysins, as mentioned in section 3.2, seem to be much less important in filamentous fungi than in yeast, and these recent studies may provide some evidence that these organisms take advantage of different forms of endocytosis for the variety of different tasks that they perform in their life cycles.

A promising method being developed to treat plant diseases is host-induced gene silencing using RNA Interference (RNAi), in which pathogens are exposed to short interfering double-stranded RNAs that mediate silencing of important genes in their genome, leading to dysfunction or death (McDonald et al., 2005; Nakayashiki et al., 2005; Nunes and Dean, 2012). How the genetic material is transported into the pathogen in this case is not currently known, and it is not effective for all fungal pathogens, but at least for *A. nidulans*, it is able to be absorbed from the media (Khatri and Rajam, 2007), and the endocytic pathway may be the method of internalization, as seen in *Drosophila melanogaster* (Saleh et al., 2006).

6. Conclusion

It is now clear that membrane flux and traffic through the PM are complex, underlying processes that define the filamentous lifestyle of filamentous fungi (**Figure 2**), but each new study brings with it new questions. How important is the coordination between exocytosis and endocytosis for development? What methods do different tip proteins use to maintain their localization (e.g. by endocytic recycling or some other mechanism), and how does this relate to protein function? What role does the plasma membrane play in localizing these processes? How do they contribute to growth inside hosts, and translocation of signaling molecules and antifungal compounds? Finally, how do proteins assemble within, or traverse through the thick cell wall? FF are ideal models in which to explore these questions, which are of interest to all of eukaryotic cell biology. With efficient gene-targeting techniques already established for model systems, and

increasingly being adapted for more economically important organisms, it is only a matter of time before these are addressed.

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750 **Figure caption**

Figure 1. Endocytic and exocytic machinery in hyphal tips. Exocytosis (**A**) drives growth at
 hyphal tips, and involves several components. Rho-GTPases including Rac1p and Cdc42p
 homologues (white) help polarize the actin cytoskeleton (green) and may directly localize the
 755 exocyst (orange). Specific lipids such as PIP₂ (yellow) that are enriched at the tip, and vesicle
 traffic may also contribute to exocyst placement (question marks). Secretory vesicles (blue)
 travel to the tip on microtubules (grey). Near the tip, vesicles associate with actin filaments and
 are tethered to the plasma membrane (black) by the exocyst. They eventually fuse with the
 membrane through interactions between vSNAREs (pink) and tSNAREs (red). Extracellular
 760 vesicles, additionally, may be released by fusion of specialized multivesicular bodies (MVB)
 with the plasma membrane. Endocytosis (**B**), is primarily seen at a subapical ring in growing
 hyphae. It involves the nucleation of actin (green) through interactions with Fimbrin (oval) and
 SlaB (pentagon) providing the force to invaginate the membrane, followed by scission, which
 involves BAR proteins such as the amphiphysins (rectangle). After internalization, endocytic
 765 vesicles travel to early endosomes (blue), and recycled cargo can then travel to late Golgi
 equivalents (yellow) to reenter the secretory pathway or possibly be sent back to the plasma
 membrane (orange arrow). Additional pathways of endocytosis in fungi include the endocytosis
 of Can1/CanA at MCCs/eisosomes (pink), and a possible clathrin-independent route regulated by
 Rho1p/RhoA (cloud) near the apex (red arrows).

Figure 2. Pathways of Membrane Flow in Fungal Hyphae. Raw materials for the plasma
 membrane, as well as membrane proteins and other molecules, are brought to hyphal tips on
 microtubules (grey). At the tip, they can traffic back to subapical compartments, or enter the
 Spitzenkörper (S) and be sent to the plasma membrane on actin filaments (green). At the apex,
 775 vesicles can fuse, causing growth to occur (orange arrows), or transiently associate with the
 plasma membrane (not shown). Molecules in the plasma membrane can be endocytosed through
 either clathrin-dependent or independent endocytosis at the collar (red arrows). Endocytosis and
 exocytosis also occurs at septa (far left), and may even occur at other places in the cell through
 an unexplored mechanism (orange and red dotted arrows).

Figure 1

FIGURES

Figure 2