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The Cytoskeleton and Polarity Markers During Polarized Growth of Filamentous Fungi

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I. Introduction

Maintaining cell polarity is essential for cells to ensure homeostasis and their proper functioning (Goehring and Grill 2013; Wu and Lew 2013). Symmetry breaking is often preceded by cytoskeleton-dependent polarization of certain key proteins as observed in epithelial cells with apical-basal polarity, neuronal differentiation from dendrites to axons, and migrating cells. Filamentous fungi are highly polarized eukaryotic cells, which continuously elongate their hyphae at the tips. Some distance back from the tip, hyphae can initiate new sites of polar growth in the process of branch formation. The establishment and maintenance of polar growth is one fascinating question in biology. Filamentous fungi are widely used as model systems for the analysis of the relationship between cell polarity and shape (Harris 2006; Fischer et al. 2008; Riquelme et al. 2011, 2018; Takeshita et al. 2014). Some filamentous fungi are pathogenic to animals and plants, and often growth in the host is accompanied by a change from hyphal growth to yeast-like growth or vice versa (dimorphism) (Garcia-Vidal et al. 2013). Other fungi are useful in biotechnology, such as enzyme production and fermentation in food industry due to their high ability of enzyme secretion (Punt et al. 2011). Thus, the analysis of polarized growth of filamentous fungi can contribute to the medical, agricultural, and biotechnological fields.

The filamentous ascomycete *Aspergillus nidulans* has been employed worldwide for more than 60 years as a model organism because it is closely related to clinically and economically important *Aspergilli* and it is easily manipulated in the laboratory. The most characteristic cell type of filamentous fungi is the vegetative hypha. This nonspecialized, syncytial (multinucleated) cell is characterized by a continuous polarized growth mode, mediated at the tip through the addition of new material that is transported from distal regions. In single-cell yeasts, such as in budding yeast *Saccharomyces cerevisiae* and in fission yeast *Schizosaccharomyces pombe*, polarized growth is restricted to certain times during the cell cycle, whereas in filamentous fungi cell extension is a continuous and indefinite process.

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The extension of hyphal tips requires the continuous enlargement of the cell membrane and the extension of the cell wall. Both are achieved through massive vesicle fusion at the tip. The vesicles transport cell wall-synthesizing enzymes and provide new membrane. Vesicle transport as well as all other dynamic processes related to polar growth, such as organelle duplication and distribution, or the transport of RNA, proteins, or lipids, requires cytoskeletal elements. Many of those components were discovered early on in mutant screenings followed by genetic and later molecular biological analyses. For instance, tubulin was discovered through a combination of biochemical analyses with the analysis of mutants with altered sensitivity against the microtubule drug benomyl (*benA*) in *A. nidulans* (Oakley 2004). Mitotic elements were isolated as temperature-sensitive mutants with a *block-in-mitosis* (*bimA*, *bimC*, *bimE*) or mutants, which never entered mitosis (*never-in-mitosis*, *nimA*, *nimX*, *nimT*) (Orr and Rosenberger 1976; Morris and Enos 1992; Osmani and Mirabito 2004). In other mutants of this screening, *nuclear distribution* (*nudA*, *nudE*) was affected (Meyer et al. 1988). In subsequent suppressor screenings using the *benA33* mutant, *tubA* and *mipA* were discovered (Morris et al. 1979; Oakley and Oakley 1989). The genes encoded beta tubulin (*benA*), alpha tubulin (*tubA*), or gamma tubulin (*mipA*), dynein (*nudA*), or kinesin (*bimC*). The mutagenesis approaches performed in *S. cerevisiae* and *S. pombe* but also filamentous fungi such as *A. nidulans* revealed a wealth of information, which could probably not have generated by other means. The improved molecular biological methods and the genome information opened the possibility of reverse-genetic approaches. With these approaches the role of proteins of conserved pathways was studied in other organisms and organism-specific functions were discovered. However, recent major advances in cost-effective sequencing of entire genomes have the great potential to revolutionize our approaches again and allow intelligent mutant screening followed by bulk sequencing of mutant genomes. This strategy reduced mutant analysis from months or years to weeks or months (Tan et al. 2014).

Polarized growth is thus studied by genetic, molecular biological, biochemical, and cell biological methods. However, this research field has benefited more than others from the combination of the still ongoing improvement of the microscopic techniques and the development of fluorescent reporter proteins in recent years. Several overviews have summarized different aspects of polarized growth in fungi (Chang and Peter 2003; Harris and Momany 2004; Penalva 2010; Berepiki et al. 2011; Steinberg 2011, 2014; Sudbery 2011; Egan et al. 2012a).

Here we review recent findings unraveling the mechanism of polarized growth with special emphasis on the roles of the actin and microtubule (MT) cytoskeletons, polarity markers linking the two cytoskeletons.

II. The Actin Cytoskeleton

The actin cytoskeleton plays a central role in cell morphology of eukaryotic cells (Dominguez and Holmes 2011). **Actin filaments (F-actin)**, which are composed of linear polymers of **G-actin** subunits, generate force against the plasma membrane and also act as tracks for myosin motors. The dynamic cycles of polymerization and depolymerization of G-actin and F-actin are involved in many different key cellular processes, such as cell motility, cytokinesis, secretion, and the control of cell morphology (Michelot and Drubin 2011).

There are three high-order **F-actin** structures with distinct functions in filamentous fungi: actin rings, patches, and cables (Berepiki et al. 2011). Studies using anti-actin chemical agents confirmed that a polymerized actin cytoskeleton is required for normal apical growth and hyphal tip shape in different fungal organisms (Torralba et al. 1998). Phalloidin conjugated to fluorescent dyes has been widely used for imaging F-actin in eukaryotic cells including fungi such as budding yeast (Amberg 1998), fission yeast (Pelham and Chang 2001), and *Ashbya gossypii* (Walther and Wendland 2004) but does not work in most filamentous fungi (Brent Heath et al. 2003). The immunostaining of actin using an anti-actin antibody and GFP-labeled actin or an actin-binding protein (AbpA

in *A. nidulans*) revealed the localization of the F-actin structures, mainly actin rings and actin patches, in filamentous fungi (Araujo-Bazan et al. 2008). However, visualization of actin cables was difficult by these methods. Recently, specific markers for actin cables, such as Lifeact and tropomyosin, were developed to visualize them (Taheri-Talesh et al. 2008; Berepiki et al. 2010; Delgado-Alvarez et al. 2010). Lifeact, which consists of 17 amino acids from the N-terminus of Abp140p of *S. cerevisiae*, has been shown to be a marker for F-actin binding and labeling in vitro and in yeast cells (Asakura et al. 1998; Riedl et al. 2008).

The **actin rings** in cooperation with class II myosin function in septum formation (Taheri-Talesh et al. 2012; Delgado-Alvarez et al. 2014) (Fig. 1a). Septum formation proceeds according to the following series of steps: actin and myosin tangle assembly at a septation site, contraction of the actin ring (or called actomyosin ring), actin-mediated invagination of the plasma membrane, and deposition of the chitinous primary septum (Momany and Hamer 1997; Delgado-Alvarez et al. 2014). Mutant analysis of class II myosin (*myoB* mutation in the converter subdomain in *A. nidulans*) suggests that the motor activity is necessary for the contraction of the actin ring (Hill et al. 2015). The actin ring assembly and septum formation are controlled through the nuclear position and cell cycle progression in *A. nidulans* (Harris 2001).

Actin patches are peripheral punctate structures where probably the endocytic machinery localizes (Araujo-Bazan et al. 2008) (Fig. 1b). The predominant localization of these patches at subapical regions suggests spatial coupling of apical exocytosis and subapical compensatory endocytosis (Penalva 2010). Class I myosins function in

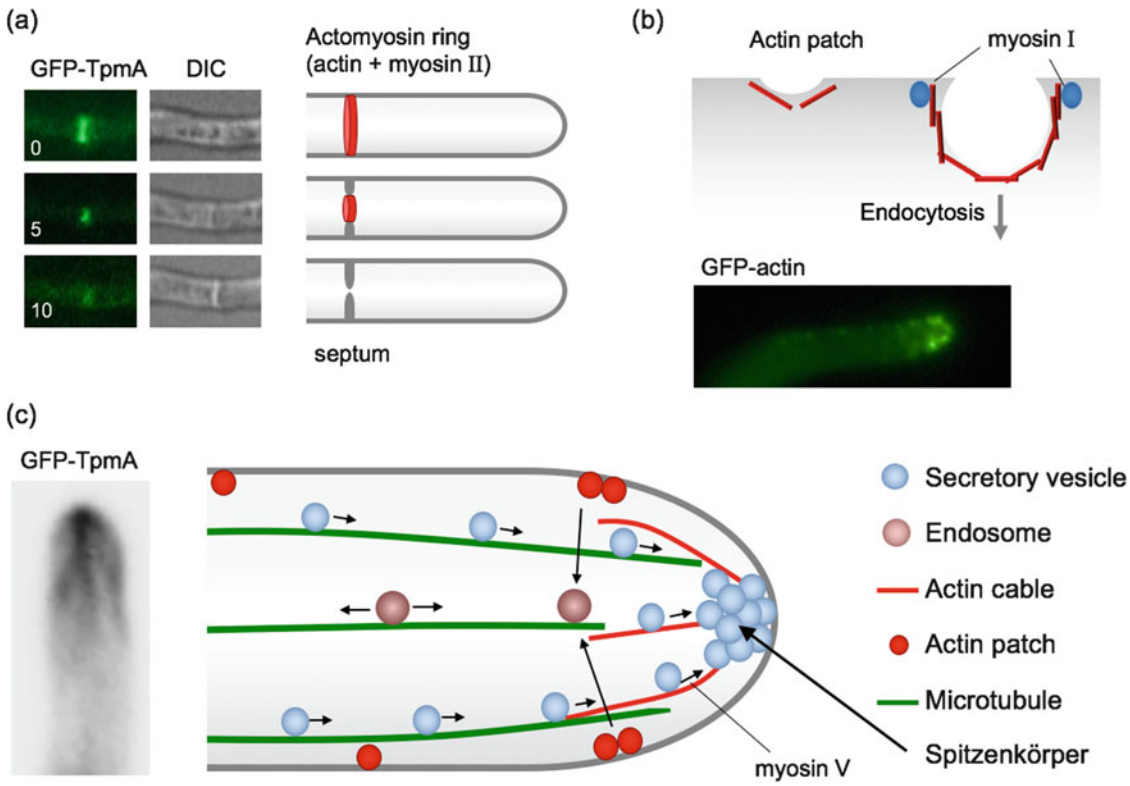

Fig. 1 Distinct roles of actin cytoskeletons and related myosin in *A. nidulans*. (**a**) Actin ring and myosin II (actomyosin ring) for septation. (**b**) Actin patch and myosin I for endocytosis. (**c**) Actin cables and myosin V for exocytosis. Scheme of tip growth in *A. nidulans* hyphae. Vesicle trafficking via the actin and MT cytoskeleton. Before fusion with the plasma membrane, secretion vesicles accumulate at Spitzenkörper

endocytosis to support invagination of endocytic vesicles (Kaksonen et al. 2006). Mutant analysis of *myoA*, the class I myosin in *A. nidulans*, revealed that the function of MyoA in endocytosis is regulated through phosphorylation by a member of the p21-activated kinase (PAK) family (Yamashita and May 1998). The mutant phenotype of genes involved in endocytosis indicates that endocytosis is essential (Araujo-Bazan et al. 2008). Besides the internalization of extracellular molecules, plasma membrane proteins, and lipids by endocytosis, endocytic recycling of polarized material at the hyphal tip and a balance between endocytosis and exocytosis at the hyphal tip are assumed to control polarized growth and cell shape (Shaw et al. 2011).

Actin cables are linear bundles of short actin filaments nucleated by formins that are present at the apex of the hyphae. As mentioned before, dynamic actin cables are generally very difficult to visualize; however, recently specific markers, such as Lifeact and tropomyosin, were developed (Taheri-Talesh et al. 2008; Berepiki et al. 2010; Delgado-Alvarez et al. 2010). In *N. crassa*, Lifeact has been used to visualize dynamic actin cables and patches (Berepiki et al. 2010; Delgado-Alvarez et al. 2010). However, it has to be considered that overexpression of the construct may cause some artifacts (Bergs et al. 2016). Tropomyosin is a conserved actin filament-binding protein and regulates the interaction between actin and myosin in response to Ca^{2+} (Gunning et al. 2005). Tropomyosin has been used as a marker for actin cables in *A. nidulans* and *N. crassa* (Evangelista et al. 2002; Pearson et al. 2004; Taheri-Talesh et al. 2008). GFP-labeled tropomyosin, TpmA in *A. nidulans*, revealed the dynamic behavior with cycles of elongation and shrinkage (Fig. 1c) (Bergs et al. 2016). Multiple actin cables were formed from the hyphal tip with each actin cable showing elongation and shrinkage in an independent manner.

Actin cables are present at the apex of hyphae and are thought to serve as tracks for class V myosin-dependent secretory vesicle transport to the tip (Fig. 1c) (Taheri-Talesh et al. 2008, 2012; Berepiki et al. 2011; Pantazopoulou et al. 2014). The "basic" growth machinery involved in the formation of actin cables, vesicle transport, and exocytosis, such as formin, the polarisome (protein complex Spa2, Pea2, Aip3/Bud6, and formin Bni1 in *S. cerevisiae*), class V myosin, and the exocyst complex (octameric protein complex involved in the tethering of post-Golgi vesicles to the plasma membrane prior to vesicle fusion), is relatively conserved among eukaryotic cells and localized to the apex of hyphae (Harris et al. 2005; Sudbery 2011). Maturation of late Golgi cisternae into exocytic post-Golgi carriers was visualized in *A. nidulans* (Pantazopoulou et al. 2014). These carriers move on a MT-based bidirectional conveyor belt relaying them to actin, which ultimately focuses exocytosis at the apex.

III. Spitzenkörper

Before fusion, the secretion vesicles accumulate at the hyphal tip in a structure called **Spitzenkörper** or vesicle supply center (VSC) (Grove and Bracker 1970; Harris et al. 2005), a special structure in filamentous fungi, which determines growth direction of the hyphae (Bartnicki-Garcia et al. 1995; Riquelme and Sanchez-Leon 2014; Riquelme et al. 1998, 2014) (Fig. 1c). A VSC in motion provides a rational basis to predict how the secretory apparatus generates morphogenesis. The Spitzenkörper is believed to function as a VSC that regulates the delivery of cell wall-building vesicles to the apical cell surface, since the simulation analysis of VSC advance as a Spitzenkörper enables to mimic the hyphal growth of *N. crassa* (Bartnicki-García et al. 1989; Riquelme et al. 2000). The exact composition and organization of the Spitzenkörper has been vigorously elucidated (Riquelme et al. 2007, 2014; Sanchez-Leon et al. 2011; Fajardo-Somera et al. 2015). In *N. crassa*, all chitin synthases localize at the Spitzenkörper core, whereas macrovesicles carrying a β-1,3-glucan synthase complex occupy the Spitzenkörper outer layer. Similar spatial distribution of DnfA and DnfB, two P4 ATPases, in the Spitzenkörper was observed in *A. nidulans* (Schultzhaus et al. 2015).

IV. Septins

As filament-forming proteins, **septins** can be considered as a part of the cytoskeleton. Septins are a conserved family of GTP-binding proteins

that form filaments in fungi and animals. Different septins form protein complexes with each other and form heteropolymers showing a variety of higher-order structures (Mostowy and Cossart 2012). In *S. cerevisiae*, five septin proteins (Cdc3, Cdc10, Cdc11, Cdc12, Shs1) form an hourglass structure associated with the plasma membrane at the mother-bud neck (Bertin et al. 2012). They act as a scaffold for proteins involved in cell division (Oh and Bi 2011). In filamentous fungi, in addition to septum formation, septins have been shown to have roles in morphogenesis, coordinating nuclear division, and organizing the cytoskeleton (Lindsey and Momany 2006; Gladfelter 2010; Bridges and Gladfelter 2014).

In the hyphal form of the human pathogen *Candida albicans*, septins form three distinct structures, a diffuse band at the base of hyphae, a bright double ring at septation sites, and a diffuse cap at hyphal tips (Sudbery 2001; Warenda and Konopka 2002). In the filamentous phytopathogen *A. gossypii*, septins form a diffuse cap at hyphal tips and rings composed of discrete bars at septation sites and at newly emerging branches (Helfer and Gladfelter 2006; DeMay et al. 2009). In *C. albicans* and *A. gossypii*, the diffuse cap initially appears at the hyphal tip and travels with the hyphal tip until an unknown signal triggers detachment from the tip and the formation of an anchored, higher-order ring. The higher-order ring encircles the hyphal cell cortex and persists at that location, while the tip then continues to grow. Septation sites are determined by positions of the high-order ring that detaches from the tip and anchors at the hyphal cell cortex in these fungi. The structural change of septins is regulated through their phosphorylation by cyclin-dependent kinases in *C. albicans* (Sinha et al. 2007) or septin-associated kinases in *A. gossypii* (DeMay et al. 2009).

In *A. nidulans* and *N. crassa*, septins form different higher-order structures, rings, filaments, bars, bands, and caps (Westfall and Momany 2002; Lindsey et al. 2010; Berepiki and Read 2013; Hernandez-Rodriguez et al. 2014). They are important for septation, germination, branch emergence, and asexual spore formation. In the basidiomycete plant pathogen *Ustilago maydis*, septins localize to a variety of structures, collars at the bud neck and filaments at growing cell tips that run along the length of the cell and partially colocalize with MTs (Boyce et al. 2005; Alvarez-Tabares and Perez-Martin 2010). Septin filaments in the basidiomycete dikaryotic hyphae of *Cryptococcus neoformans* have also been shown to occasionally colocalize with MTs (Kozubowski and Heitman 2010). These septins have roles in morphogenesis and host infection. In the plant pathogen *Magnaporthe oryzae*, the location of the appressorium septum is determined by the site of septin ring assembly (Saunders et al. 2010). The septin ring functions as a scaffold for proteins required for appressorium formation (Dagdas et al. 2012). The recent reports on septins in filamentous fungi have revealed new roles for these cytoskeletal polymers.

V. The Microtubule Cytoskeleton

MTs play a crucial role during mitosis but serve also additional functions in interphase in filamentous fungi. They are important for the distribution of nuclei and other organelles and serve as tracks for endosomes and other vesicles; thus they are important for rapid hyphal growth (Xiang and Fischer 2004; Horio and Oakley 2005; Egan et al. 2012a; Steinberg 2014).

The rather stable minus end of MTs is located at the **MT-organizing center (MTOC)**, whereas the plus end is facing to the cell periphery with alternate growing and shrinking phases. In filamentous fungi, spindle pole bodies (SPBs) serve as MTOCs (Fig. 2a) (Oakley et al. 1990). They contain γ-tubulin, first discovered in *A. nidulans*, which is required for nucleation of MTs (Oakley and Oakley 1989; Oakley et al. 1990). Furthermore, there is good evidence that areas close to the septa act as MTOCs in *A. nidulans* (**sMTOCs**) (Fig. 2a) (Veith et al. 2005; Xiong and Oakley 2009; Zekert and Fischer 2009; Zhang et al. 2017). The composition of those MTOCs, their role, and their tethering to the septal membrane remain to be elusive. However, there is also good evidence for several MTOCs in *S. pombe*, in *U. maydis*, and in higher eukaryotes. In *S. pombe* equatorial (**eMTOC**) and interphase MTOCs (**iMTOC**) have been described (Sawin and Tran 2006) but appear to be only temporally active during certain stages of the cell cycle, and their exact nature also is enigmatic. In higher eukaryotes the Golgi apparatus had some MT-forming activity (Sutterlin and Colanzi 2010).

In the tip compartment of *A. nidulans*, most MTs are oriented with their dynamic plus ends toward the hyphal tip (Fig. 2a, b) (Konzack et al. 2005). There are only a few MTs found in interphase compartments, and nuclei migrate probably along MTs until they reach a certain

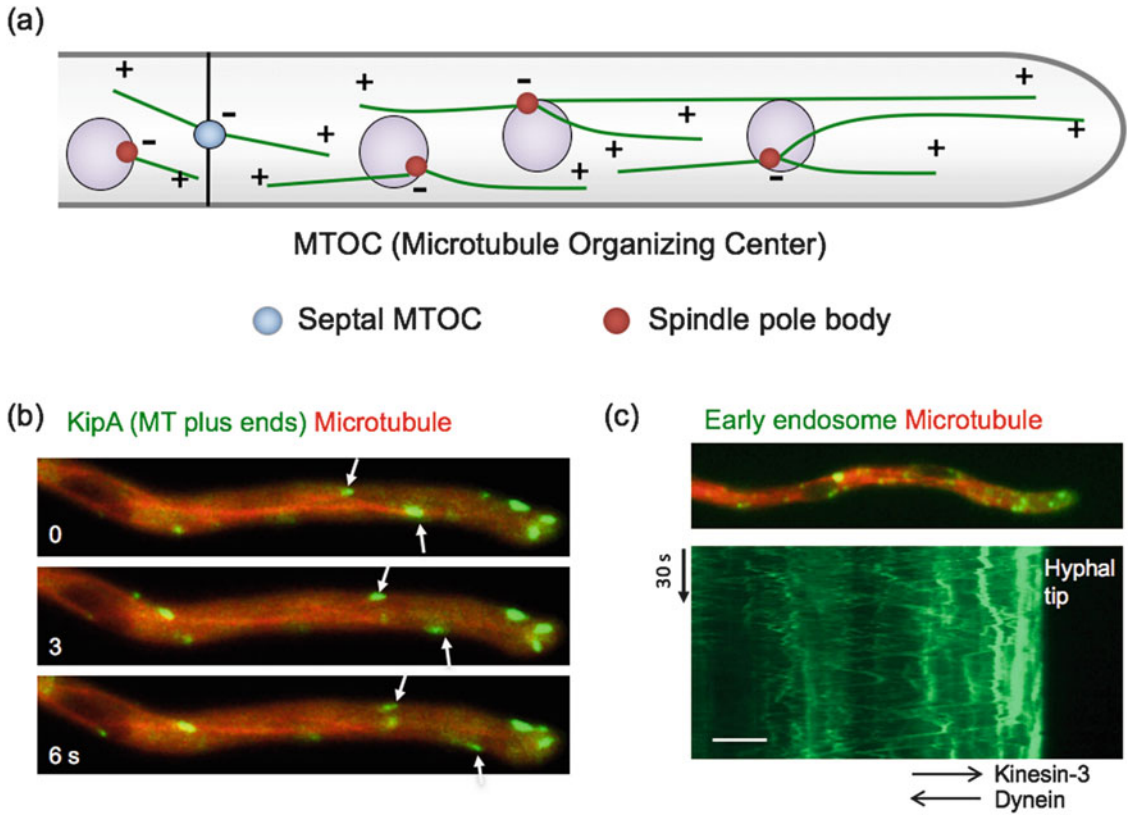

Fig. 2 Microtubule cytoskeleton in *A. nidulans*. (**a**) MT-organizing center (MTOC) at spindle pole bodies and septa. Most MTs are oriented with their dynamic plus ends toward the hyphal tip in the tip compartment. (**b**) Time lapse of GFP-KipA (MT plus end marker) and mCherry-TubA (MT) in *A. nidulans* hyphae. (**c**) Image of GFP-RabA (early endosome marker) and mCherry-TubA (MT) and kymograph of GFP-RabA

position. The entire hypha looks therefore very organized with evenly spaced nuclei.

Two classes of MT-dependent motors, the minus end-directed **dynein** and the plus end-directed **kinesins**, are involved in positioning of organelles and transport of membranes. Whereas genomes of filamentous fungi contain a single dynein motor, they usually encode 10–12 kinesins (Schoch et al. 2003). The function of kinesin-3 and the dynein motor in the transport of early endosomes have been analyzed deeply (Fig. 2c) (Steinberg 2011, 2014; Egan et al. 2012a). In *A. nidulans* hyphae, most MTs between the hyphal tip and the most proximal nucleus were polarized with their plus ends oriented toward the growing hyphal tip. Dynein and kinesin-3/UncA are the opposite motors responsible for bidirectional transport of endosomes and peroxisomes (Abenza et al. 2009; Egan et al. 2012b). Studies of *A. nidulans* kinesin-3 implicated indirect evidence for the existence of a subpopulation of detyrosinated MTs (Zekert and Fischer 2009; Seidel et al. 2012). However, a final proof for the existence of posttranslationally modified tubulin in fungi is yet still missing.

Endocytic recycling at subapical regions supports fungal tip growth. The bidirectional motility of early endosomes is thought to be involved in sorting the endocytic cargo to the subapical vacuole for degradation (Wedlich-Soldner et al. 2000). An unexpected new role for early endosome motility was revealed in *U. maydis*. The RNA-binding protein Rrm4 transports various mRNAs on moving early endosomes, suggesting that early endosome motility toward the cell ends supports polar delivery of Rrm4-bound RNAs (Konig et al. 2009; Bau-

mann et al. 2012). In addition, it was shown that the mRNA of the septins *cdc3* and *cdc12* is actively translated on moving early endosomes, and both proteins bind to early endosomes (Baumann et al. 2014). In fact, it was confirmed that ribosomes attach to early endosomes and are translationally active during the transport (Higuchi et al. 2014). It is suggested that bidirectional early endosome motility constantly stirs the translation machinery, which diffuses passively in the cytoplasm and thereby contributes to distribute the ribosomes throughout the cell (Steinberg 2014; Haag et al. 2015). Indeed, endosomal transport of heteromeric septin complexes along microtubules is crucial for formation of higher-order structures in *U. maydis* (Zander et al. 2016).

The deletion of **conventional kinesin (kinesin-1)** decreased the growth rate and caused defects in Spitzenkörper stability, protein secretion, and pathogenicity (Lehmler et al. 1997; Seiler et al. 1997, 1999; Requena et al. 2001; Schuster et al. 2012). These results suggest a possible conserved role in vesicle transportation similar to higher eukaryotic cells. High-speed imaging revealed vesicle transport of chitin synthases (Takeshita et al. 2015). The frequency of the transport was clearly decreased in the absence of *kinA*, kinesin-1 in *A. nidulans*. Secretory vesicles are thought to be transported by kinesin-1 along MTs for long distances toward hyphal tips in filamentous fungi, although the localization of the ER and the Golgi close to hyphal tips raises questions about the function and cargo of kinesin-1 (Markina-Inarrairaegui et al. 2013; Pinar et al. 2013). Possibly long-distance transport of secretion vesicles is less important and that actin-dependent movement is rather sufficient. Indeed, hyphal extension can occur quite long without functional MTs but is immediately stopped as soon as the integrity of the actin cytoskeleton is disturbed (Torralba et al. 1998; Horio and Oakley 2005). Although the role of MTs and the different cytoskeletons could be diverse in different fungi, vesicle movement and delivery to the tip plasma membrane likely depend on the cooperation of actin- and MT-dependent motors (Zhang et al. 2011; Schuster et al. 2012; Taheri-Talesh et al. 2012).

VI. Cell-End Markers for Polarity Maintenance

Cell polarity is essential for the proper functioning of many cell types. During cellular morphogenesis—from fission yeast to human cells—MTs deliver positional information to the proper site of cortical polarity (Siegrist and Doe 2007; Li and Gundersen 2008). The polarization of the actin cytoskeleton and signal transduction cascades and continuous membrane transport toward the growth site depend on MTs.

Because MT dynamics and many MT functions are conserved among eukaryotes, lower eukaryotes can serve as excellent models. In ***S. pombe***, the **kelch-repeat protein Tea1** is delivered by growing MTs to the cell ends (Fig. 3a) (Mata and Nurse 1997). Tea1 reaches the MT plus end with the kinesin-7, **Tea2** (Browning et al. 2000, 2003), and is anchored at the cell end membrane through the interaction with the prenylated protein, **Mod5** (Snaith and Sawin 2003). At the cell end, Tea1 interacts with additional components, which ultimately recruit the **formin For3** (Martin and Chang 2003; Feierbach et al. 2004; Tatebe et al. 2008). For3 forms actin cables required for exocytosis and polarized growth. **Cell-end markers**, Tea1 and Mod5, thus transmit positional information regulated by MTs to the actin cytoskeleton and thereby contribute to polarized growth. The cell-end marker genes were identified after analysis of polarity mutants (T-shaped or bent cells). The mutants of *tea1* and *tea2* (tip elongation aberrant) and *mod5* (morphology defective) exhibit T-shaped or bent cells as a result of the mislocalization of the polarity site away from the center of the cell end.

The molecular mechanism of MTs to regulate polarity maintenance is principally conserved from *S. pombe* to filamentous fungi (Fig. 3a) (Riquelme et al. 1998; Fischer et al. 2008; Takeshita et al. 2008; Higashitsuji et al. 2009; Takeshita and Fischer 2011). Tea1 and Tea2 are conserved in ***A. nidulans*** as **TeaA** (Tea1) and **KipA** (Tea2), respectively (Konzack et al. 2005; Takeshita et al. 2008). Although the Mod5 sequence is not conserved in filamentous fungi, a functional counterpart, **TeaR**, was discovered by screening for proteins that harbor a C-terminal prenylation motif in *A. nidulans* (Takeshita et al. 2008). TeaA is conserved in *Ascomycetes* and some *Basidiomycetes*, such as *Cryptococcus neoformans* and *Puccinia graminis*,

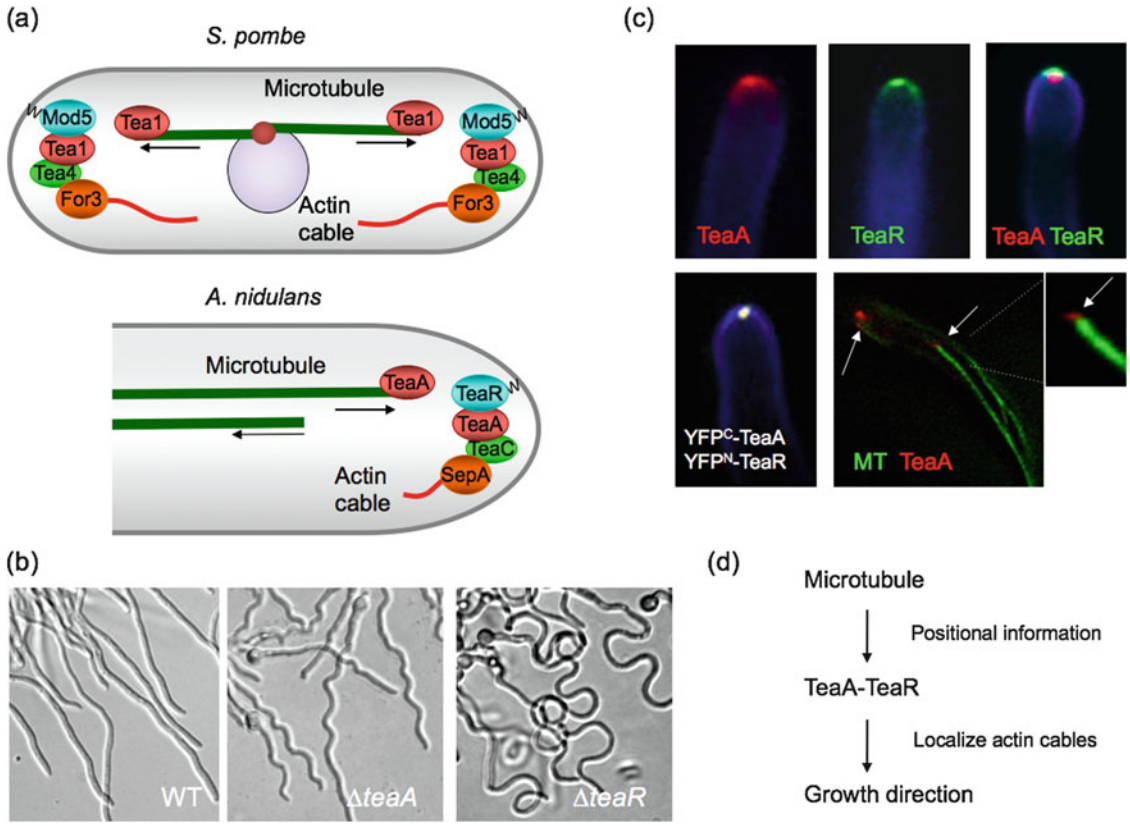

Fig. 3 Role of cell-end markers in *A. nidulans*. (**a**) Scheme of the function of cell-end markers in *S. pombe* and *A. nidulans*. (**b**) Differential interference contrast images of wild type, Δ*teaA*, Δ*teaR* strains. Δ*teaR* strains exhibited curved hyphae and Δ*teaA* strains exhibited zigzag hyphae. (**c**) Localization of mRFP1-TeaA and GFP-TeaR (upper) at the hyphal tips. Bimolecular fluorescence complementation (BiFC) assay of TeaA and TeaR at the hyphal tip (lower left). Localization of mRFP1-TeaA at the hyphal tip and MT plus end (lower right). (**d**) Scheme of growth direction regulated by cell-end markers and cell cytoskeletons

but not in *Zygomycetes*. TeaR is generally conserved in *Ascomycetes* except in *Hemiascomycetes*. The *A. nidulans kipA*, *teaA*, and *teaR* mutants show defects in polarity maintenance, which leads to curved (Δ*kipA* or Δ*teaR*) or zigzag growing hyphae (Δ*teaA*) (Fig. 3b). The two cell-end markers, TeaA and TeaR, localize at hyphal tips interdependently (Fig. 3c). TeaA is delivered to hyphal tips by growing MTs (Takeshita and Fischer 2011) and anchored to the hyphal tip cortex though the interaction with TeaR (Takeshita et al. 2008). TeaA, along with TeaC, recruits the formin, SepA, to the growth zone (Higashitsuji et al. 2009). The conserved mechanism of "cell-end markers," TeaA and TeaR, to transmit positional information from MTs to the actin cytoskeleton is required for maintenance of polarity and the growth direction of hyphae (Fig. 3d). Although the cell-end marker proteins appear to be conserved in other filamentous fungi, it remains to be studied if the roles assigned to them in *A. nidulans* are also conserved. For instance, the MT cytoskeleton is much more complex in *N. crassa*.

MTs in *A. nidulans* are necessary to maintain the polarity at the tip of hyphae through cell-end markers. Besides this function, MTs have additional functions in filamentous fungi, such as nuclear distribution and the movement of vesicles and other organelles; thus they are important for rapid hyphal growth. In contrast, MTs in *S. pombe* are required for polarity maintenance through cell-end markers but are not necessary for

vesicle trafficking. Actin cables grow toward the growing cell ends, and Myo52, a myosin V, is responsible for polarized secretion of vesicles along actin cables and hence membrane enlargement and secretion of cell wall-synthesizing enzymes (Montegi et al. 2001; Win et al. 2001; Mulvihill et al. 2006).

VII. Rho GTPase

The small Rho-type GTPases Cdc42 and Rac1 are key regulators of eukaryotic cell polarity (Jaffe and Hall 2005; Virag et al. 2007). The switching between inactive GDP-bound and active GTP-bound states is controlled by guanine nucleotide exchange factors (GEFs) (Schmidt and Hall 2002) and GTPase-activating proteins (GAPs) (Bernards and Settleman 2004). Active GTPases stimulate multiple effector molecules, such as p21-activated kinases (PAKs), mitogen-activated protein kinases (MAPKs), formin, and subunits of the exocyst complex, which regulate numerous cellular processes including the rearrangement of the actin cytoskeleton, targeted vesicle transport, and exocytosis (Bishop and Hall 2000). In *S. cerevisiae*, two positive feedback loops are thought to contribute to Cdc42 polarization. One pathway involves recruitment of GEFs and effector complexes from the cytoplasm in a cytoskeleton-independent manner (Johnson et al. 2011). The other one is a vesicle-recycling feedback loop, where Cdc42 orients actin cables, which in turn deliver Cdc42 as cargo on secretory vesicles (Wedlich-Soldner et al. 2003).

In *S. cerevisiae* and *S. pombe*, Rac1 orthologs are not conserved, and Cdc42 alone is necessary and sufficient to control polarized growth (Adams et al. 1990; Miller and Johnson 1994). In contrast, filamentous fungi require both Rho GTPases to regulate their hyphal growth (Mahlert et al. 2006; Virag et al. 2007; Lichius et al. 2014). Both Cdc42 and Rac1 share at least one overlapping function that is required for polarity establishment. The combination of Δ*cdc42* with Δ*rac1* appeared synthetically lethal in *A. nidulans* (Virag et al. 2007). The cell-end marker deletion strains in *A. nidulans* lose the axis of polarity, although the hyphae still continue polarized tip growth. Although the polarization of Cdc42 and Rac1 by positive feedback loops as known in *S. cerevisiae* remains elusive in filamentous fungi, the Rho GTPases are possibly involved in polarity establishment independently of the cell-end markers in *A. nidulans*.

VIII. Cell-End Markers for Polarity Establishment

MTs and cell-end markers are not essential for spore germination itself but only for **site selection of germination** (Takeshita and Fischer 2011). During conidia germination, TeaA appeared at growth sites prior to the appearance of a small germination bud, and then MTs contact the cortex of the tip, while the polarized MTs likely deliver more TeaA and other proteins to the bud site, enforcing polar growth. Wild-type spores always germinate at only one site to form one hypha; in contrast, spores of Δ*teaA* often germinated at two (40%) or three sites (3%) simultaneously (Fig. 4a). A similar phenotype of multi-germtube formation was observed using the MT-destabilizing drug benomyl. It had been shown already that MTs are not essential for the germination process itself (Oakley and Morris 1980). TeaA and MTs are not necessary for the emergence of the germtube but probably rather for restricting germination to a specific place.

When the spores start germination, they grow isotropically at first, and then they switch to polarized growth with new material added to the tip of an emerging germtube. In *A. nidulans*, temperature-sensitive ***swo* (swollen cell) mutants** representing genes involved in polarity establishment and polarity maintenance were isolated (Momany et al. 1999). *swoC* (putative pseudouridylate synthase) and *swoF* (N-myristoyl transferase) (Shaw et al. 2002) are required to establish polarity, while *swoA* (O-mannosyltransferase) is required to maintain polarity. Besides that, the terminal phenotype of Δ*cdc42*Δ*rac1* indicates their functions are required to establish polarity (Virag et al. 2007). In addition, the morphological abnormalities of mutants involved in endocytosis indicate the importance of endocytosis in polarity establishment and polarity maintenance (Lee et al. 2008; Hervas-Aguilar and Penalva 2010; Shaw et al. 2011).

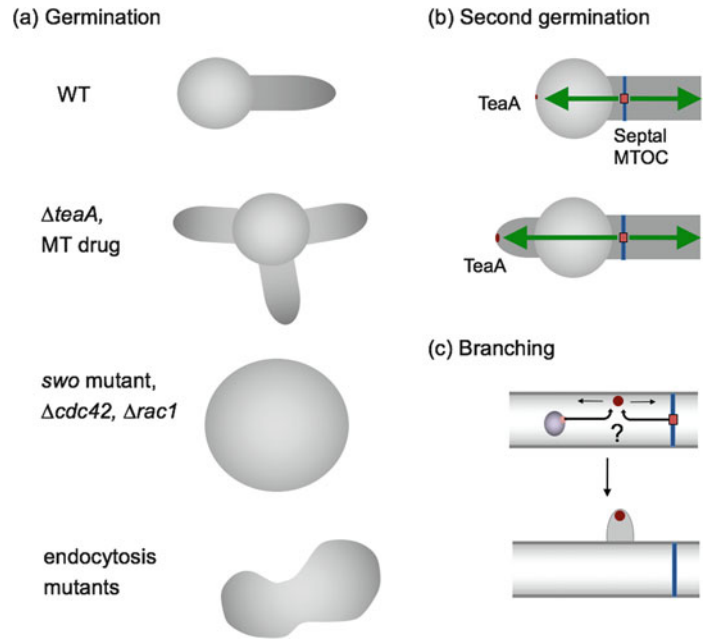

Fig. 4 Cell-end markers for polarity establishment. (**a**) Germination in wild type, and several mutants. (**b**) Scheme of second germination site selection by MTs from septal MTOC and TeaA. (**c**) Branching site selection

Once the first hypha reaches a determinate length, a second germtube appears on the spore. This **second germination site** normally lies opposite of the first hypha (Harris et al. 1999). The second germtube appeared after the first septum at the base of the first hypha was formed. TeaA appeared at the second germination site, opposite the first hypha, after septation in the first hypha. In *A. nidulans*, MTs are formed from SPBs and from septal MTOCs (Konzack et al. 2005; Veith et al. 2005; Xiong and Oakley 2009). MTs emanating from the septum of the first hypha grew toward the first germtube as well as in the direction of the spore and reached the second germination tip where TeaA localized (Fig. 4b). MTs originating from the septal MTOCs are thus important for TeaA delivery and may explain the bipolar germination pattern (Takeshita and Fischer 2011).

There appears to be two patterns of **lateral branching**: branches associated with septa and random branching. In several fungi including members of the *Saccharomycotina* (*A. gossypii*), zygomycetes (*Basidiobolus ranarum*), and basidiomycetes (*Coprinus* species), new branches emerge adjacent to septa (Trinci 1974). A random pattern of branching is observed in *A. nidulans*. Although the cell-end markers localize to branching sites prior to branching emerges, the mechanism of branch site selection remains largely unknown (Fig. 4c) (Harris 2008).

IX. Cell-End Markers for Polarity Focusing

The role of MTs in transmitting **positional information** through delivery of cell-end markers to the growth machinery is conserved in both *S. pombe* and *A. nidulans*. A Spitzenkörper, however, can only be observed in filamentous fungi but not at cell ends of fission yeast (Fig. 5a). This difference could be due to different growth speeds (Kohli et al. 2008). Another possible reason is that the cell-end markers concentrate at the apex of hyphae in *A. nidulans*, whereas the cell-end markers localize at multiple sites along cell ends in fission yeast (Dodgson et al. 2013). The positive feedback loop defined through the interdependence of TeaA and TeaR could be important for their concentration, but not sufficient because this

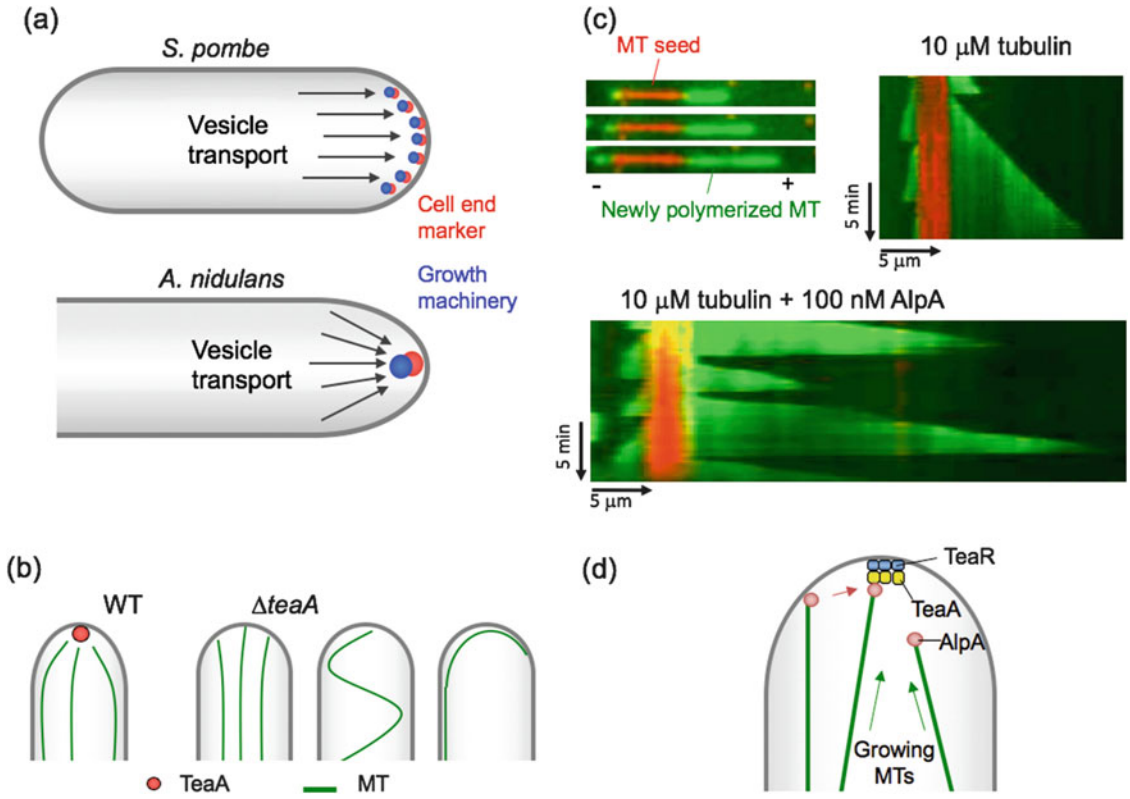

Fig. 5 Cell-end markers for polarity focusing. (**a**) Comparison of the localization of cell-end markers and the growth machinery in *S. pombe* and *A. nidulans*. (**b**) Behavior of MTs at hyphal tips in *A. nidulans* wild type and Δ*teaA* strains. (**c**) In vitro MT polymerization assay. Images of a seed MT (red) with a dynamic MT lattice growing from the plus end (green). Kymographs of MTs in the absence of AlpA (right) and presence of 100 nM AlpA (lower). (**d**) Scheme of the interaction between TeaA at the hyphal tip cortex and AlpA at MT plus ends

mechanism is conserved in *S. pombe* as well (Snaith and Sawin 2003; Takeshita et al. 2008; Bicho et al. 2010). MTs in *A. nidulans* elongate toward the tips and tend to converge in the apical region (Konzack et al. 2005), which is not observed in *S. pombe*. The central position of TeaA at the tip correlated with the convergence of the MT plus ends to a single point. In the absence of TeaA, MTs often contacted the membrane off the center of the apex (Fig. 5b) (Takeshita et al. 2008, 2013).

A recent study showed that a functional connection between TeaA and the **MT polymerase AlpA** is required for proper regulation of MT growth at hyphal tips (Takeshita et al. 2013). AlpA is a member of the XMAP215/Dis1 family whose conserved TOG domains, which contain multiple HEAT repeats, are known to bind tubulin from yeast to human (Al-Bassam et al. 2007). XMAP215 from *Xenopus laevis* catalyzes the addition of tubulin dimers to the growing plus ends (Brouhard et al. 2008; Al-Bassam and Chang 2011). *A. nidulans* AlpA decorates MT filaments and accumulates at MT plus ends (Enke et al. 2007). Deletion of *alpA* resulted in a drastic reduction of the MT array and dynamics. MT in vitro polymerization assays with purified tubulin from porcine brains and recombinant AlpA have revealed the activity of AlpA as a MT polymerase (Fig. 5c) (Takeshita et al. 2013). The MT growth speed in vitro was comparable with that of XMAP215 of *X. laevis* and approximately fourfold higher than that of Alp14, the orthologue in *S. pombe* (Brouhard et al. 2008; Al-Bassam et al. 2012). The rate of MT

polymerization in vivo in *A. nidulans* leading hyphae is approximately threefold higher than in *S. pombe*, consistent with the ratio in vitro (Drummond and Cross 2000; Efimov et al. 2006). However, AlpA-dependent MT growth speed in vitro was approximately only half of the one determined in vivo (6 μm/min compared to 13 ± 3 μm/min). Therefore, other microtubule plus-end-tracking proteins are likely to enhance the AlpA activity for MT growth in vivo.

As a difference to *S. pombe*, *A. nidulans* TeaA is involved in the convergence of MT plus ends at the tip apex, suggesting specific interactions of the MT plus end with the cortex. One possibility is an interaction between TeaA and AlpA (Takeshita et al. 2013). MT polymerization assays in vitro showed that TeaA increased the catastrophe frequency of MTs in the presence of AlpA, and TeaA reduced the in vitro AlpA activity significantly. From these results it was concluded that AlpA promotes MT growth at MT plus ends until MTs reach the hyphal tip, where TeaA blocks the AlpA activity and induces MT catastrophe (Fig. 5d). The interdependence of TeaA and MTs could act as a positive feedback loop to concentrate TeaA at the apex resulting in well-focused vesicle secretion for the organization of the Spitzenkörper (Bartnicki-Garcia et al. 1995; Riquelme et al. 2014).

X. Transient Polarity Marker Assembly

The membrane-associated cell-end marker TeaR is highly dynamic at growing hyphal tips (Ishitsuka et al. 2015). A MT grows toward the hyphal tip, pauses in close contact with the apical membrane, and then undergoes a catastrophe event resulting in retraction. TeaR accumulates at the hyphal tip membrane and then decreases immediately after a MT plus-end touches the tip membrane and starts to shrink. Colocalization studies indicate that TeaR clusters represent zones of exocytosis at the apical membrane. In general, membrane-binding polarity markers are delivered to the plasma membrane by vesicle transport and exocytosis. After exocytosis, vesicles fuse with the plasma membrane, leading to its extension. Simulation analysis had predicted that membrane insertion by active exocytosis would dilute and/or disperse membrane-binding polarity markers during fast hyphal growth (Savage et al. 2012). There was no clear answer to the question of how cell polarity is maintained during incessant vesicle exocytosis, especially for rapidly growing systems such as filamentous fungi. One of the problems to capture the complex process is that conventional live cell imaging methods lack the resolution.

The localization of TeaR cluster at hyphal tips has been analyzed by super-resolution microscopy PALM (photoactivation localization microscopy) (Ishitsuka et al. 2015). The resolution of conventional light microscopy techniques is limited to about 250 nm due to light diffraction. Super-resolution microscopy techniques, such as PALM, have overcome the diffraction limit, resulting in lateral image resolution as high as 20 nm and providing a powerful tool for the investigation of protein localization in high detail (Sahl and Moerner 2013). The PALM imaging analysis revealed that TeaR transiently assembles (approximately 120 nm, 20 TeaR proteins per cluster on average) at the hyphal tip membrane and disperses along the membrane after exocytosis, which inserts a new membrane that results in local membrane extension (Fig. 6a) (Ishitsuka et al. 2015).

These findings gave rise to a "transient polarity assembly model" to explain how fungal tip cells extend through repeated cycles of TeaR assembly/disassembly, actin polymerization, and exocytosis rather than by constant elongation (Fig. 6b) (Takeshita 2016). The findings of colocalization studies support the notion that TeaR clusters represent zones of exocytosis and are prerequisite for apical membrane extension. In this model, the interaction between the MT plus end, where TeaA is located, and TeaR at the apical membrane initiates the recruitment of other polarity markers, resulting in the assembly of TeaR polarity site. The accumulated cell-end markers induce actin polymerization followed by active exocytosis.

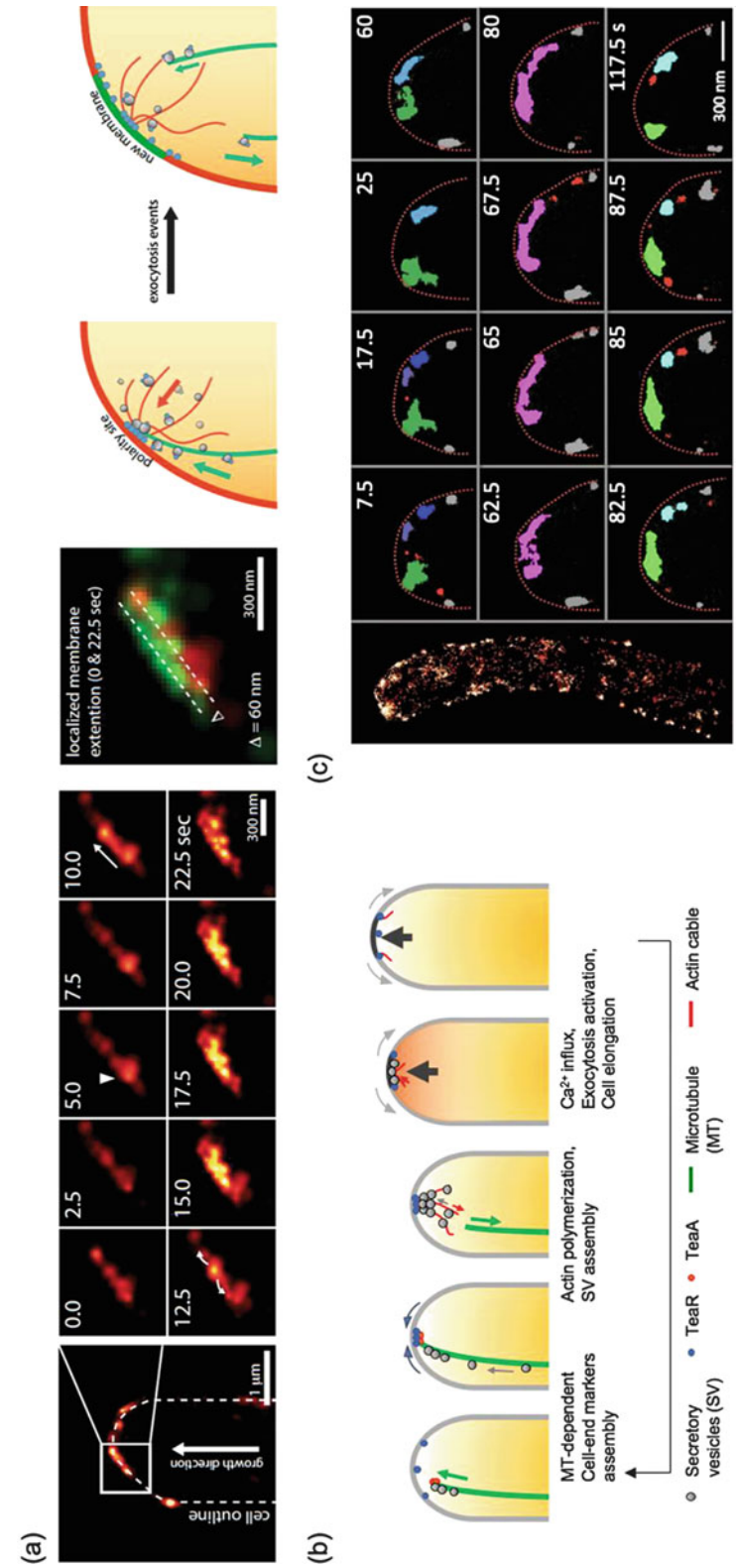

Fig. 6 Oscillatory fungal cell growth. (**a**) Time-lapse PALM of TeaR shows the appearance of a new cluster ($t = 5$ s), a translational movement ($t = 10$ s), and a spreading of the signal along the plasma membrane. Local membrane extension ($t = 0$ in red, $t = 22.5$ s in green). (**b**) Scheme of transient polarity model and temporally controlled actin polymerization and exocytosis coordinated by pulsed Ca^{2+} influx. (**c**) Time-lapse cluster analysis of PALM images of chitin synthase ChsB. (Left) Localization image of a hypha with mEosFPthermo-ChsB clusters (500 frames). (Right) Sequence of ChsB cluster images (clusters in different colors) rendered from images reconstructed by moving window binning (cluster images of 2.5 s time intervals for a total period of 125 s)

Newly synthesized TeaR is delivered to the tip membrane on secretory vesicles through MT and actin cables. The plasma membrane extends locally at the site of vesicle fusion, and, subsequently, the TeaR polarity site is dispersed or displaced along the membrane. Once the polarity site is disassembled, however, next MT comes to the tip and gathers TeaR floating in the membrane through the interaction with TeaA at the MT plus end and the cycle starts over.

In line with this model, recent work on *N. crassa* has identified bursts of exocytotic events at various sites within the apical membrane rather than a persistent exocytosis site (Riquelme et al. 2014). Mathematical simulation analysis confirmed the validity of the transient polarity assembly model (Ishitsuka et al. 2015). The simultaneous visualization of actin cables and MTs suggests temporally and spatially coordinated polymerization and depolymerization between the two cytoskeletons (Bergs et al. 2016). Interaction between MigA, a MT plus-end localizing protein, and a class V myosin suggests that an active mechanism captures MTs and pulls the ends along actin filaments (Manck et al. 2015). These results also support the model.

XI. Oscillatory Fungal Cell Growth

Many dynamic cellular processes that appear continuous are driven by underlying mechanisms that oscillate with distinct periods. For example, eukaryotic cells do not grow continuously but rather by pulsed extension of the periphery. Stepwise cell extension at the hyphal tips of several filamentous fungi was discovered 20 years ago (Lopez-Franco et al. 1994), but only a few molecular details of the mechanism have been clarified since then. The "transient polarity assembly model" also implies the stepwise cell extension. Indeed, the time-lapse PALM imaging revealed that the cell extension rate of hyphae is not constant but varies in an oscillatory manner (Fig. 6c) (Zhou et al. 2018).

In addition, a recent study has provided evidence for molecular mechanism of oscillatory fungal cell growth. Live cell imaging analyses revealed oscillations of actin assembly and exocytosis in growing hyphal tips (Takeshita et al. 2017). Intracellular Ca^{2+} levels are known to regulate actin assembly and vesicle fusion (Janmey 1994; Schneggenburger and Neher 2005). Ca^{2+} levels also pulsed at the hyphal tips (Kim et al. 2012). The **fluorescence-based Ca^{2+} biosensor R-GECO** varies the emission according to Ca^{2+} concentrations and has enabled the visualization of pulsatile Ca^{2+} concentrations in growing hyphal tips of *A. nidulans* (Takeshita et al. 2017). Intracellular Ca^{2+} levels pulse at ~30-second intervals. These positively and temporally correlate with amounts of F-actin and secretory vesicles at hyphal tips. Orthologues of Ca^{2+} channels at the plasma membrane in *A. nidulans* are required for proper tip growth and the oscillation of F-actin, secretory vesicles, and Ca^{2+} level pulses (Wang et al. 2012; Takeshita et al. 2017). These results suggest that pulsed Ca^{2+} influx coordinates the temporally controlled actin polymerization and exocytosis that drive stepwise cell extension (Fig. 6b). The oscillatory fungal growth could be important for dynamic responses to external and internal signals in chemotropism, cell-cell fusion, microbial interaction, and the fungal penetration of plant and animal cells (Takeshita 2018).

XII. Conclusion

The establishment and maintenance of cell polarity in fungi—as in higher eukaryotes—require the interplay between the actin and MT cytoskeletons and landmark proteins at the cortex (Siegrist and Doe 2007; Li and Gundersen 2008). This rather complex arrangement of components may be necessary to guarantee robust polar growth. Coordinated oscillations of actin polymerization, exocytosis, and Ca^{2+} levels associated with cell growth seem to be a conserved phenomenon shared among various organisms, including fungi (Das et al. 2012), mammalian cells (Wollman and Meyer 2012), and plant root hairs (Monshausen et al. 2008) and pollen tubes (Kroeger and Geitmann 2012). The oscillatory cell growth allows cells to respond more quickly and often to internal and external cues such as chemical or mechanical environmental signals.

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