

Cell Biology of Hyphal Growth

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ABSTRACT Filamentous fungi are a large and ancient clade of microorganisms that occupy a broad range of ecological niches. The success of filamentous fungi is largely due to their elongate hypha, a chain of cells, separated from each other by septa. Hyphae grow by polarized exocytosis at the apex, which allows the fungus to overcome long distances and invade many substrates, including soils and host tissues. Hyphal tip growth is initiated by establishment of a growth site and the subsequent maintenance of the growth axis, with transport of growth supplies, including membranes and proteins, delivered by motors along the cytoskeleton to the hyphal apex. Among the enzymes delivered are cell wall synthases that are exocytosed for local synthesis of the extracellular cell wall. Exocytosis is opposed by endocytic uptake of soluble and membrane-bound material into the cell. The first intracellular compartment in the endocytic pathway is the early endosomes, which emerge to perform essential additional functions as spatial organizers of the hyphal cell. Individual compartments within septated hyphae can communicate with each other via septal pores, which allow passage of cytoplasm or organelles to help differentiation within the mycelium. This article introduces the reader to more detailed aspects of hyphal growth in fungi.

INTRODUCTION

Filamentous fungi are a large and ancient clade of microorganisms that occupy a broad range of ecological niches (1, 2). Fungi are recyclers, being major decomposers of plant debris (3); they form mycorrhizal symbiosis with 93% of all flowering plant families (4), and they serve in the industrial production of proteins (5). However, fungi pose a threat to public health, the ecosystem, and our food security (6, 7). The success of filamentous fungi is largely due to their elongate hypha, a chain of cells separated from each other by septa (8).

Hyphae grow rapidly by polarized exocytosis at the apex (9–11), which allows the fungus to extend over long distances and invade many substrates, including soils and host tissues. Hyphal tip growth is initiated by establishment of a growth site and the subsequent maintenance of the growth axis, with transport of growth supplies, including membranes and proteins, delivered by motors along the cytoskeleton to the hyphal apex (12). Among the enzymes delivered are cell wall synthases that are exocytosed for local synthesis of the extracellular cell wall (13). Exocytosis is opposed by endocytic uptake of soluble and membrane-bound material into the cell (14). The first intracellular compartment in the endocytic pathway is the early endosomes (EEs), which emerge to perform essential additional functions as spatial organizers of the hyphal cell (15). Individual compartments within septated hyphae can communicate with each other via septal pores, which allow passage of cytoplasm or organelles (16) to help differentiation within the mycelium (17). This article introduces the reader to more detailed aspects of hyphal growth in fungi.

Received: 28 November 2016, **Accepted:** 8 December 2016,
Published: 21 April 2017

Editors: Joseph Heitman, Department of Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC 27710; Neil A. R. Gow, School of Medical Sciences, University of Aberdeen, Fosterhill, Aberdeen, AB25 2ZD, United Kingdom

Citation: Steinberg G, Peñalva MA, Riquelme M, Wösten HA, Harris SD. 2017. Cell biology of hyphal growth. *Microbiol Spectrum* 5(2): FUNK-0034-2016. doi:10.1128/microbiolspec.FUNK-0034-2016.

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REGULATION OF HYPHAL MORPHOGENESIS

Hyphal morphogenesis refers to the complex biological processes that directly contribute to the formation of highly polarized hyphae by filamentous fungi. The two fundamental processes that underlie hyphal morphogenesis are polarity establishment and polarity maintenance. The regulation of these processes over time and space presumably accounts for much of the variation in hyphal morphology and growth patterns observed in the filamentous fungi (18). Additional processes that contribute to the complexity of hyphal morphology in those filamentous fungi that belong to the Dikarya include septation and the formation of septal pores (19). The regulation of these processes must also play an important role in specifying the overall organization of hyphae in these fungi. Nevertheless, we are still at an early stage in understanding the temporal and spatial regulation of hyphal morphogenesis. Progress has been achieved in determining how hyphae maintain a polarity axis (20, 21), but we still know little about how polarization sites are initially established in germinating spores and during branch formation. In addition, much remains to be learned about when and where septa are made. The following sections will address these points, but before doing so, it is perhaps useful to focus on the broader regulatory issue of how morphogenesis is coordinated with hyphal growth.

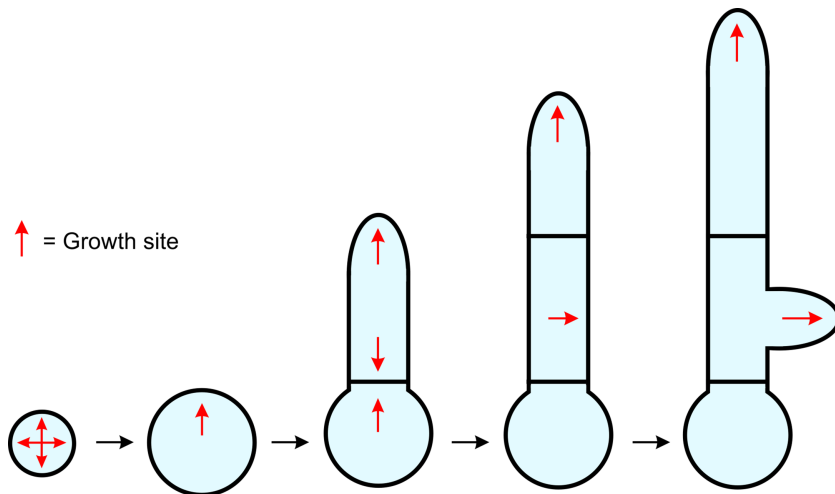
Experimentally, we typically grow filamentous fungi and assess morphogenesis under conditions that are largely homogeneous and in which all needed nutrients are available. This is unlikely to be representative of conditions that hyphae would normally experience in their natural environment (though there are likely to be important exceptions). In reality, most hyphae propagate in a spatially heterogeneous environment that is characterized by a patchy distribution of nutrients. Accordingly, two possible strategies could conceivably be invoked to account for hyphal extension under variable conditions. First, hyphal growth could be a “brute-force” mechanism by which a hypha harnesses the morphogenetic machinery (i.e., the cytoskeleton and vesicle trafficking machinery) and turgor pressure to plough ahead with colonization of the substrate. Extension rates and the frequency of branching may only be minimally adjusted to account for a changing environment. Alternatively, hyphae may fine-tune extension rates and morphogenesis to reflect the local environment at the tip and at incipient sites of branch formation. It seems reasonable to speculate that some combination of both strategies is deployed within a given mycelium. However, the latter strategy would seemingly rely more on

the ability of hyphae to adjust the timing and location of polarization events, as well as to modulate the degree of polarity maintenance, in response to local environmental conditions. Insight into how this might occur comes from the model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Both normally utilize an internal program to specify the position of localized cell surface expansion and cell wall deposition but are able to override the program in response to external signals such as mating pheromones (22). Accordingly, it is tempting to speculate that the signaling pathways that mediate growth and stress responses in filamentous fungi (e.g., TOR, PKA, HOG) likely interface with the mechanisms that temporally and spatially regulate hyphal morphogenesis. To date, little is known about how these pathways might impact hyphal morphogenesis, though there is growing evidence that they do mediate tropic responses that influence the directionality of hyphal extension (23). Characterization of the links between these pathways and the morphogenetic machinery would appear to be fertile territory for future investigation.

Spatial Regulation of Hyphal Morphogenesis

The key events underlying the spatial regulation of hyphal morphogenesis are the initial specification of a polarity axis (polarity establishment) and the subsequent stabilization of the axis (polarity maintenance). Two types of polarity establishment events are commonly observed in a typical hypha: spore polarization and branch formation (Fig. 1). In both cases, specification of a polarity axis occurs in a cell that is otherwise displaying isotropic or apolar growth (i.e., germinating spores or an existing hyphal compartment). Stabilization of the resulting axis requires the recruitment of the morphogenetic machinery to the specified site. As a result, cell surface expansion and cell wall deposition are subsequently confined to a discrete cortical site, which ultimately leads to the formation of a new hypha.

Detailed studies of *S. cerevisiae* and *S. pombe* have provided significant insight into the nature of the spatial landmarks that direct polarity establishment (22). *S. cerevisiae* utilizes a set of cortical landmark proteins (the Bud proteins) to specify new polarity axes (24), whereas *S. pombe* relies on microtubule-based delivery of a set of marker proteins (the Tea proteins) to position new growth sites (25). By contrast, comparatively little is known about mechanisms that specify new polarity axes in filamentous fungi. The Tea proteins are reasonably well conserved (26) and play a fundamental role in regulating the directionality of hyphal extension (see below). A subset of the Bud proteins is also present in



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FIGURE 1 Growth patterns in fungal hyphae. Growth occurs in an isotropic fashion during spore germination. Specification of a polarity axis ultimately results in the formation of a hypha that continues to grow at the tip. While tip growth is maintained, the specification of additional polarity axes enables the formation of septa and lateral branches. Whereas septum formation is transient, branching results in the formation of a secondary hypha that also continues to grow at the tip. Red arrows designate polarity axes.

filamentous fungi (27, 28), but instead they regulate septum formation (see below). Accordingly, at this time, the identities of any landmark/marker proteins that mediate polarity establishment in filamentous fungi remain a mystery. There is even some question as to whether a marking system is indeed necessary. Few examples of spatially biased spore polarization have been observed (e.g., reference 23), and when reported, it is in response to signals emanating from a potential plant host. Indeed, for those fungi whose spores are capable of producing multiple hyphae, it appears to be more important to ensure that the second polarity axis is opposite to the first. This type of bipolar spore polarization pattern is often observed and can be perturbed by disruption of the vesicle trafficking machinery, microtubules, and Tea marking system (29, 30).

A unique feature of filamentous fungi that enables the formation of mycelia is the ability to simultaneously sustain multiple axes of hyphal polarity. These axes result in the formation of branches, either lateral branches that emerge from subapical compartments or apical branches that form by “splitting” of the hyphal tip (31). As with spore polarization, branch formation requires the initial specification of a polarity axis followed by its stabilization. To date, the extent to which branching occurs in a defined pattern has not been fully explored. Furthermore, relatively little is known about mechanisms potentially involved in spatial regulation of branch formation in filamentous fungi. Nevertheless, key clues have emerged from a limited number of studies. For example, in *Aspergillus nidulans*, the absence of the Cdc42 GTPase or the FadA heterotrimeric G protein complex

largely abolishes the formation of lateral branches and suggests signaling pathways that might be required for the specification of branch sites (32) (S. Harris, unpublished results). More intriguingly, the well-characterized septins appear to play a critical role in the specification and/or stabilization of new polarity axes at lateral branch sites (33). Elegant work using *Ashbya gossypii* suggests that the pivotal function of septins at incipient branch sites is to recognize membrane curvature (34). Lastly, evidence suggests that some fungi (e.g., *A. nidulans*, *Epichloe festucae*) actively suppress lateral branch formation from regions adjacent to hyphal tips through the localized accumulation of reactive oxygen species mediated by NADPH oxidase complexes (35, 36). This presumably ensures that growth is directed toward the tip and facilitates migration away from depleted substrate when nutrients are scarce.

A key feature that distinguishes hyphae from yeast cells is their ability to sustain polarized growth over a considerable distance. This implies that the regulatory systems that stabilize polarity axes must be much more stringent in filamentous fungi to support the long-range vectorial delivery of secretory vesicles to the hyphal tip. Although several components of the morphogenetic machinery have been implicated in the maintenance of hyphal polarity (20, 21), the nature and identity of any regulatory system that organizes the machinery and marks the hyphal tip remain elusive. It has been firmly established that spatially coupled exocytosis and endocytosis are essential for the stabilization of polarity axes (37, 38), and it has been suggested that this could reflect the need to recycle one or more cell surface markers

that identify the hyphal tip (39, 40). It should also be added that a secondary role for the coupling of endocytosis with exocytosis might be to enable the spontaneous generation of local asymmetries that are then amplified to define a polarity axis if no functional marker is present. To date, the most compelling candidate for a hyphal tip marker is the *A. nidulans* TeaR protein (a homologue of the *S. pombe* mod5 cell end marker). TeaR is associated with exocytic vesicles that are transported on microtubules and delivered to the cell surface at the hyphal tip, where it mediates the local recruitment of the morphogenetic machinery (41). Recent evidence suggests that membrane-associated TeaR is rapidly dispersed at hyphal tips, only to be replenished again via microtubules (42). This result supports the existence of a dynamic feedback loop that continually establishes transient polarity axes at the hyphal tip that serve to maintain the overall direction of hyphal extension. The presence of lipid microdomains at hyphal tips and their importance in the formation of stable polarity axes raise the possibility that additional regulatory systems may operate in parallel with TeaR to mark the hyphal tip (43, 44).

Filamentous fungi exhibit tropic responses to chemical, mechanical, electrical, and other environmental stimuli (see references 45 and 46). The dynamic nature of polarity maintenance provides a satisfying explanation for how the direction of hyphal extension can be rapidly reoriented in response to external signals. Moreover, it seems likely that the perception and transduction of external signals can override internally programmed polarity systems such as that mediated by TeaR. The demonstration that a chemotropic response is mediated by a cell surface pheromone receptor and a mitogen-activated protein kinase signaling pathway in the root colonizing fungus *Fusarium oxysporum* (23) provides some insight into how an existing polarity axis can be subverted by external signals.

Temporal Regulation of Hyphal Morphogenesis

In uninucleate fungal cells, the maintenance of nuclear content requires precise coordination of cellular morphogenesis with nuclear division. Mechanisms that couple these two processes have been described in some detail in *S. cerevisiae* and relatives. For example, in *S. cerevisiae*, regulatory mechanisms focused on the cyclin-dependent kinase (CDK) Cdc28 coordinate polarity transitions with nuclear division at multiple points in the cell cycle (47). *Candida albicans* is a polymorphic fungus that forms uninucleate hyphal cells, and here

also, CDK plays a critical role in controlling the timing of polarity establishment, septum formation, and the transition from budding to hyphal growth (48). Key CDK targets include the septins and polarisome protein Spa2 (49, 50). The importance of CDK for the temporal coordination of morphogenesis with the cell cycle has also been documented in the plant pathogen *Ustilago maydis* (51).

In multinucleate fungal hyphae, the need to coordinate the timing of polarity establishment with nuclear division is not so apparent. Indeed, *A. nidulans* mutants incapable of nuclear division are able to form elongated hyphae (52), and mutants that fail to undergo spore polarization are able to complete multiple rounds of nuclear division (29). Despite this apparent lack of dependency, there is some evidence in *Aspergillus* species that polarity establishment and septum formation are under normal circumstances coordinated with nuclear division (29, 53, 54). This presumably reflects the need to maintain a preferred ratio of cytoplasmic volume per nucleus in growing hyphae. The underlying process, which has been termed the duplication cycle (53), remains poorly characterized, and it is not even clear whether the phenomenon is broadly conserved. For example, hyphal compartments in *Neurospora crassa* and *A. gossypii* can possess >100 asynchronously dividing nuclei, and there is no apparent evidence that hyphal morphogenesis is temporally coupled to the division of these nuclei (e.g., reference 55). One final observation potentially refutes the notion that hyphal morphogenesis is not dependent on nuclear division in filamentous fungi. In particular, the temperature-sensitive *A. nidulans* *nimL*, *nimM*, and *nimN* (never-in-mitosis) fail to undergo nuclear division and fail to establish polarity (unlike other *nim* mutants; references 29, 52, 56). These mutants are very sensitive to the DNA synthesis inhibitor hydroxyurea and share their polarization phenotype with wild-type conidia exposed to hydroxyurea (29). These observations suggest that completion of an as yet to be defined step in the S phase of the cell cycle might be necessary for spore polarization to proceed.

FUNGAL EXOCYTOSIS

Hyphal growth occurs by apical extension. Cell wall-modifying enzymes, substrates, and the membrane required for cell expansion are delivered to the apex by exocytosis, which suggests that the filamentous habit of growth is strongly dependent on the secretory pathway. Such an apparently simple fact has major implications both in the fungal pathogenicity field (host invasion is

dependent on apical extension) and in the field of biotechnology (filamentous fungi are major producers of industrial enzymes). The hyphal mode of growth and its dependence on exocytosis represent an experimental advantage, because mutations impairing exocytosis even to a minor extent often result in reduced colony growth, making them easily scorable (57). Moreover, mutations impairing exocytic regulators delay or preclude polarity establishment (58, 59) or, when inactivated by conditional mutations in rapidly growing hyphae, often result in a tip swelling (60), which can be used as a cell-autonomous indicator of exocytic deficit.

The Golgi and Its Regulation

Although an increasing body of evidence supports the contention that unconventional protein secretion pathways do exist in fungi (61), these are relatively poorly understood. Therefore, this article will focus on the Golgi as the central hub in the conventional secretory pathway that sorts protein cargoes to their final destination, be it the extracellular milieu, the plasma membrane, or the endovacuolar system. Several recent reports testify to the interest that the mechanistic understanding of biosynthetic traffic is spawning. For brevity we will consider here reports using the genetic models *A. nidulans* and *N. crassa*. Pioneering work on motors that move carriers toward their destinations, carried out with *U. maydis*, will be considered below.

As in *S. cerevisiae*, in the filamentous ascomycetes *A. nidulans* and *N. crassa* the Golgi consists of isolated membrane-bound structures that do not pile up to form the characteristic stacks of mammalian cells (62, 63). Even though these isolated membranous structures were initially denoted Golgi “equivalents,” they are *bona fide* Golgi “cisternae,” equivalent to those in other eukaryotes. Their nonstacked organization represents a major experimental advantage for live microscopy studies because cisternae can be resolved by diffraction-limited optical microscopy (64–66). When imaged with fluorescent proteins, the extensively studied Golgi of *A. nidulans* is seen as a dynamic network of ring-shaped and fenestrated cisternae, often interconnected by tubular structures (64, 67, 68). This morphology is consistent with electron microscopy studies (summarized in reference 63).

The Golgi is an intrinsically transient and compositionally heterogeneous membranous entity that is constantly fed by anterograde coat protein complex II (COPII) carriers budding from transitional endoplasmic reticulum (ER) domains/ER exit sites (Fig. 2). An unanswered question in cell biology is how the protein cargo that exits the ER traffics across the Golgi apparatus and

is sorted into plasma membrane- and endosome-bound carriers (69). It is widely accepted that this traffic occurs, at least in part, by cisternal maturation rather than by vesicle-mediated connections between stable cisternae. According to this view, early cisternae, which are formed by ER-derived traffic, progressively change their lipid and protein content, becoming not only gradually enriched in cargo but also, in the end, compositionally competent (see below) to break up into carrier vesicles destined for the plasma membrane and endosomes (Fig. 2). Cisternae at this final stage of maturation are denoted “late” or “TGN” cisternae (see below). The progressive attainment of late composition is thought to be mediated by retrograde COPI vesicle traffic retrieving to cisternae in earlier stages of maturation those components that do not belong to mature (i.e., later) stages (Fig. 2).

Strong support for the cisternal maturation model comes from ground-breaking studies of *S. cerevisiae* (70, 71) and from our own work in *A. nidulans* (Pantazopoulou and Peñalva, unpublished observations; see also below). However, readers should note that the maturation model very likely requires some modification to accommodate the involvement of tubular connections that are often observed between metazoan cisternae (and between fungal ones [68]). These connections could represent highways for certain types of cargo. The model also needs to accommodate the experimentally supported possibility that rapid partitioning mediated by domains of different lipid composition enables the budding of carriers at all levels (72; for overview see references 69, 71). In a useful attempt to relate the compositional and morphological variety of the Golgi with specific roles, Glick and coworkers (73) have proposed that the Golgi consists of cisternae in three different functional stages (Fig. 2). We will use this scheme for further discussion.

In stage I, denoted “cisternal assembly,” ER-derived COPII carriers coalesce to form cisternae (Fig. 2). During this stage, ER protein residents that escape to the Golgi incorporated into COPII carriers recycle to their normal destination using COPI-coated retrograde vesicles (74). This is the case, for example, for TM receptors such as *A. nidulans* RerA^{Rer1}, which sorts soluble cargo at the ER into anterograde COPII carriers, and for the syntaxin SedV^{Sed5}, a key component of the SNARE machinery mediating membrane fusion at the Golgi (60, 64, 75). Membranes in stage I contain a network of peripheral proteins recruited to them by Golgi-specific GTPases that tether membranes, thereby facilitating fusion. Among them are *A. nidulans* GrhA^{Grh1} (68) and *N. crassa* USO-1 (65). As cisternae increase in size at the

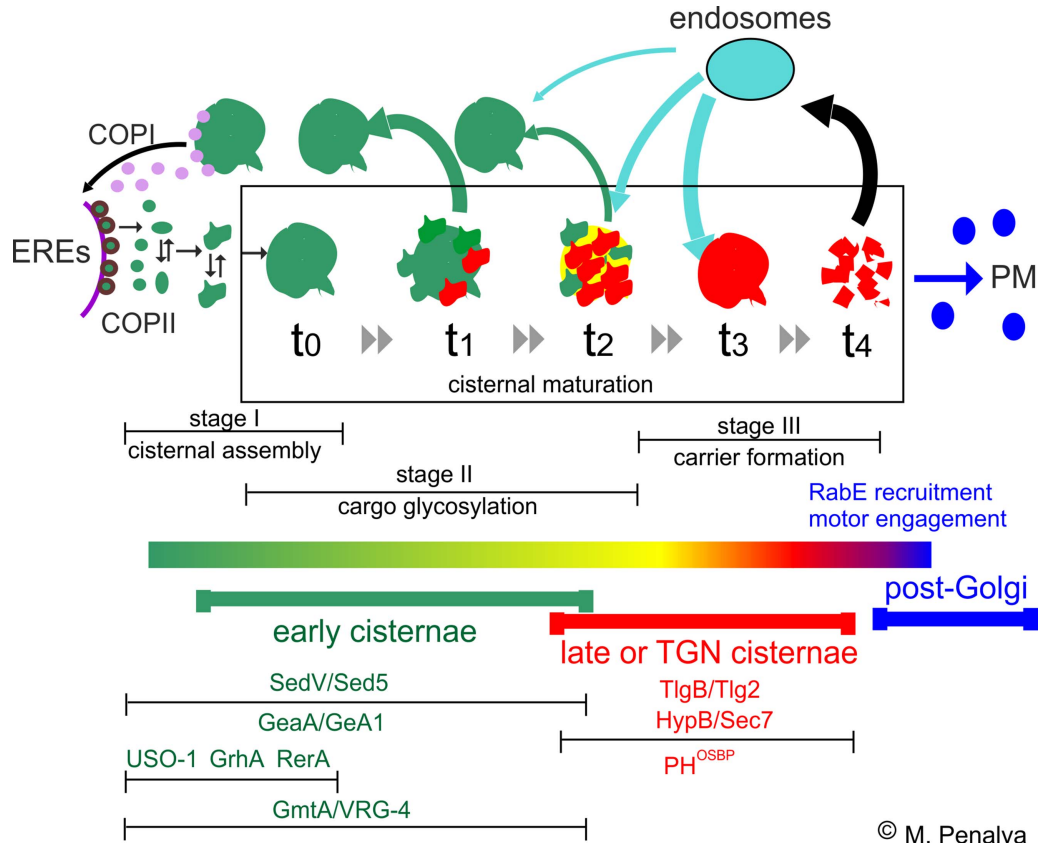


FIGURE 2 Highly schematic representation of the cisternal maturation process in the nonstacked fungal Golgi, with indication of the different functional stages. COPII-coated vesicles (green) bud off specialized domains of the ER denoted ER exit sites (ERES) or transitional ER (left). COPII vesicles coalesce to form an early Golgi cisterna, represented here as a green fenestrated structure that depicts actual Golgi structures often visible in EM micrographs. Retrograde COPI traffic (violet vesicles) retrieves back to the ER proteins such as cargo receptors that need to be recycled. Early cisternae are equipped with cargo glycosylation enzymes (t0). As time passes (double arrowheads) an early Golgi cisterna becomes progressively enriched in cargo and late Golgi components (represented in red) by delivering early Golgi ones (e.g., glycosylating enzymes) to cisternae in earlier stages of maturation, in a process which is likely mediated by COPI retrograde traffic (t1 and t2). Eventually, late Golgi components become predominate (TGN, t3) and the cargo-enriched cisterna becomes competent to tear off into carriers destined for the plasma membrane (PM) and the endosomes (t4). TGN cisternae also receive traffic from the endosomal system (blue arrows). In the route (dark blue) connecting the cisternae with the PM, the transition between late Golgi and post-Golgi identity is dictated by the recruitment of RabE^{RAB11} to the membranes, which is critically regulated by TRAPP^{II} (see text). Proteins that have been shown by microscopy to localize to specific stages are indicated, with green lettering indicating early Golgi and red lettering indicating TGN. The image summarizes work performed with *A. nidulans*.

expense of heterotypic and homotypic fusion, they move on to functional stage II (Fig. 2).

Stage II is the glycosylation stage (73). Golgi cisternae at this stage, during which the definitive carbohydrate decoration of protein cargo is laid, are equipped with glycosidases and glycosyltransferases. Golgi glycosyl-

transferases extend the *O*-mannose core that is attached in the ER to a subset of cargoes, giving rise to the diverse species-specific *O*-glycans decorating fungal secreted proteins. *N*-glycosylated proteins are remodeled, after modification of core *N*-glycan structures also added at the ER, by attachment of further polysaccharide chains

of variable size and composition (76). These stage II cisternae are also armed with the transporters needed to import nucleotide diphosphate-activated monosaccharide precursors from the cytosol into the cisternal lumen. GDP-mannose transporters GmtA of *A. nidulans* (77) and VRG-4 of *N. crassa* (65), homologues of budding yeast Vrg4p used in seminal studies on Golgi maturation (70), are useful to define this compartment. Cisternae at this stage no longer receive anterograde COPII carriers. However, stage II cisternae are also furnished with tethers and with the SedV^{Sed5} syntaxin to accept retrograde traffic (presumably COPI-mediated) that retrieves to them carbohydrate-modifying enzymes that escape to cisternae in a posterior stage (stage III) of maturation. The COG (conserved oligomeric Golgi) complex (78) also mediates the tethering of these intra-Golgi carriers to stage II cisternae, such that deficient COG function results in glycosylation defects (79). The physiological importance of two *A. nidulans* Golgi COG components, PodB^{Cog2} and Swop^{Cog4}, has been demonstrated by showing that two classical *ts* alleles, *podB1* (polarity defective) and *swop1* (swollen cell), cause polarization, cell wall, and protein glycosylation defects (80). The role of GmtA was established with the hyperbranching and hypomannosylating *ts* mutation *cali11* (i.e., *gmtA11*), which results in calcofluor white sensitivity (77).

Cisternae that progress to stage III, denoted “carrier forming” (73), acquire a composition that enables them to generate anterograde carriers destined for the plasma membrane and the endosomes (Fig. 2) but that incapacitates them to generate retrograde COPI traffic destined for cisternae at earlier stages of maturation. Cisternae in this carrier-forming stage have been denoted the *trans*-Golgi network (TGN), by analogy to the most plasma membrane-proximal (*trans*) cisternae of the mammalian Golgi stacks. For all fungal purposes TGN is synonymous with “late Golgi.” Traffic exiting the TGN toward endosomes is mediated by clathrin-coated carriers that use AP-1, GGA (Golgi-localized, gamma adaptin ear-containing, ARF-binding), and epsin adaptors to sort appropriate cargo into the clathrin cage. In contrast, as discussed below, carriers connecting the TGN with the plasma membrane are atypical in that they do not appear to involve any protein coat. TGN membranes contain PtdIns4P and the late-Golgi Arf1 GEF (guanine nucleotide exchange factor) Sec7, which has been intensively studied in *A. nidulans* (60, 81–84) and used as a prototypic TGN marker in both *A. nidulans* (68) and *N. crassa* (65). However, the most robust marker of the TGN is a fusion protein between

mRFP and the PH domain of the human oxysterol binding protein (PH^{OSBP}). This chimera is recruited very efficiently and specifically to TGN cisternae by coincidence detection of active (i.e., GTP-loaded and membrane bound) Arf1 and PtdIns4P (85).

The cisternal maturation model implies that TGN cisternae must dissipate as they break up into transport vesicles. A study of *A. nidulans* documented the process by which TGN cisternae give rise to post-Golgi carriers destined for the apical plasma membrane (82). During this process, cisternal membranes progressively lose Golgi identity (i.e., they lose PH^{OSBP} [i.e., Arf1/PtdIns4P] and HypB^{Sec7} [the late Golgi Arf1 GEF]) as they become increasingly rich in RabE^{RAB11} (Ypt31/32p in budding yeast), the determinant of post-Golgi identity (82). By the time RabE^{RAB11} peaks at a maternal cisterna, the signal of Sec7/PH^{OSBP} has virtually disappeared. At this point, RabE^{RAB11}-rich membranes undergo rapid movement to the Spitzenkörper (SPK), using kinesin and myosin-5 motors that power their long-distance transport to the SPK (82), consistent with a model in which RabE^{RAB11} participates directly or indirectly in the recruitment of the motors (Fig. 2). The fact that cisternae that have acquired RabE^{RAB11} and undergone movement toward the apex leave little signal of TGN markers behind strongly suggests that TGN cisternae largely dissipate into RabE^{RAB11} exocytic carriers, which represents strong evidence for a maturation-based mechanism determining the biogenesis of these carriers (82). The biogenesis of carriers for endosomes, which had been filmed in *S. cerevisiae* (86), has been recently documented in *A. nidulans* (87).

Unlike early Golgi cisternae (stages I and II), TGN cisternae contain the t-SNARE TlgB^{Tlg2}, which colocalizes with PH^{OSBP} (60). TlgB^{Tlg2} is a Qa syntaxin that forms SNARE bundles with Vti1 as Qb-, TlgA^{Tlg1} as Qc-, and SynA as R-SNARE (75). In all likelihood, this SNARE bundle mediates the fusion of retrograde traffic connecting the earliest endosomal compartment, to which SynA arrives by endocytosis, with the TGN. This is the pathway that is followed by proteins that, like SynA itself, are efficiently taken up by the subapical endocytic ring and pass through the TGN before returning by way of apex-directed exocytosis to the plasma membrane (37, 64, 88). This earliest endosome, which for convenience is designated here as a “sorting endosome,” should not be confused with the EEs, which are readily identifiable by their high motility on microtubule and the presence of the GTPase Rab5 (see below).

The SynA pathway is the one that must be responsible for the strong labeling of the SPK with the endocytic

tracer FM4-64 (89). Another passenger of this pathway is the *A. nidulans* phospholipid flippase DnfA^{Dnf1} (39). *cis*-acting mutations abolishing endocytosis of SynA (64) or DnfA^{Dnf1} (39) result in a uniform rather than polarized distribution of the cargo, showing that rapid endocytic uptake/recycling combined with slow diffusion across the plane of the membrane generates polarity (90). In *A. nidulans*, traffic of cargoes between the sorting endosome and the TGN requires RAB6 (RabC^{RAB6}) (64) and its effector Vps52 (39). Another flippase, DnfB^{Drs2}, is a TGN resident that partially localizes, like DnfA^{Dnf1}, to the SPK. Remarkably, DnfA^{Dnf1} and DnfB^{Drs2} reside in different regions of the SPK (39). The role of flippases in exocytosis is not understood, but it may have major practical implications: ablation of the *Magnaporthe grisea* APT2 gene (homologue of DnfB^{Drs2}) does not result in hyphal growth or sporulation defects but precludes the secretion of several extracellular enzymes and, importantly, prevents pathogenicity (91).

TlgB^{Tlg2} is notable in that its genetic ablation is inconsequential for growth and results in a minor effect on SynA localization (75). However, *tlgBΔ* is synthetically lethal when combined with a hypomorphic mutation in *sedV*, the gene encoding the early Golgi syntaxin (75). This indicates that the Golgi can be organized with only one syntaxin (SedV^{Sed5}), provided that this syntaxin is fully functional. A plausible explanation is that when TlgB^{Tlg2} is absent, proteins can recycle from sorting endosomes to early Golgi cisternae in a SedV^{Sed5}-dependent manner and then move forward to the TGN by cisternal maturation. This interpretation is supported by the observation that SedV^{Sed5}, like TlgB^{Tlg2}, can form SNARE bundles with SynA, Vti1, and TlgA^{Tlg1} (75). If this interpretation were correct, the only role of TlgB^{Tlg2} would be in the fusion of retrograde carriers derived from the endosomes with the TGN.

The Master Regulators of the Golgi: RABs, Arf1, and Their Regulators

The budding from the ER of COPII carriers that feed the Golgi, the different stages of Golgi maturation, and the exit of carriers from the TGN are governed by small GTPases of the ARF and RAB families. SarA^{Sar1} is an ER-located ARF that plays a fundamental role in the budding of COPII carriers (92, 93). Temperature shift experiments with *A. nidulans* *sarA^{ts}* mutations have been used to impair ER exit. As predicted by the cisternal maturation model, in cells shifted to the restrictive temperature, early Golgi residents such as SedV^{Sed5}, which have access to COPI-mediated retrograde traffic, rapidly

relocalize to the ER, consistent with their Golgi localization being determined by an equilibrium between anterograde and retrograde traffic. In contrast, the TGN (late Golgi) resident TlgB^{Tlg2} does not rapidly relocalize to the ER, relocalizing instead to a diffuse cytosolic haze (92). This different behavior of early and late Golgi membranes (SedV^{Sed5} and TlgB^{Tlg2} are integral membrane proteins) is consistent with the cisternal maturation model. Notably, prolonged impairment of ER exit by *sarA^{ts}* mutations results in the formation of apical balloons surrounded by a thick wall of chitin. One possible explanation is that chitin biosynthetic enzymes are less dependent on SarA^{Sar1} function than other cell wall biosynthetic activities (92).

ARF1 (ArfA in *A. nidulans*) is an essential regulator of the Golgi (94) that plays different roles at the levels of the early and late cisternae. Two essential ArfA GEFs, GeaA and HypB^{Sec7}, localizing to the early and late Golgi membranes, respectively, ultimately mediate these roles (81). A major (and as yet unexplained) finding that illustrates the power of random classical genetic screens was that *geaA1* resulting in a single Y1022C substitution within a conserved domain of GeaA makes vegetative growth independent of HypB^{Sec7} and thus of the TGN (81). Remarkably, besides the early Golgi, GeaA^{Y1022C} localizes to the apical plasma membrane, suggesting that under these conditions early Golgi membranes can undergo exocytosis.

Three RAB GTPases are needed to organize the Golgi: RAB1 (RabE^{RAB11}, YPT-31), RAB6 (RabC^{RAB6}), and RAB11 (RabE^{RAB11}, YPT-31). RabO^{RAB1}/YPT-1 (60, 65) and RabC^{Rab6} (64) are present in both the early and the late Golgi. Notably, the three Golgi RABs are present also in the SPK (see below).

In *A. nidulans*, RabO^{RAB1} and RabE^{RAB11} are essential (60, 82). The acute inactivation of RabO^{RAB1} rapidly disorganizes both the early and the late Golgi cisternae (60). In addition to exocytosis, *A. nidulans* RabO^{RAB1} plays a crucial role in autophagy (84). The absence of RabC^{RAB6}, albeit not lethal, is severely debilitating and leads to fragmented cisternae (64). It results in delocalization of the VpsT^{VPS10} vacuolar hydrolase receptor, which recycles between the endosomes and the Golgi (64), an observation consistent with the role generally attributed to RAB6 homologues as key regulators of retrograde traffic in the secretory pathway.

RAB11 has been studied in detail in *A. nidulans* (82, 83). As noted above, RabE^{RAB11} is not a Golgi resident, but it is instead recruited to TGN membranes to determine their acquisition of post-Golgi identity. This recruitment must be governed by its GEF, which

is therefore crucial for exocytosis. A long-standing question in the literature concerned the identity of the RabE^{RAB11}/Ypt31 GEF and the roles of the TRAPP (oligomeric complex transport protein particle) as a Golgi organizer. TRAPP has been isolated in three versions. TRAPPI (composed of Trs33, Ber3, Bet5, Trs23, Trs31, and Trs20 subunits) is a demonstrated GEF for Ypt1 (yeast RAB1), acting in the ER/early Golgi interphase. TRAPPIII is TRAPPI plus Trs85 and acts as GEF for Ypt1 in the preautophagosome. TRAPPII is TRAPPI plus three additional subunits: Trs120, Trs130, and Trs65. TRAPPII had been proposed to be the Ypt31 GEF acting in the TGN (95), but this role was disputed by others, who concluded that TRAPPII was the late Golgi version of the GEF that activates Ypt1 in early Golgi (96). *hypA1* is a *ts* mutation in the gene encoding *A. nidulans* Trs120 (97). The characterization of extragenic suppressors of *hypA1* combined with biochemical assays established that the physiological substrate of TRAPPII is RabE^{RAB11} (the Ypt31 orthologue). TRAPPII, as visualized with Trs120-GFP, peaks at TGN cisternae preceding their dissipation into carriers, thereby determining the Golgi to post-Golgi transition by governing RabE^{RAB11} recruitment (83). It remains to be established whether TRAPPII is assembled *de novo* in the TGN. Alternatively, TRAPPII might be assembled using as a scaffold the TRAPPI complex already present in the early cisternae that would arrive at the TGN by maturation. If this were the case, anterograde traffic across the Golgi might be mediated by a RAB1 to RAB11 cascade governed by maturation of TRAPPI into TRAPPII (95).

The Fungal SPK and Delivery of Cell Wall-Building Enzymes

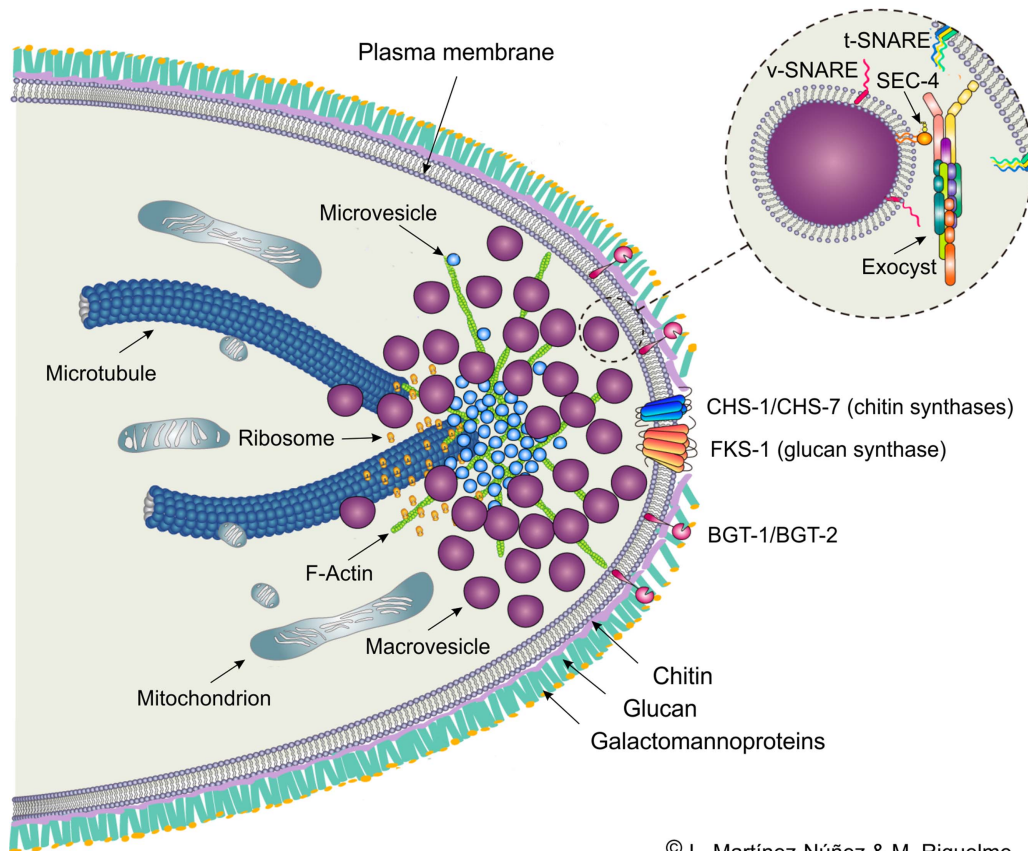
The SPK is a conspicuous apical body observed at the growing apices of hyphae in fungal species belonging to the subkingdom Dikarya (Ascomycota and Basidiomycota). The SPK was first discovered in fixed cells of the basidiomycetes *Coprinus sterquilinus* and *Coprinus narcoticus* stained with iron-hematoxylin (10). It was later observed in live cells of *Polystictus versicolor*, where it was shown to be important for maintaining hyphal tip elongation, morphology, and growth direction (98, 99). The subsequent characterization of the SPK in diverse fungal species by phase-contrast and transmission electron microscopy identified up to nine SPK patterns (100). Mating projections of *S. cerevisiae*, mating projections and invasive pseudohyphae of *C. albicans*, dikaryotic hyphae of *U. maydis*, and germ tubes of *Uromyces phaseoli* and *Puccinia graminis* dis-

play an apical cluster referred to as an SPK-like structure thought to operate as the SPK in hyphae (101–103).

Among the non-Dikarya fungi, only *Allomyces* and *Basidiobolus* have been found to display an SPK (104, 105). Early studies reported the absence of an SPK in the Zygomycetes (106). More recently, several zygomycetous fungi were analyzed (*Gilbertella persicaria*, *Phycomyces blakesleanus*, *Rhizopus oryzae*, *Coemansia reversa*, *Mucor indicus*, *Gigaspora* spp., and *Mortierella verticillata*), and it was shown that they present instead an apical vesicle crescent (13, 107, 108). The apical vesicle crescent corresponds mainly to an accumulation of apical vesicles of different sizes, as for the SPK. However, practically nothing is known about the composition or regulation of the apical vesicle crescent. In contrast, extensive studies conducted on ascomycetous species have made it possible to identify the main components of the SPK (see below).

Hyphal growth occurs at the apices (10). Autoradiographic analysis of *M. indicus* (formerly *rouxii*) showed accumulation of tritiated *N*-acetyl-D-glucosamine (GlycNAc), a monomeric unit of chitin, preferentially at hyphal tips, indicating that apical growth was correlated to a polarized mechanism of cell wall assembly (9). Several authors proposed that this assembly of cell wall at the apex must include the participation of synthetic as well as lytic (plasticizing, softening) activities (109–111). By contrast, an alternative model not involving lytic activity proposed a plastic cell wall at the apex that would become rigid at the subapex, where remodeling glycosyltransferases would cross-link the cell wall material and rigidify it (11). Recent work with *N. crassa* has revealed two glycosyltransferases belonging to the CAZy family GH17 localized at the plasma membrane of the hyphal dome, excluding the foremost apical region immediately across from the SPK (112) (Fig. 3), in support of cell wall remodeling occurring at the subapex.

At the ultrastructural level the SPK corresponds to an aggregation of vesicles, actin microfilaments, and ribosomes and an amorphous unidentified material (113, 114) (Fig. 3). A computer simulation exercise led to the formulation of the vesicle supply center model for fungal morphogenesis (115). This model proposed that the SPK behaves as a vesicle supply center, from which vesicles travel to and fuse with the apical plasma membrane, providing the enzymatic machinery needed for cell wall expansion. Once inserted in the plasma membrane, (i) chitin synthases take *N*-acetyl glucosamine subunits (GlcNAc) from the cytoplasmic side and incorporate them into a growing chain of chitin microfibrils (β -1,4-GlcNAc), and (ii) the glucan synthase takes glucose



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FIGURE 3 Illustration of a hyphal tip with the main organelles and subcellular components involved in apical cell wall growth. The diagram is based on work with *N. crassa*. (Art: Leonora Martínez-Núñez).

subunits (Glc) from the cytoplasmic side and incorporates them into an extending chain of β -1,3-glucan (116, 117). The sequencing of fungal genomes allowed the construction of translational fusions of specific genes with fluorescent proteins enlightening many components of the SPK.

The ultimate evidence for the suggested link between the SPK and cell wall assembly at tips was confirmed when cell wall biosynthetic enzymes such as chitin and glucan synthases were identified at the SPK in *N. crassa* (118, 119). Significantly, coexpression of differentially fluorescently tagged enzymes identified chitin synthases and components of the glucan synthase complex at the core and at the outer layer of the SPK, respectively, coinciding with the distribution of microvesicles (chitosomes) and macrovesicles reported at the transmission electron microscopy level (119, 120) (Fig. 3). Small GTPases YPT-1/RabO (Rab1) and YPT-31/RabC (Rab6) have been found occupying the inner and the peripheral layers of the SPK in *N. crassa* and *A. nidulans*, respectively (64, 65). All the above suggests different popula-

tions of vesicles containing different cell wall synthesizing activities and being regulated by different molecular switches. We are only starting to discern the lipid composition of membranes and its role in establishing membrane asymmetry and in vesicle traffic. In *A. nidulans* flippases DnfA (Dnf1) and DnfB (Drs2) also occupy distinct strata of the SPK, in support of existing different populations of vesicles (39, 121).

The SPK has been suggested to be a transfer station from cytoplasmic microtubules to actin microfilaments (122, 123). Cytoplasmic microtubules which extend through the cytoplasm and reach the SPK region are thought to participate in the long-distance traffic of vesicles to the SPK, whereas the actin cytoskeleton, found at the SPK core would mediate the flow of vesicles to and from the SPK (124, 125) (Fig. 3). In *A. nidulans* the microtubular and actin cytoskeletons are connected at the hyphal tips by cell-end markers (26). TeaA is delivered by microtubules to the tip plasma membrane, where it interacts with TeaR and other components, subsequently leading to the recruitment of the formin SepA,

which polymerizes actin cables (126). Interestingly, while cytoplasmic microtubules are important to maintain SPK stability, hyphal morphology, and fast growth rates, they seem dispensable for vesicles to reach the SPK (127, 128).

We lack information on the biogenesis of the vesicles that constitute the SPK. While several fungal organelles have been characterized, we lack information about the post-ER and/or post-Golgi processes relative to the formation and traffic of vesicles that concentrate at the SPK (see above). It has been shown that the exocyst complex has a role in determining the site at the plasma membrane for delivery of cell wall-synthesizing enzymes (129). In *N. crassa* the exocyst components were found at two locations within the hyphal tip: at the apical plasma membrane and at the frontal region of the SPK outer layer. It is well established that an intact SPK is needed to maintain hyphal morphology and growth directionality in ascomycete fungi (130). Recently, it was found that only the aggregation of macrovesicles (not of the microvesicles) at the SPK is dependent on an intact exocyst (120). Finally, it is worth noting that the lipophilic dye FM4-64 stains the SPK in living hyphae (89). FM4-64 is taken up by the cell via endocytosis at the plasma membrane (131) (for more details see below), suggesting a connection between the exo- and endocytosis.

Myosin-Chitin Synthases in Apical Exocytosis

Our knowledge concerning the delivery of vesicles and enzymes to the hyphal tip is largely based on intensive studies of *N. crassa* and *A. nidulans*. Both organisms belong to the phylum Ascomycota, which accounts for ~64% of all fungi (2). The second major group is the basidiomycetes, which provide 34% of all described species (2). Despite the economic and ecological importance of this group, our understanding of secretion pathways in basidiomycetes is restricted to the plant pathogen *U. maydis*. In hyphae of *U. maydis*, microtubules are required to deliver chitin synthase-containing vesicles to the apical growth region (132). *U. maydis* does not contain a typical SPK, comprised of macro- and microvesicles, but similar to *N. crassa* and *A. nidulans*, vesicles accumulate near the hyphal tip in this fungus (103). How this vesicle cluster is formed is not fully understood, but class V chitin synthases may participate in this process. Class V chitin synthases are fungal-specific cell wall-forming enzymes that carry an N-terminal myosin motor domain. This domain is even found in ancient Cryptomycota (133), suggesting that it is a hallmark of the Fungi. It was shown that the myosin motor domain of the *U. maydis* class V chitin synthase (Mcs1) (134) is

not involved in apical delivery of the enzyme. Instead, this is achieved by coordinated activity of the motor proteins kinesin-1 along microtubules and myosin-5 along filamentous actin (135); the myosin-motor domain of the class V chitin synthase tethers delivered vesicles to apical actin. The transient interaction of the motor and actin increases the pausing time of the vesicles at the hyphal apex, thereby supporting polar exocytosis (135).

Interestingly, Mcs1-tethered vesicles also carry a class VII chitin synthase and a β -glucan synthase (a homologue of glucan synthase Fks1 in *N. crassa*) (136). After exocytosis, these cell wall enzymes are immobilized by newly formed polysaccharide chains, suggesting that codelivery and coexocytosis help the coordination of enzymatic activity in building the complex fungal cell wall. Myosin-chitin synthases have also been described in numerous ascomycetes, including *A. nidulans*, (137), *Aspergillus fumigatus* (138, 139), *F. oxysporum* (140), *Colletotrichum graminicola* (141), *Magnaporthe oryzae* (142), and *N. crassa* (143). Ascomycete myosin-chitin synthases have been shown to be required for morphogenesis, development, and pathogenicity (140, 141, 143, 144). As in *U. maydis*, kinesin-1 delivers *A. nidulans* myosin-chitin synthase along microtubules to the hyphal apex (145), where its myosin motor domain interacts with F-actin (146). However, whether ascomycete myosin-chitin synthases act as vesicle tethers at sites of apical exocytosis remains to be determined.

FUNGAL ENDOCYTOSIS AND EE MOTILITY

Fungal Endocytosis

Hyphal tip growth has been described as being due to the tip-ward delivery and the apical release of Golgi-derived vesicles (11, 122). In animal cells, this release (i.e., exocytosis) is balanced by the uptake of material into vesicles that form at the plasma membrane (i.e., endocytosis). These vesicles then fuse with early endosomes, and the contents can be recycled back to the surface or dispatched to the lysosome (i.e., the fungal vacuole) for degradation. For many years, endocytosis was thought to be absent in fungi (147). However, over the past 2 decades, fungal endocytosis has been recognized to be pivotal for cell function. The initial breakthrough came with the use of the lipophilic marker dye FM4-64 in the 1990s in the yeast *S. cerevisiae* (131) and then in the filamentous fungi *Uromyces fabae* (148) and *U. maydis* (149). This fluorescent dye inserts into the plasma membrane from where it is internalized by the formation of endocytic transport vesicles (131). Subse-

quently, studies of *M. grisea* (150), *A. nidulans* (151), *N. crassa*, and six additional fungi (89) confirmed the endocytic uptake of FM4-64 into fungal cells. These results were complemented by live-cell imaging and mutants studies in numerous fungi, including *U. maydis*, *A. oryzae*, *A. nidulans*, *C. albicans*, *A. gossypii*, and *N. crassa* (37–39, 152–160). Collectively, these studies revealed a critical role for endocytosis and endocytic recycling in hyphal growth of filamentous fungi (for overview see references 15, 57).

The Molecular Machinery for EE Motility

Fungal EEs were first described in the dimorphic fungus *U. maydis* (160). In this fungus, shortly after internalization, the dye FM4-64 concentrates in small, rapidly moving vesicular organelles (160). A putative t-SNARE, Yup1, binds these organelles via a PHOX-domain (i.e., phosphoinositide binding domain) (160, 161) that interacts with the lipid PIP3, characteristic of EEs (reference 162). Consistent with this, the FM4-64-stained organelles carry the small GTPases Rab4 and Rab5 (155, 161), both shown to reside on EEs in mammalian cells (163, 164). Thus, there is little doubt that the rapidly moving FM4-64-positive vesicles are EEs. Rab5-positive EEs were also described in *A. nidulans* (88), *N. crassa* (165), and *Zygomycetia tritici* (166) but are not found in the yeast *S. cerevisiae*. Thus, motile EEs are a hallmark of filamentous fungi.

Microtubules are biopolymers of tubulin-dimers that support long-distance transport of organelles and vesicles in fungal cells (12, 167). They elongate at their plus end, while the minus end is usually embedded in a nucleation site, such as the fungal spindle pole body. Motility along microtubules is mediated by molecular motors, which use the polarity of microtubules to transport their cargo. In general, kinesins are known to move toward the plus end of a microtubule, while cytoplasmic dynein motors are minus end-directed. Both types of motors are “mechanoenzymes” that hydrolyze ATP to power a conformational change in the motor domain. Repeating cycles of ATP hydrolysis result in a sequence of ~8-nm steps along the microtubule (168, 169). In filamentous fungi, the first membrane trafficking motors identified were the cytoplasmic dyneins in *N. crassa* (170) and *A. nidulans* (171), which were recognized to play a role in nuclear migration. Shortly after, kinesin motors were identified in *N. crassa* (172), *U. maydis* (149), *Nectria haematococca* (173), and the zygomycete *Syncephalastrum racemosum* (174). Interestingly, all kinesins belong to the same subgroup of kinesin motors (kinesin-1), which is thought to support tip-ward transport of fungal secretory vesicles

(135) and to deliver dynein to microtubule plus ends (175, 176) (for more details on general motor function see references 12, 167 and 177). The first insight into the machinery of EE motility was provided by studies of *U. maydis*, where kinesin-3 was identified to be the motor for plus end-directed motility of EEs (178). A similar role of kinesin-3 in EE motility was discovered in *A. nidulans* (179, 180) and *N. crassa* (165). Kinesin-3 motor proteins comprise 8 of the 45 kinesins in the human genome (181) but are absent from *S. cerevisiae*. This emphasizes the similarity of membrane trafficking in filamentous fungi and elongated mammalian cells such as neurons (167, 182).

Fungal EEs have been shown to move in a bidirectional fashion along microtubules (88, 175, 183), suggesting that plus end-directed kinesin-3 gets opposed by minus end-directed cytoplasmic dynein. Indeed, deletion of the gene for kinesin-3 resulted in the accumulation of EEs at microtubule minus ends (178), whereas inactivation of cytoplasmic dynein results in a cluster of EEs at microtubule plus ends in the hyphal apex (175, 179). Interestingly, the deletion of kinesin-1 also led to EE clustering at the hyphal tip. This is due to a role of this motor in delivery of dynein to microtubule plus ends (175, 176) (Fig. 4). Dynein motors accumulate at the plus end to form a “dynein comet” (175, 184). Studies of *U. maydis* have shown that the apical dynein comets consist of ~55 dynein motors. Two mechanisms cooperate in the formation of the comet, namely binding of the dynein-associated complex dynactin to EB1-like proteins at microtubule plus ends and by a stochastic crowding effect (185). Dynein is released from the dynein comet and, while moving toward the subapical minus ends, can randomly pick up kinesin-3-delivered EEs (186) (Fig. 4). The large numbers of dyneins at plus ends increase the probability of such interaction. This ensures that EEs do not fall off the microtubule when they arrive at plus ends, but rather are transported back toward minus ends (185) (Fig. 4).

After dynein takes over, moving EEs still bind kinesin-3 (179, 186). Surprisingly, the amount of kinesin-3 on these organelles is ~4 times higher than that of dynein (186). This suggests that kinesin-3 activity is repressed upon dynein binding. While the exact mechanism underpinning this regulation is not known, the recent identification of an EE-located motor adapter complex provides the first insight into the complex interplay between kinesin-3 and dynein during bidirectional EE motility. The key protein in this EE adapter complex is a hook protein, which was identified in a genetic screen for mutants defective in EE motility in *A. nidulans* (187) and

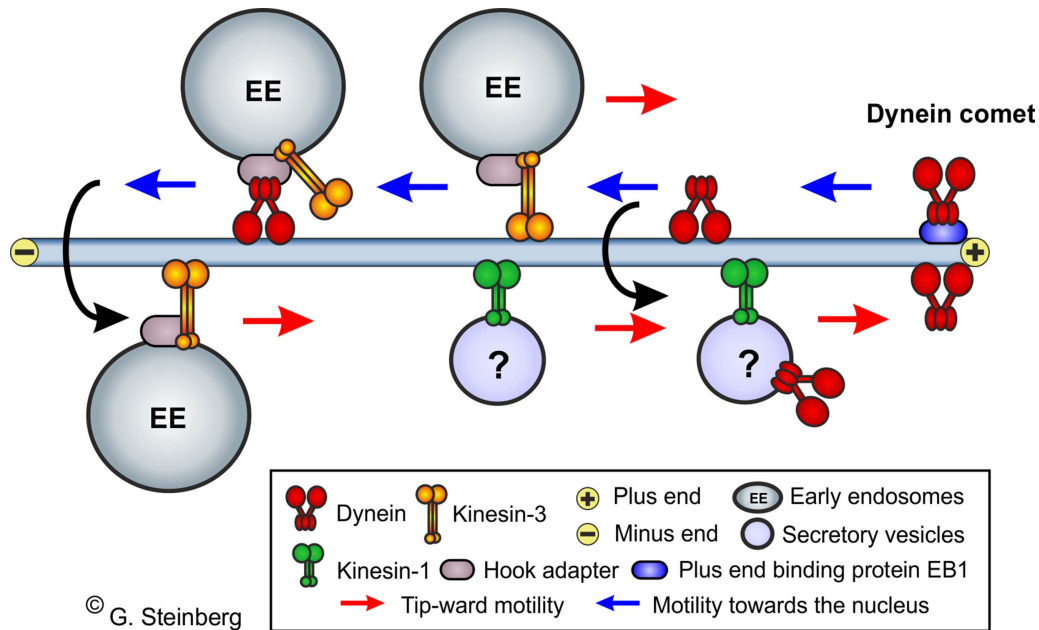


FIGURE 4 Diagram showing the cooperation of molecular motors in bidirectional EE motility. The illustration is based on results obtained from studies of *U. maydis*. See text for detailed description.

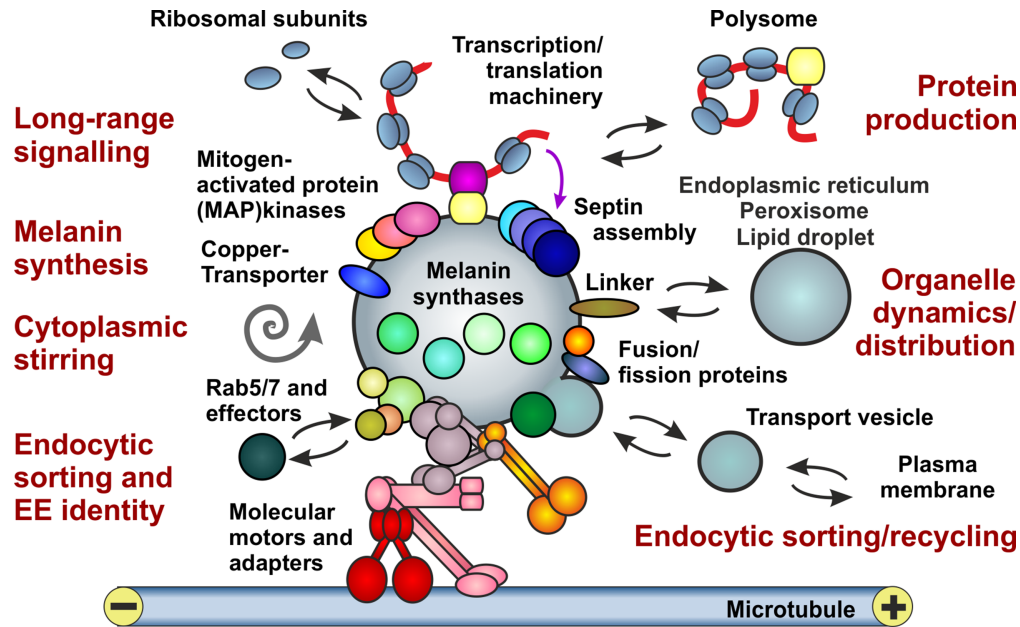
U. maydis (188). Hook forms a complex with two associated proteins, named FTS and FHIP (188), that help anchor the adapter complex to the EE membrane (189). Hook itself interacts with dynein (187, 188) and kinesin-3 (188) and is therefore a good candidate for controlling the activity or the attachment of both motors during bidirectional EE motility (15). This notion is supported by the finding that some kinesin-3 is released from EEs shortly before dynein binds (188). It is likely, however, that more proteins participate in this regulation, because for example, the dynactin subunit p25 is needed for dynein-dependent EE motility (190). However, EE motility may also be regulated by proteins that are not part of the transport machinery or the EE adapter complex. This is illustrated by the vezatin-like protein *Veza* in *A. nidulans* (191). Mutants in *vezA* show defects in dynein-hook interaction and, consequently, are impaired in retrograde EE motility. Surprisingly, *Veza* is not located on EEs, and the mechanism by which it controls interaction of dynein and EEs is not fully understood. Furthermore, the microtubule tracks themselves could participate in regulation of EE motility. It was shown in *N. crassa* and *A. nidulans* that kinesin-3 moves cargo preferentially along a subset of modified and less dynamic microtubules (165, 180). However, such selective transport of kinesin-3 was not found in the basidiomycete *U. maydis* (192).

Multiple Functions for Fungal EEs

The discovery of kinesin-3 as the motor for plus end-directed motility of fungal EEs (178) allowed the generation of mutants where EE motility was inhibited. This opened new avenues to investigate the role of these organelles in filamentous fungi. Over the past 10 years, intensive studies of *U. maydis* and *A. nidulans* have revealed multiple roles of EEs in fungal growth and virulence (Fig. 5).

The role of EEs in the endocytic pathway

EEs are the first compartment that sorts incoming material for recycling back to the plasma membrane or for degradation to the vacuole. It was shown that endocytic recycling supports tip growth of hyphae in *A. nidulans*, *A. oryzae*, *N. crassa*, and *A. gossypii* (37, 38, 152, 153, 156, 157). In addition, endocytic receptor recycling is essential during the early steps of pathogenicity in *U. maydis* (155). A role of EEs in recycling is also indicated by the fact that fungi contain the small GTPase Rab4 (155), which is involved in endocytic recycling at mammalian EEs (164). However, experimental evidence for such a role of Rab4 in filamentous fungi is missing. The second pathway from the EEs involves a maturation process, where Rab5 is replaced by Rab7, as shown in mammals (193), *A. nidulans* (194), and *U. maydis* (161). This maturation process from an EE to a late endosome



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FIGURE 5 EEs as multifunctional platforms. Proteins that associate with the organelles are shown as colored symbols and described in black; functions are indicated in dark red. The diagram is based on work with *U. maydis*, *A. nidulans*, and *A. fumigatus*. See text for detailed description.

and finally delivery to the vacuole involves EE motility (161, 194) and a complicated machinery of additional protein factors (75, 195). *A. nidulans* mutants defective in such factors (e.g., Vps33 or PepA^{Pep12}) (75) do not form normal colonies, which demonstrates that sorting to the vacuole is crucial for normal hyphal growth.

Hitchhiking of the protein translation machinery and organelles on EEs

Fungal EEs are constantly moving, and their motility is fueled by hydrolysis of ATP (15). The costs for the cell to maintain EE motility are best illustrated by a simple calculation, using parameters from *U. maydis*. Here, ~100 EEs are constantly moving (M. Schuster and G. Steinberg, unpublished results) at a rate of ~2 $\mu\text{m/s}$ (186). Considering a step size of 8 nm (168, 169) and consumption of one ATP per step (196), these 100 EEs consume 90 million ATPs per hour. While EE motility is required to sort endocytosed material to the lysosome (see above), this high energy cost suggests additional functions of EE motility. Indeed, recent studies in *U. maydis* and *A. nidulans* have shown that other cellular cargos can “hitchhike” on moving EEs. The first report of such a mechanism demonstrated that the *U. maydis* mRNA-binding protein Rrm4 travels on EEs (197). Rrm4 links various mRNAs to a FYVE-domain-

containing protein, Upa1, which itself is expected to bind to PIP3 lipid (198, 199). While it was initially suggested that this hitchhiking mechanism delivers mRNAs to the ends of the hyphal tip cell (200), a more recent report shows that entire polysomes (ribosomes and mRNA) bind and unbind to EEs, which distributes the protein translation machinery in the cell (161). Thus, transient hitchhiking supports the spatial organization of the translational machinery in the fungal cell.

Interestingly, it was reported that this mechanism is also used to relay organelles. In *U. maydis* (201) and *A. nidulans* (202), peroxisomes also transiently hitchhike on moving EEs. Moreover, lipid droplets and, to lesser extent, ER are distributed by moving EEs (201). Interestingly, when EE motility was blocked, organelles clustered at the hyphal tip (187, 201, 202). It was shown in *U. maydis* that this is due to a pole-ward drift of these organelles, probably powered by myosin 5-dependent delivery of secretory vesicles to the hyphal apex (203). Thus, hitchhiking on moving EEs maintains an even distribution of organelles and polysomes, which is vital for protein production and organelle function. It is worth noting that the process of hitchhiking is not restricted to filamentous fungi but may be a more general mechanism of cargo distribution in eukaryotic cells (204).

Local protein translation on moving EEs

Recent studies of *U. maydis* revealed a novel aspect of mRNA hitchhiking on EEs. It was shown that polyosomes, while bound to moving EEs, are translationally active (161, 205). Initially, it was thought that this mechanism may ensure binding of ribosomes to EE-anchored mRNA for cellular distribution (161). However, recent results in *U. maydis* show that all core septin proteins (Cdc3, Cdc10, Cdc11, Cdc12) travel on EEs in an Rrm4-dependent way (206). This suggests that septin mRNA is traveling on EEs and subsequently being translated on the organelles to septin proteins, which assemble into a heteromeric septin building block. Thus, local translation on EEs may help to assemble and transport protein complexes in the hyphal cell.

Mixing the cytoplasm to enhance organelle interactions

EEs are constantly moving along microtubules (186). This raises the possibility that the EE trafficking stirs the fungal cytoplasm. Evidence for such a function was recently provided in *U. maydis*. Here, bidirectional EE motility increases the diffusion of peroxisomes and lipid droplets (203). This is most likely due to perturbation of the cytoplasm or direct collision between EEs and organelles. These unspecific interactions are further fostered by dynein-driven bending of microtubules (207, 208), which allow moving EEs to explore all regions of the cytoplasm within the hyphal cell tube. Mathematical modeling shows that these perturbations increase the local mobility of peroxisomes and lipid droplets, thus raising the chance of organelle-organelle interactions, which are essential for the cellular roles of both organelles (209). Interestingly, EEs in *A. nidulans* were shown to support the aggregation of proteins, which are formed in response to a heat shock (210). How EEs support protein aggregation is not known, but transient interactions between both were reported (210), suggesting that either hitchhiking (see above) or passive collisions support protein aggregate formation.

Participation of EEs in melanin biosynthesis

Melanin is a negatively charged hydrophobic polymer pigment. This polymer mechanically strengthens the fungal cell wall and protects microorganisms against microbicidal peptides, reactive oxygen species, and antifungal drugs. Consequently, melanin is associated with fungal virulence, in both human and plant pathogens (211–213). A genetic screen, designed to identify conidial pigmentation mutants in *A. fumigatus* (214), revealed a novel role for EEs in melanin biosynthesis.

In one of the mutants, all melanin synthesis genes were unaffected, but a conserved sorting nexin, Mvp1, was mutated (215). This protein contains a PHOX domain, known to bind to lipids enriched in EEs (162). Subsequent studies led to the discovery that early enzymes of the melanin biosynthesis pathway (Alb1, Arp1, Arp2, Ayl1) are recruited to EEs. This, and the role of the sortin nexin Mvp1 in conidia pigmentation, strongly suggest that fungal melanin biosynthesis is initiated in the endosomal system, from where melanin intermediates are secreted and further processed by late biosynthetic enzymes (Abr1, Abr2) in the cell wall. Thus, EEs may govern aspects of secondary metabolism in filamentous fungi.

Endosomes in Long-Range Signaling

Mammalian neurons and elongate fungal hyphae share a common challenge in long-range signaling from the cell pole to the nucleus. In *U. maydis*, the nucleus is located ~50 μm behind the growing tip (216), and perception of external cues by tip-located receptors requires signaling over this distance. In neurons, such retrograde signaling is supported by moving EEs (217), and it was speculated that fungal EEs also take part in long-range transmission of signals (218). Evidence for this hypothesis comes from work with *U. maydis*. When the fungus enters into its plant host, it secretes effector proteins to suppress plant defense system activation (219–222). But how does the fungal nucleus know that effector genes need to be transcribed? It was shown that retrograde EE motility bridges between the invading hyphal tip and the subapical nucleus and that this motility is required to increase effector gene transcription and subsequent effector protein secretion (219). This supports the notion that EE motility transmits the signal for effector production from the hyphal tip to the subapical nucleus. Neither the plant cues that trigger effector transcription nor the detailed mechanism underpinning signal relay is known. However, the mitogen-activated protein kinase Crk1, described in the control of morphogenesis in *U. maydis* (223), is located on EEs and participates in regulating effector transcription (219). While our understanding of long-range signaling in fungi is still very fragmentary, these findings demonstrate a role for moving EEs in signal transduction.

Since the discovery of EEs in 2000 in *U. maydis* (160), our knowledge of the molecular machinery and cellular role of EE motility has increased significantly. It has emerged that EEs are motile multipurpose platforms that are involved in various essential aspects of fungal biology (Fig. 5). Initially recognized as sorting organelles,

involved in recycling and degradation of endocytosed cargo, EEs have now been shown to spatially organize the hyphal cell. They enable long-range signaling and serve as platforms for local protein translation. Thus, motile EEs are pivotal to the fungal cell.

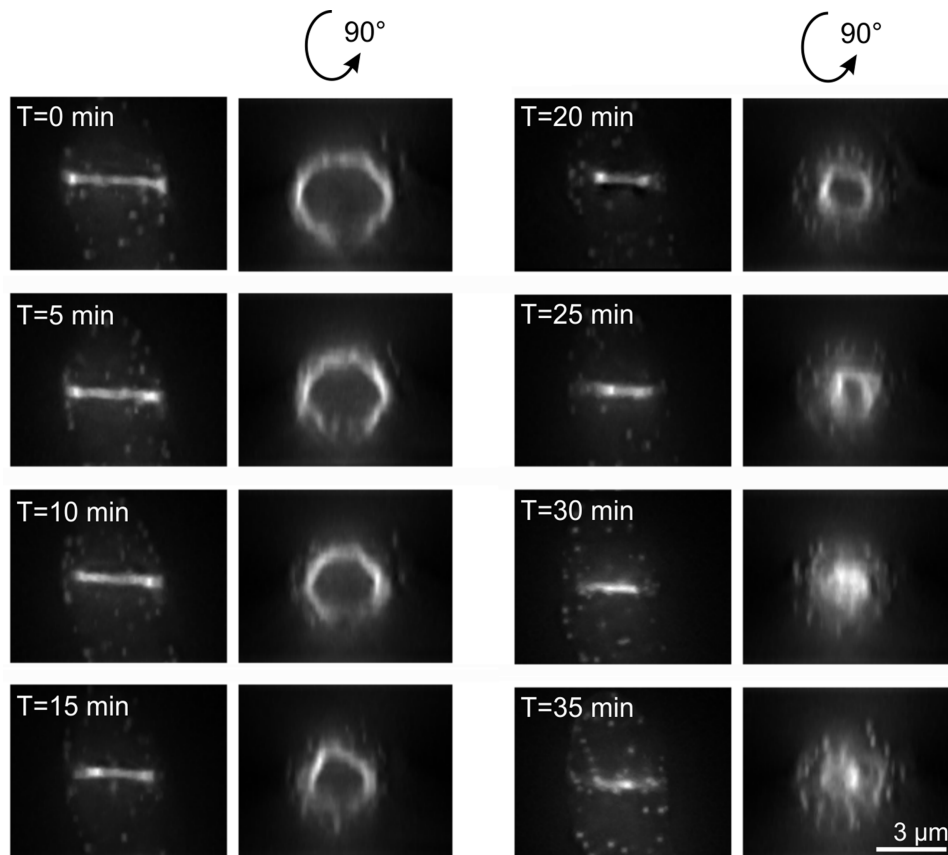
SEPTATION IN FUNGAL HYPHAE

Septum Formation

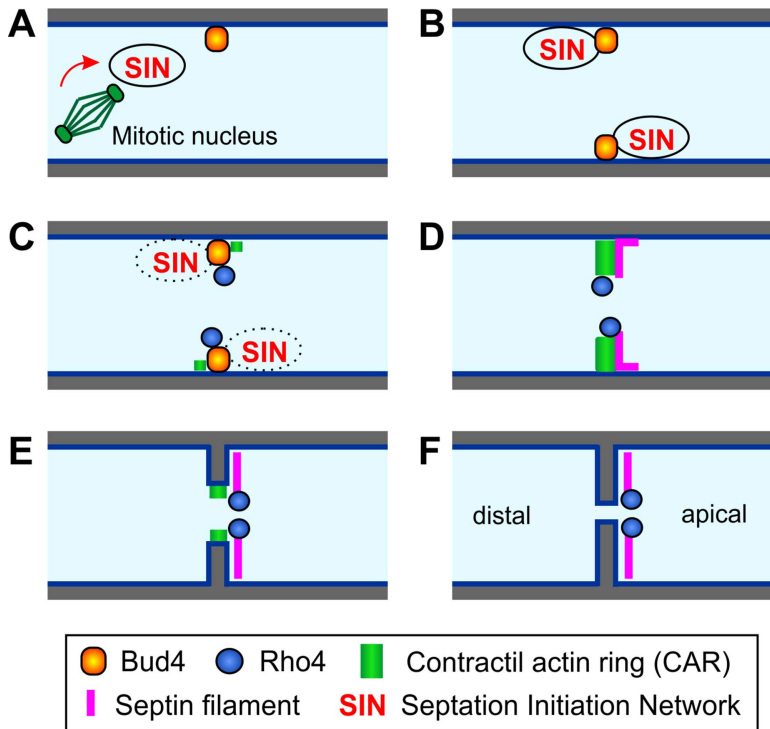
Fungal cells divide via the process of septation, whereby the localized synthesis of a cross-wall divides an existing cell into two distinct cells. In yeasts, dissolution of the septum physically separates the two cells. However, they remain attached in multicellular hyphae and often retain some degree of cytoplasmic continuity through septal pores. It is well established that the process of septum formation in fungal hyphae requires the presence of intact actin filaments, which assemble into a ring (i.e., the

contractile actin ring [CAR]) (Fig. 6) that constricts in a process resembling cytokinesis in animal cells (224, 225). In yeasts, and presumably hyphae as well, the CAR guides deposition of the septal wall material (224, 226). Much of our understanding of the mechanisms that underlie assembly and constriction of the CAR is based on the functional characterization of gene products identified on the basis of their homology to yeast proteins with well-defined roles in septation (e.g., references 226–228). In general, the roles of these gene products appear to be largely conserved in filamentous fungi (224). Additional studies have provided insight into the regulation of septum formation in fungal hyphae, and in some cases they have yielded unexpected surprises (229). These surprises to a large extent reflect novel features of septum formation in multicellular hyphae compared to yeasts. This includes, for example, the precise spacing of septa so that the appropriate compartment size is

FIGURE 6 Time course of the contraction of the CAR during septum formation in the wheat pathogen *Z. tritici*. The side view of the three-dimensional image stack shows that the CAR is closing with time. Time in minutes is shown in the upper-left corners. The CAR was labeled using an F-actin-specific GFP-LifeAct probe.



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maintained (230). Achieving a greater understanding of the mechanics and regulation of septum formation in fungal hyphae will go a long way toward revealing key functional features that underlie the unique organization of fungal hyphae.

The CAR and deposition of the septum

Like in animal cells, assembly and constriction of the CAR underlies septum formation in fungal hyphae (224, 225). The CAR initially appears at the incipient septation site as a tangle of actin filaments that is associated with myosin and tropomyosin. This structure subsequently consolidates into a thin “proto-CAR” that circumscribes the hypha. Constriction of the CAR coincides with ingrowth of the plasma membrane and the deposition of the new cell wall material that will become the septum. The CAR remains positioned at the advancing edge of the invaginating plasma membrane, which is also the active site of cell wall deposition that is mediated by the delivery of exocytic vesicles. During constriction, the appearance of actin patches that flank the CAR is consistent with localized endocytosis. Unlike cytokinesis in yeast cells, the CAR does not fully close, thereby retaining cytoplasmic continuity between adjacent hyphal cells through the resulting septal pore.

Other than tropomyosin and myosin, other components of the CAR include the anillin-like protein Bud4 (27, 224, 231), formin (224, 232), the actin-nucleating protein Bud6 (233), alpha-actinin (234, 235), paxillin (A. Virag and S. D. Harris, unpublished), and chitin synthase (224, 236). Notably, Bud4 and chitin synthase appear just prior to the initiation of constriction (224) (Fig. 7). Depending on the specific fungus, the absence of any of these components completely abolishes septum formation or results in the formation of abnormal septa. In general, formins, Bud6, and alpha-actinin are directly needed to assemble microfilaments into a CAR (232, 233, 235). The role of Bud4 is particularly intriguing, because Bud4 homologues in *S. cerevisiae* and *S. pombe* associate with septins (237, 238). In filamentous fungi such as *A. nidulans*, *A. fumigatus*, and *N. crassa*, septins also appear as rings at septation sites (e.g., references 239–241). In addition, fungal septins are subject to posttranslational modification such as phosphorylation (242), which likely plays a key role in determining their association with diverse partner proteins. Nevertheless, the relationship of septin localization and modifications to CARs and the timing of their appearance remain to be determined.

Studies using *A. nidulans* and *N. crassa* show that the monomeric GTPase Rho4 coordinates assembly of

the CAR at septation sites (28, 243). Strikingly, Rho4 orthologues are conserved in filamentous fungi and *S. pombe*, but not in *S. cerevisiae* (which possesses a different GTPase known as Rho4). Activated Rho4 appears to recruit formins to the septation site (28), which is presumably an early step in the formation of the proto-CAR. It is interesting to note that weak homologues of *S. cerevisiae* Bud3 serve as guanine nucleotide exchange factors that activate Rho4 (27, 28). Accordingly, *rho4* and *bud3* mutants fail to recruit formins and thus do not assemble CARs or form septa. These results suggest that the Bud3-Bud4 module that provides a septin-associated spatial landmark for polarity establishment in budding yeast could conceivably have been co-opted from an ancestral function in directing CAR assembly at septation sites in filamentous fungi.

Regulation of septum formation in fungal hyphae

The formation of CARs at septation sites is subject to both spatial and temporal regulation (53). The existence of spatial regulation is supported by the uniform distribution of septa in fungal hyphae and their general exclusion from apical regions. Temporal regulation is likely based on the coordination of septum formation with mitosis at least in those filamentous fungi that exhibit parasynchronous nuclear division. Collectively, both forms of regulation presumably ensure that the compartment sizes in a hypha are appropriate for the extant growth conditions.

In *A. nidulans*, results from the use of mitotic inhibitors support the view that signals emanating from dividing nuclei (e.g., the mitosis spindle or spindle poles) trigger the local formation of CARs (225). A promising source for such a signal is the septation initiation network (SIN), which coordinates CAR assembly and constriction with mitosis in *S. pombe* (244) (Fig. 7). Elements of the SIN pathway are conserved in filamentous fungi and are needed for CAR formation and septation (e.g., references 245, 246). However, they appear to function in a manner that is different from *S. pombe*, because for example, prior association with spindle poles is not required for SIN recruitment to septation sites (247). Although these observations implicate the SIN in the temporal regulation of CAR assembly or maturation, the specific nature of the signal remains to be determined. The timing of septum formation is also subject to regulation by DNA damage and other forms of genotoxic stress (229, 248). For example, low levels of DNA damage permit nuclear division but block

septation in *A. nidulans* and *Fusarium graminearum*. In *A. nidulans*, this effect is mediated by inhibition of the same CDK that regulates mitosis (229).

Even though signals emanating from mitotic nuclei are presumably capable of triggering CAR assembly or maturation, it is also evident that not all mitoses are capable of activating formation of a septum. Otherwise, hyphal cells would in principle contain only a single nucleus, which is not the case for many filamentous fungi. Indeed, the regular spacing of septa and the relatively uniform number of septa per hyphal compartment in these fungi imply that septum formation is spatially regulated. That is, the activation of CAR assembly by a given mitosis event seemingly precludes adjacent dividing nuclei from also triggering septation. This could be simply due to titration of one or more key components needed for CAR assembly, but it might also reflect the active repression of septum formation at sites flanking a new CAR even if mitotic nuclei are nearby. How this might occur remains a puzzle, though it is tempting to speculate that cortical landmarks might specify “pre-septation” sites that must be activated by an adjacent mitosis event to enable CAR formation or they are otherwise disassembled.

Communication and Differentiation of Hyphal Compartments

In basidiomycetes and ascomycetes, septa divide the hypha into compartments. These septa contain pores of 50 to 500 nm (249–252) that allow intra- and inter-hyphal translocation of metabolites, proteins, RNA, and even organelles. The rate of mass flow of cytoplasm in the mycelium of the basidiomycetes *Armillaria mellea* and *Serpula lacrimans* is in the range of 3 to 70 $\mu\text{m s}^{-1}$ (253), while a rate of $\sim 5 \mu\text{m s}^{-1}$ was observed in *N. crassa* (254). These rates underpin the potential for fast mixing of cytoplasm through the mycelium, implying that the mycelium of ascomycete and basidiomycete fungi is continuous like that of lower fungi. This paradigm is, however, changing. Studies have shown that fungal septa with growing hyphae can be closed, thus preventing cytoplasmic exchange between compartments (see “The Mechanism of Septal Pore Closure,” below), which allows heterogeneity of hyphal cells within the fungal mycelium.

Hyphal heterogeneity

Zones within fungal mycelia show heterogeneous gene expression patterns and heterogeneity in growth and secretion (255–260; overview in reference 17). For instance, growth is mainly limited to the periphery of

colonies of *Aspergillus niger* (255), while protein secretion occurs throughout the colony, with the exception of an intermediate zone, that is primed to sporulate under particular environmental conditions (260). There is differential gene expression across these zones (256, 259), and different proteins are secreted at the periphery and central zones of these colonies (259). Even hyphae within a given mycelial zone are heterogeneous with respect to cytosolic composition (17, 255, 257, 258, 261–265). For instance, part of the hyphae at the outer zone of *A. niger* colonies have high transcriptional and translational activity, which is accompanied by secretion of proteins that are involved in substrate degradation (255, 263–265).

Hyphae show highly polarized apical tip growth. They have at least four regions. The most apical region of hyphae (i.e., the first 1 to 5 μm) contains the Spitzenkörper, some mitochondria, and some smooth endoplasmic reticulum (266, 267). The tip region is also rich in actin filaments but sparse in ribosomes (262, 268). The second region is 2 to 4 times as large and contains mitochondria and some ER cisternae (104). The third region, which ends at the first septum, contains the complete collection of organelles. The fourth and following regions make up the second and following compartments. They contain all organelles, but their structure and abundance are often different from those in the third region. For instance, vacuoles are more abundant in subapical compartments, while 18S rRNA is less abundant, at least in the basidiomycete *Schizophyllum commune* (262).

Mechanisms of cytoplasmic flow

Cytoplasmic streaming depends on the architecture of the mycelium and is the result of turgor pressure gradients, diffusion, and/or turbulence due to movement of organelles along the cytoskeleton. Hyphal fusion plays an important role in long-distance translocation of nutrients in *N. crassa* (269). This is evident from the finding that transport of nutrients is highly affected in a soft mutant (Δso), which is a mutant strain that lacks the ability to undergo hyphal fusion. Even a 50% reduction in hyphal fusion frequency, as occurs in the $\Delta\text{prm-1}$ mutant, results in decreased nutrient translocation rates from the center toward the periphery of the colony (269).

Turgor pressure-mediated translocation has been proposed as a mechanism of cytoplasmic streaming in several fungi (253, 269–271). For instance, long-distance translocation of the nonmetabolizable glucose analogue 3-O- ^{14}C methyl glucose through the mycelium of *Morchella esculenta* would depend on this mechanism.

Translocation of 3-O-methyl glucose depends on the formation of sclerotia. A mutant unable to form these survival structures does not transport this glucose analogue through the mycelium (270). To explain these results, it was postulated that nutrients and solutes lower the water potential of the cytosol in the vegetative hyphae, resulting in water uptake from the medium. This uptake generates a higher turgor pressure in the hyphae. However, it is insufficiently high to create a flow through the mycelium. For this to occur, solutes are converted into high-molecular-weight polymers upon their arrival at the sclerotia or are released as exudate into the medium. These processes decrease the turgor within the sclerotia and thus act as a sink by attracting water (i.e., cytosol) from the vegetative hyphae. Addition of the metabolic inhibitor azide strongly reduces translocation of 3-O-methyl glucose, showing that metabolic activity is a prerequisite for turgor pressure gradient-mediated translocation to occur.

Mass transport driven by pressure gradients also dominates intrahyphal transport toward the hyphal tips of *N. crassa* (272). The pressure gradient required for this flow is very low (10^2 to 10^4 Pa cm^{-1}) compared with the 4 to 5 10^5 Pa turgor pressure within these hyphae (273). This low required value of the gradient is caused by the micrometer-range width of the hyphae. Pressure differences in micro-channels in the range of 10^2 to 10^3 Pa result in translocation rates of 50 to 500 $\mu\text{m s}^{-1}$, with mixing of the fluid dominated by diffusion (274). This velocity agrees with the maximal rate of bulk flow in *N. crassa* hyphae of 60 $\mu\text{m s}^{-1}$, although average flow rates are only ~ 5 $\mu\text{m s}^{-1}$ (254). The intrahyphal osmotic gradients required for mass flow in *N. crassa* are likely to result from ion transport, as indicated by experiments using extracellular osmotic gradients. The high concentration of organelles in the cytosol of *N. crassa* hyphae impedes bulk flow of the cytoplasm, causing a so-called partial plug flow (272).

Cytoplasmic streaming in *N. crassa* occurs at ~ 5 $\mu\text{m s}^{-1}$ (254). In contrast, the *N. crassa* kinesin motor Nkin transports its cargo at 2.1 to 3.8 $\mu\text{m s}^{-1}$ (275). Thus, it is very unlikely that fast cytoplasmic streaming is a direct consequence of motor activity. Indeed, movement of nuclei toward the outer part of the colony was still observed in strains with mutations in microtubule-related motors (dynein and kinesin) and after treatment of wild-type strains with molecules disrupting the cytoskeleton (276). However, motor proteins move organelles in hyphal cells (overview in references 12, 167, 277, 278). Such motor-driven motility of organelles was recently shown to mix the cytoplasm and increase diffusion

within the cytoplasm (203), which could support the exchange between adjacent hyphal cells. This notion is supported by findings in *A. niger*, in which depolymerization of actin and tubulin with cytochalasin A and nocodazole, respectively, reduced the rate of cytoplasmic streaming by about 25% (279). While cytoplasmic streaming was shown to support apical translocation of organelles (272, 280), it may also result in an uneven distribution of organelles, as was shown in *N. crassa* (281). Cytoplasm streams toward the apex in this fungus. The stream narrows in the septal pores, resulting in microfluidic eddies on the upstream side of the septum, where nuclei can be trapped. The presence of septa in hyphae thus creates subcellular domains within *N. crassa* hyphae.

It is currently not known if the mechanism underlying cytoplasmic streaming in *N. crassa* applies to all its hyphae or to other fungi. Most of the work on streaming in *N. crassa* has been done with so-called trunk hyphae. These hyphae are wider than the majority of hyphae in the mycelium. Such trunk hyphae have also been observed in other fungi such as *A. niger* (282), where they also represent the minority; cytoplasmic streaming in *A. niger* hyphae has a rate 2,000 times lower than that observed in *N. crassa*, while its growth rate is about 10-fold lower (279). The cytoplasm of fungal cells is able to contract, which was shown in *A. niger*, *N. crassa*, and *Trichoderma atroviride* (283, 284). Thus, motor-driven contractions of the cytoskeleton may contribute to cytoplasmic streaming in filamentous fungi.

Selective transport across closed septa

Fungi have the ability to close their septa, which limits exchange between hyphal compartments (see “Communication and Differentiation of Hyphal Compartments,” above). In *A. niger* it was shown that the newly formed compartments are in exchange with neighbor-

ing compartments, while older parts of the mycelium are closed off by plugging of the septal pore, with ~90% of the 4th to 8th subapical septa closed, followed by complete sealing of the 9th and 10th septa (279). Closure of these septa is mainly caused by a peroxisome-like organelle, the Woronin body (see “Closing Pores by Woronin Bodies,” below). Due to the closure of old septa in the center of the colony, transport of nutrients through the cytoplasm cannot occur. Indeed, measurement of a fluorescent glucose analogue provided no evidence for glucose transport from the periphery to the colony center (282). Surprisingly, however, glucose was found to be transported from the center to the periphery of the mycelium. Because the fluorescent glucose analogue accumulated in the cross-walls of the septa, it was concluded that glucose is efficiently transported across the plasma membrane lining the septal cross-walls (282). This could be mediated by transporters that allow the passive passage of sugars down a concentration gradient. Indeed, a hexose transporter, *mstE*, localizes to septa in *A. nidulans* (285). Such a septal pore-independent and selective transport mechanism would operate independently of septal pore plugging (Fig. 8). Indeed, transport of glucose between different zones of the colony of *A. niger* was not increased in mutants that were devoid of Woronin bodies (282).

The Mechanism of Septal Pore Closure

Filamentous fungi grow by apical tip growth of their hyphae. In ascomycete and basidiomycete fungi and a few subgroups of the zygomycetes, septa partition the hypha into subcompartments. Communication between these compartments is mediated by septal pores, which range in size from 25 nm in *C. albicans* (286) to 360 nm in *N. crassa* (287). The width of the septal pore allows exchange of cytoplasm, including small molecules, ribosomes, and proteins, but also entire organelles such as

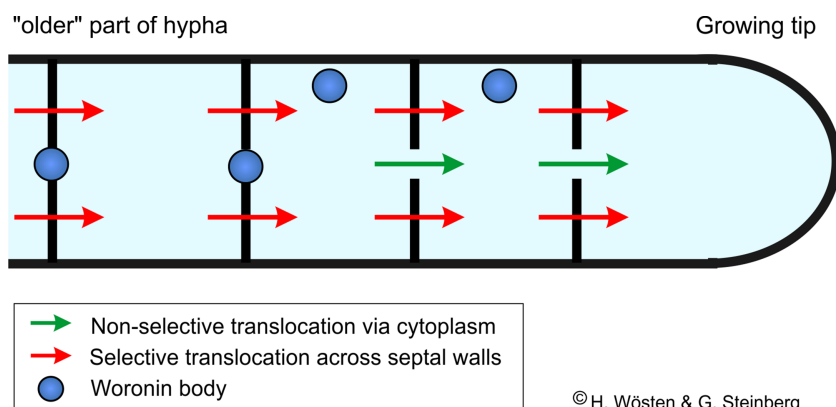


FIGURE 8 Model for tip-ward translocation in *A. niger*. Newly formed hyphal compartments are in cytoplasmic contact with neighboring cells. The Woronin body is not plugging the septal pore, and cytoplasmic streaming, as well as diffusion through the septal pore, is possible (green arrow). Older septa are plugged by Woronin bodies, which prevents exchange of cytoplasm. However, selective transport of molecules such as glucose toward the growth region is still possible (red arrows). This may involve septum-associated transporters.

mitochondria and nuclei (252, 254, 288). However, it is essential for the hyphal cell to keep this exchange under tight control and thus be able to close the septal pore upon stress or cell injury or during particular developmental stages and differentiation. To this end, fungi have developed various ways to plug the septal pore (289). In most cases, insight into the process of pore closure is based on ultrastructural studies, which show closure by organelles, small crystalline bodies, or electron-dense proteinaceous material (289). However, recent research has begun to shed light on the molecular mechanisms underpinning pore closure in filamentous fungi.

Closing pores by proteinaceous material

Plugging of the septal pore by a deposit occurs independently of hyphal injury and is based on *de novo* deposition of proteinaceous material (289). Basidiomycete fungi contain a complex septal pore, the dolipore (252), which was shown in *S. commune* to be plugged by electron-dense proteinaceous material in response to environmental stress (e.g., osmotic shock) or hyphal wounding (290, 291). Such deposition appears to be the only mechanism for pore plugging in basidiomycetes (subphylum Agaricomycotina). Not much is known

about the nature of this electron-dense material. However, it has been suggested that the septal pore cap (i.e., parenthesomes; Fig. 9) (252) of the basidiomycete dolipore provides a reservoir from which plugging proteins are released (289). The septal pore cap is derived from the endoplasmic reticulum and flanks both sides of the pore channel. Septal pore caps are usually perforated and were suggested to act as sieves that limit passage of organelles through the dolipore (292); passage of nuclei through dolipores requires its degradation and conversion to a simple pore (293). Support for an additional role of the septal pore cap in pore plugging comes from the recent identification of protein SPC18 in *Rhizoctonia solani* (294). SPC18 localizes to septal pore caps but was also found in the pore channels, suggesting that the septal pore cap provides the material for plugging the dolipore. This notion is further supported by a mutant in a septal pore cap protein SPC33 in *S. commune* (295). This mutant lacks septal pore caps and is unable to close the dolipore channel upon wounding. Interestingly, this results in defects in growth and fruiting body formation, indicating that the ability to close the septal pore is a prerequisite for differentiation and development in basidiomycetes.

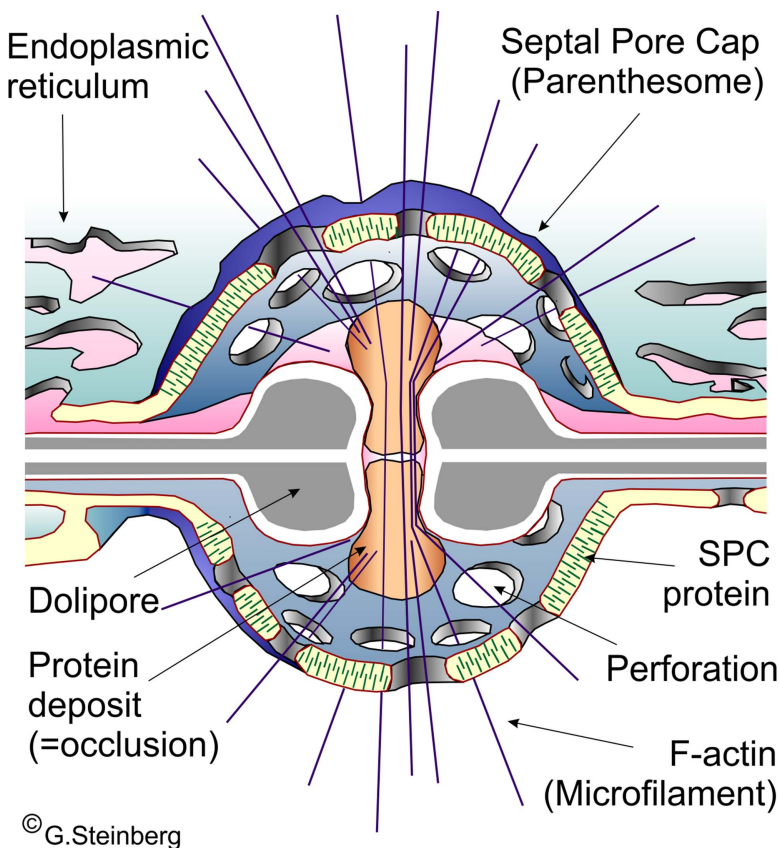


FIGURE 9 Schematic drawing of a dolipore in the basidiomycete *Polyporus biennis*. The image was redrawn from a reconstruction of electron micrographs, first published in reference 346.

In older parts of hyphae in the ascomycete fungus *N. crassa*, septal pores contain accumulations of electron-dense material (287), suggesting that protein deposition limits flow between hyphal compartments. Work in *N. crassa*, *Sordaria macrospora*, and *Aspergillus oryzae* revealed a role for the SOFT/PRO40/SO1 protein (hereafter named SO) in plugging septal pores (296–298). SO is a cytoplasmic protein that, upon cell injury or environmental stress, relocates to the septal pore. Mutants lacking SO show slower plugging after cell injury (297) or are significantly impaired in septal closure (298). This suggests that deposition of SO proteins participates in sealing septa. Interestingly, only the C-terminal half of SO is involved in plugging septal pores in *A. oryzae*, whereas the N-terminal half appears to serve a septum-independent role in cell-cell fusion (299). This functional diversity may explain the broad phenotypic variation in SO mutants, which show defects in cell-cell fusion, aerial hyphae formation, and conidiation (298, 300).

Whether SO protein represents the electron-dense material in septal pores of ascomycetes (e.g., references 287, 301) is not known. In *A. nidulans*, the NIMA kinase locates at the septum and the septal pore is open (302). Upon entry into mitosis, NIMA leaves the septum to control early steps in mitosis (303). Upon this relocation of NIMA, the septal pore closes, and this isolates the mitotic cell from neighboring interphase cells (302). Thus, NIMA appears to orchestrate septal pore opening during interphase. Surprisingly, this NIMA-controlled plugging occurs independently of SO and of Woronin bodies (see below), suggesting that additional mechanisms exist which close a septal pore. Indeed, the recent identification of 17 additional septal pore-associated (SPA) proteins (304) suggests that various proteins cooperate in pore closure. A subset of these SPA proteins localize to the lumen of the septal pore channel (SPA type II: SPA3, SPA5, SPA6). Thus, these proteins may participate in controlling communication and exchange between hyphal compartments. Such a role in pore plugging is further supported by biochemical data that show that recombinant SPA5 self-assembles into gel-like aggregates (304). However, the precise cellular role of most SPA proteins needs to be elucidated.

Closing pores by Woronin bodies (WBs)

Filamentous ascomycetes (subphylum Pezizomycotina) and imperfect fungi (Deuteromycota) have developed an additional mechanism for septal pore plugging. This is based on a fungus-specific organelle, the WB. WBs were named by A. H. Reginald Buller (305) after

the Russian botanist Mikhail Strepanovich Woronin, who first described *kleine Körnchen* (small granules) adjacent to septa in *Ascobolus pulcherrimus* (306). Woronin's observations were confirmed in various fungi, where these highly refractive particles were found to move within the cytoplasm (see reference 305 for references). Subsequent electron microscopy studies revealed that WBs are spherical membrane-bound organelles of 100 to 750 nm in diameter (289). Fungal cells contain usually 3 to 6 WBs adjacent to their septa (305, 307), but a large number of additional WBs are found in the cytoplasm of *A. nidulans* and *A. fumigatus* (302, 308, 309). Septum-associated WBs are nonmobile and anchored at the septal pore (301, 308, 310). In *Neurospora* and *Sordaria*, WBs are larger and are anchored at the cell periphery, which is thought to be an adaptation to the prominent and rapid cytoplasmic streaming seen in these fungi (311).

The main purpose of WB-based septal pore closure is to prevent catastrophic loss of cytoplasm after hyphal injury (289). The first insight into such a function in septal pore plugging was provided by ultrastructural studies that showed WBs sealing septa in wounded hyphae (312–314). A breakthrough came with the identification of the protein Hex1 in WBs of *N. crassa* (315, 316), which was subsequently reported in *M. grisea* (317), *Trichoderma reesei* (318), *A. oryzae* (301), and *A. fumigatus* (308). Hex1 is the main component of the crystalline matrix of WBs (319, 320), and deletion of *hex* genes results in the absence of WBs (301, 308, 315, 316). Strikingly, these null mutants are impaired in sealing their septa after injury, which strongly indicates a role of WBs in septal pore plugging. However, WBs also seal 5 to 13% of septal pores in unwounded hyphae (321). This suggests that WBs have additional roles in restricting flow through septal pores in healthy hyphae, and this may generate heterogeneity within the mycelium (261).

Ultrastructural studies of *F. oxysporum* suggested that WBs originate from microbodies (i.e., peroxisomes and glyoxysomes; reference 322). This conclusion gained support by the finding that antibodies against the peroxisomal targeting signal recognize WBs (323). Hex1 from *N. crassa* contains a peroxisomal targeting signal, and live-cell imaging confirmed that Hex1 crystals are formed at peroxisomes (324), where a Woronin sorting complex (WSC) supports assembly of Hex1 and formation of WBs, as well as cortical anchorage of the organelle (325). A role for the WSC in WB formation was confirmed in *A. fumigatus*, although WSC was not involved in anchorage of WBs near the septum (326).

Finally, it was shown that mutants deleted in peroxisomal *pex*-genes (*pex6*, *pex14*, *pex11*), required for proliferation or biogenesis of peroxisomes in *M. grisea*, *N. crassa*, and *A. oryzae*, lack WBs (327–329). Taken together, these studies provide compelling evidence for the conclusion that WBs derive from peroxisomes.

It was recognized early that WBs, once in position, are stationary and even resist fast cytoplasmic streaming (301, 305, 308, 324, 325). Interestingly, when WBs were misplaced by an optical laser trap in *N. haematococca*, they snapped back to their original position at the septum (330). This simple experiment strongly suggests the existence of a physical tether that holds WBs in position. Indeed, ultrastructural studies suggested a fibrous connection between WBs and the septal pore (309). Again, it was work with *N. crassa* that moved the field forward and revealed the molecular nature of this tether. A genetic screen for mutants defective in WB segregation identified the *leashin* (*lah*) locus, which is involved in WB tethering (331). This locus encodes two proteins, LAH-1 and LAH-2. While LAH-1 tethers WBs to the cell cortex, LAH-2 is located at the hyphal apex and the septal pore, where it performs unknown functions. In most other ascomycetes, only LAH-2 homologues, consisting of 6,000 to 7,500 amino acids, are found that have been shown to tether WBs to the septum. In *A. oryzae* and *A. fumigatus* mutants that lack Lah proteins, WBs are no longer tethered to the septum and hyphae fail to plug their septal pores after wounding (326, 332). Most strikingly, a shorter version of Lah reduces the distance of WBs to the septal pore (from 99 to 50 nm) (332), which adds further evidence for a role of Lah in tethering WBs to septal pores. The molecular details of how Lah proteins tether the WB proteins may differ between fungi, because Lah was shown to interact with WSC in *N. crassa* (331), whereas Lah was reported to bind directly to HexA in *A. fumigatus* (326). How the cytoplasmic tether interacts with the WB core protein HexA is not understood.

The mechanism by which WBs move into the septal pore during plugging remains elusive. A tempting hypothesis is that hyphal injury results in high pressure in the adjacent cells, and bulk flow of cytoplasm moves WBs into the pore channel (310, 313). Indeed, sealing of septal pores takes several seconds, and hyphal cells often show some cytoplasmic bleeding before the pore is plugged (261, 297). However, such a passive mechanism does not explain why only one WB seals the septal pore while the others remain unaffected (307). Alternatively, it was suggested that an active, maybe cytoskeleton-dependent or contractile mechanism could draw WBs

toward the septum (307, 310). The nature of such a mechanism is not known, but it may involve the tether protein Lah. Indeed, Lah shares short sequence motifs with the muscle protein titin (331, 332), which were shown to respond to changes in intracellular calcium, thereby changing the elasticity of the protein (333). Hyphal wounding increases calcium levels at the septal pore (304), which supports the idea that Lah could act as an elastic molecular spring that drags WBs into the septal pore.

In *A. nidulans* and *A. oryzae* the vast majority of the WBs are not associated with the septum (302, 308), but rather move rapidly along the cytoskeleton at a speed of $\sim 1.4 \mu\text{m/s}$ (302). Similar behavior was described by early light microscopists (305 and references therein), suggesting that WB motility is a common feature. Currently, neither the molecular machinery for this motility nor the biological role of these moving WBs is understood. One possibility is that immature WBs are motile until they settle at the septum. Alternatively, WBs may have additional and septum-independent functions in the fungal cell. Assessing this question is further complicated by the fact that contradictory conclusions were drawn from *hex1* mutants in various fungi. While Hex1, and therefore WBs, in *A. oryzae* was found to be essential for conidiation (301), *N. crassa* mutants deleted in *HEX-1* either showed no defect in conidiation (315) or exhibited poor conidiation (316). Moreover, in the plant pathogen *M. grisea*, the Hex1 homologue MVP1 was reported to be dispensable for virulence (317), whereas another study concluded that Hex1 in this fungus is highly important for its full virulence (334). The reason for such phenotypic variation is currently not known.

Consolidation

The final step after plugging the pore by protein deposit and/or WBs is the development of a permanent seal of the pore. This process, called consolidation, is found in asco- and basidiomycetes (289, 335). It involves synthesis and deposition of electron-lucent cell wall material at the septum and occurs within 30 min to 3 h after hyphal wounding (290, 307, 314). Cell wall formation requires delivery of secretory vesicles, which carry cell wall synthases along the cytoskeleton (135, 136, 336, 337). Indeed, large numbers of vesicles (314), as well as actin filaments and associated myosin-5 motors, concentrate at the septal pore of injured hyphae (304). Thus, consolidation results in permanent sealing, which follows the rapid emergency plugging of septal pores in response to hyphal cell injury.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

In this article, we have summarized our current knowledge of important processes underlying hyphal growth in filamentous fungi. We have shown that hyphal morphogenesis and septum formation are distinct morphogenetic processes that rely on the cytoskeleton and vesicle trafficking machinery to direct localized cell wall deposition to a precise location. Vesicle trafficking also characterizes the conventional secretory pathway in fungi. We have summarized our understanding of the processing of secretory cargo across the Golgi, coat-independent secretory vesicle budding from the trans-Golgi network, and the composition of the Spitzenkörper. Recent identification of nonclassical pathways and the role of fungal-specific myosin-chitin synthases in exocytosis add to the complexity of the secretory pathway in filamentous fungi. In addition, it has emerged that EEs are not only involved in the endocytic pathway; they are also crucial for the spatial organization of the hyphal cell and perform specialized roles in long-range signaling, melanin biosynthesis, septin filament assembly and organizing protein translation in the cell. Finally, we have summarized our current knowledge of the mechanism of septum plugging and its role in controlling communication between hyphal cells.

While our understanding of various proteins and structures involved in these critical processes is growing rapidly, little is known about the regulatory mechanisms that orchestrate them. Current research is focused on a few model fungi; this limits comparative studies. However, comparative genomics among fungal species belonging to different taxa will allow identification of conserved key core determinants of hyphal tip growth. Furthermore, establishing molecular tools for cytological studies in nonmodel fungi, such as the wheat pathogen *Z. tritici* (166, 338–343) or the grass pathogen species *Ustilago bromivora* and *Brachypodium* spp. (344), will enable more detailed comparison of the cytology of hyphal growth and fungal pathogenicity.

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