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Review

Hyphal morphogenesis in *Aspergillus nidulans*

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ABSTRACT

The formation of hyphae that grow solely by apical extension is a defining feature of filamentous fungi. Hyphal morphogenesis involves several key steps, including the establishment and maintenance of a stable polarity axis, as well as cell division *via* the deposition of septa. Several filamentous fungi have been employed in attempts to decipher the mechanisms underlying these steps. Amongst these fungi, *Aspergillus nidulans* has proven to be a particularly valuable model. The genetic tractability of this fungus coupled with the availability of sophisticated post-genomics resources has enabled the identification and characterization of numerous genes involved in hyphal morphogenesis. Here, we summarize current progress towards understanding the function of these genes and the mechanisms involved in polarized hyphal growth and septation in *A. nidulans*. We also highlight important areas for future investigation.

1. Overview

Despite their apparent simplicity, fungal hyphae are remarkable structures that allow filamentous fungi to colonize a diverse array of habitats. The characteristic feature of a hypha is the localization of growth to the extreme tip, leading to the formation of an elongated tube capable of impressive extension rates. The formation of apical and lateral branches increases the surface area colonized by a hyphal network. The partitioning of hyphae into cellular units by cross-walls known as septa permits compartmentalization of functions and is thought to play a key role in supporting the development of reproductive structures that bear spores. A deeper understanding of the molecular basis of hyphal morphogenesis is important at two levels. First, it would yield meaningful insight that could be exploited to allow better control of fungal growth, whether limiting the growth of a pathogen or optimizing the growth of an industrial strain that produces valuable compounds. Second, it would permit comparison to analogous processes in animals and plants. This might be particularly relevant to other highly polarized cell types in these kingdoms, including neurons and pollen tubes, with a view towards the elucidation of common principles underlying this unique mode of growth. Accordingly, there is increasing interest in the identification and characterization of functions required for the establishment and maintenance of hyphal polarity, the formation of branches, and septation. One of the fungi that has proven to be a veritable 'workhorse' in this effort is *Aspergillus nidulans*, which is a widely recognized model fungus known for its genetic tractability and ease of manipulation. In this review, we summarize progress towards understanding the molecular basis of hyphal morphogenesis in *A. nidulans*.

2. *A. nidulans* as a model organism

A. nidulans (teleomorph *Emericella nidulans*) is an ascomycete fungus that belongs to the class *Eurotiomycetes* and the order

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Eurotiales. Over the past ~50 y, the seminal efforts of a long list of notable research scientists have elevated *A. nidulans* to the status of a model organism. Befitting this status, numerous methods have been developed to facilitate the efficient analysis of gene function in *A. nidulans*. Foremost amongst these is the ability to use classical genetic approaches to identify and characterize interesting sets of mutants (Todd *et al.* 2007a,b), including conditional mutations that affect essential functions. Additional methods, such as PCR-mediated gene replacement and heterokaryon rescue, permit the targeted analysis of specific genes, including those whose deletion might be lethal (Osmani et al. 2006; Szewczyk *et al.* 2006). Finally, a diverse collection of fluorescent reagents and probes (i.e., GFP-based markers) enable the real-time imaging of several important proteins in growing hyphae. Collectively, through the use of these methods, numerous *A. nidulans* proteins have been functionally implicated in some aspect of hyphal morphogenesis (Harris *et al.*, 2009). In many cases, these proteins were selected based on their homology to proteins known to be involved in the polarized morphogenesis of other organisms, primarily the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. In other examples, the proteins were identified as a result of unbiased genetic screens that focused on mutants that exhibit defects in polarity establishment, polarity maintenance, septum formation, or nuclear division. Notably, these screens often lead to the identification of proteins with no previously suspected role in hyphal morphogenesis.

3. Features of hyphal morphogenesis in *A. nidulans*

Like most filamentous fungi, *A. nidulans* initiates a new round of growth through the process of spore germination. The events underlying the germination of asexual conidiospores leading to the growth of a mature hypha have been characterized extensively (Harris 1997; d'Enfert 1997; Osherov and May 2001; Momany 2002; Harris 2006). It is presumed that a similar sequence of events accompanies the germination of sexual ascospores, though this has not been investigated in any detail.

In *A. nidulans*, the first step in spore germination is the breaking of dormancy, which is accompanied by spore rehydration, initiation of translation, resumption of metabolic activity, and isotropic expansion of the cell surface. The next step is the establishment of a polarity axis upon which subsequent cell surface expansion and cell wall deposition are directed. The stabilization of this axis results in the maintenance of polarity and enables the formation of a germ tube that ultimately matures into a hypha.

Hyphae are populated by multiple nuclei due to a series of parasynchronous nuclear divisions (note that conidiospores are uninucleate, whereas ascospores are binucleate). Nuclear division is coordinated with growth such that each division is coupled to a doubling of cell mass, and the entire process is referred to as the duplication cycle (Fiddy and Trinci 1976; Harris 1997). Once hyphae reach a certain volume, which appears to vary depending on growth conditions, they are partitioned by the formation of the first septum. Notably, septation is coordinated with nuclear division and the first septum typically forms nears the basal end of a hypha near the junction with the conidiospore. Following the first septation event, each passage through the duplication cycle is terminated by the formation of septa in the hyphal tip compartment. On the other hand, sub-apical compartments enter a period of mitotic quiescence that is eventually broken by the formation of a branch that generates a new hypha. Branch formation requires the establishment and maintenance of a new polarity axis, and likely recapitulates many of the events involved in spore germination.

For the remainder of this review, we will focus on specific features of hyphal morphogenesis in *A. nidulans*, with emphasis placed on what is known about the underlying mechanisms.

4. Isotropic growth

The primary trigger for conidiospore germination in *A. nidulans* appears to be glucose, whereas nitrogen and phosphorus are dispensable (it is not known if this is also true for ascospores). The presence of glucose is seemingly sensed by a G protein-coupled receptor (GPCR), because a constitutively active (i.e. GTP bound) Gα protein GanB causes precocious germination of conidia, even in the absence of a carbon source (Chang *et al.* 2004). One downstream effector of GanB is likely to be CyaA, an adenylate cyclase necessary for cyclic AMP (cAMP) production. cAMP acts as a secondary messenger that binds to the regulatory subunit of protein kinase A (PKA), thereby activating the catalytic subunit. In *A. nidulans*, both CyaA and PKA are required for efficient spore germination (Fillinger *et al.* 2002). Additional studies also implicate a Ras signaling pathway in glucose sensing. In particular, a dominant activating mutation in the Ras homologue RasA enables conidiospores to initiate germination in the absence of a carbon source. Although mutant spores undergo isotropic expansion and nuclear condensation/division, they do not proceed to germ tube emergence (Osherov and May 2000). This implies that the level of active RasA must diminish for later stages of germination to proceed, and suggests that Ras activity might determine the extent of isotropic expansion based on nutrient conditions. The upstream activator of RasA remains unknown, though likely candidates include GPCRs as well as glucose transporters (Fig 1).

What are the physiological responses necessary for isotropic expansion that rely on the above signaling pathways? During the isotropic growth phase of conidiospores, water uptake is likely necessary to increase the volume of the spore and maintain turgor pressure. However, the mechanisms underlying the transport of water, and whether aquaporins are specifically involved, have not yet been investigated. One strategy that fungi use to increase water uptake is the synthesis or uptake of compatible solutes (osmolytes) that increase the water potential of the spore. Trehalose metabolism has been linked to glycerol accumulation in germinating spores of *A. nidulans*, suggesting the possibility that glycerol serves as an osmolyte (d'Enfert *et al.* 1999; d'Enfert and Fontaine 1997; Fillinger *et al.* 2002). However, glycerol cannot be the sole osmolyte contributing to the water potential of swelling conidia because the deletion of glycerol

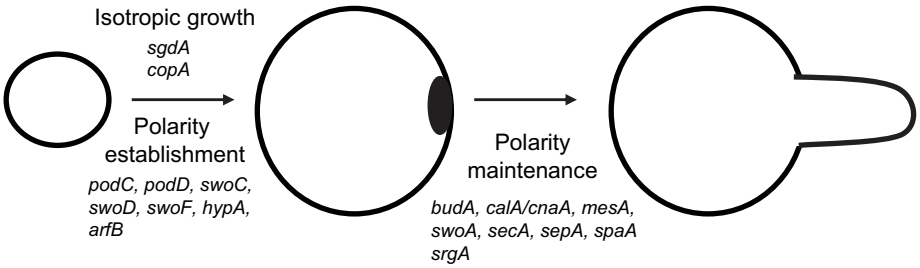

Fig. 1 – Genes that contribute to isotropic growth, polarity establishment and polarity maintenance during the germination of *Aspergillus nidulans* conidiospores. Conidiospores undergo a period of isotropic expansion before a polarity axis is established (black spot) upon which the incipient germ tube will be released. The release of the germ tube and its subsequent growth are dependent upon the ability of *A. nidulans* to maintain several protein complexes at the point of polarity establishment (see text for details).

dehydrogenase genes and subsequent reduction in intracellular glycerol levels does not preclude isotropic expansion and spore germination (de Vries *et al.* 2003; Fillnger *et al.* 2001). Cellular mannitol, trehalose, and perhaps proline may serve as additional osmolytes. Indeed, a gene encoding the proline transporter *prnB* is upregulated during conidial germination (Tazebay *et al.* 1995; Tazebay *et al.* 1997).

Common housekeeping functions also appear to be strongly associated with isotropic expansion. Multiple genetic screens for conditional polarity mutants have revealed that disruption of protein translation and folding arrests conidiospore germination during the isotropic expansion phase (Lin and Momany 2003; Osherov and May 2000; Osherov and May 2001). Similar effects are observed when conidiospores are treated with inhibitors of translation (Osherov and May 2000). These observations suggest that increased metabolic activity is needed to support isotropic expansion. The TOR signaling pathway is an attractive candidate for mediating this effect. In *S. cerevisiae*, TOR appears to act in combination with PKA to regulate the growth response to nutrient repletion (Slattery *et al.* 2008). Coupled with the known capacity of TOR to regulate actin organization (Schmidt *et al.* 1996), this observation hints at a possible mechanism for the coordination of cell surface expansion with metabolism in germinating conidiospores.

5. Establishment of a polarity axis

A prerequisite for the successful emergence of a germ tube from a swollen spore is the establishment of a polarity axis. Polarity establishment encompasses the processes of specifying a new polarity axis and using the resulting positional information to spatially organize the morphogenetic machinery. This results in the termination of isotropic expansion, such that cell wall deposition no longer occurs around the entire circumference of the spore, and is instead confined to a specific site that will eventually become the hyphal tip. Despite considerable interest in the mechanisms underlying polarity establishment in *A. nidulans*, they remain poorly defined. Nevertheless, genetic analyses have provided some insight into how new polarity axes are specified and have also implicated several cellular functions in the establishment of polarity.

The best understood paradigm for the specification of a polarization site in fungi is the *S. cerevisiae* bud site selection system. This system is based on the use of distinct cortical markers that specify one of two possible budding patterns. The resulting positional information is subsequently relayed to the GTPase Cdc42 *via* a Ras-related GTPase Rsr1/Bud1 (Park and Bi 2007). Locally active Cdc42 then promotes recruitment of the morphogenetic machinery to the presumptive bud site. Critical components of this regulatory system are either absent from the *A. nidulans* genome (e.g., Bud8, Bud9) or, if present, are very poorly conserved (i.e., Axl2, Bud3, Bud4, Axl1) (Harris and Momany 2004). Furthermore, functional characterization of the poorly conserved homologues shows that they have no obvious role in the establishment or maintenance of hyphal polarity (H. Si and S. Harris, manuscript submitted). Based on this evidence, the bud site selection system does not appear to perform an analogous function during spore germination in *A. nidulans*. Nevertheless, results from studies using *A. fumigatus* implicate a Ras GTPase, RasB, in the spatial regulation of polarized hyphal growth (Fortwendel et al. 2005), and cortical markers that generate positional information in *A. nidulans* have also been identified (i.e., TeaR; see below for more detail). Accordingly, a cortical marking system might yet specify the polarity axis in swollen *A. nidulans* spores, though the potential involvement of RasB and TeaR, as well as the identification of other vital components, remains to be investigated (Fig 2).

An alternative paradigm for the specification of the polarity axis is provided by the *S. cerevisiae* mating pheromone response. Binding of mating pheromone to its cognate GPCR leads to activation of an associated heterotrimeric G protein, such that the liberated $\beta\gamma$ complex is able to serve as a positional marker that locally recruits components of the Cdc42 GTPase module (Park and Bi 2007). Local activation of Cdc42 then reorganizes the morphogenetic machinery in a manner that overrides existing bud site selection signals. Most components of the pheromone response pathway are conserved in *A. nidulans*, and furthermore, at least one GPCR and a heterotrimeric G protein have been implicated in the regulation of spore germination. Thus, it is entirely conceivable that

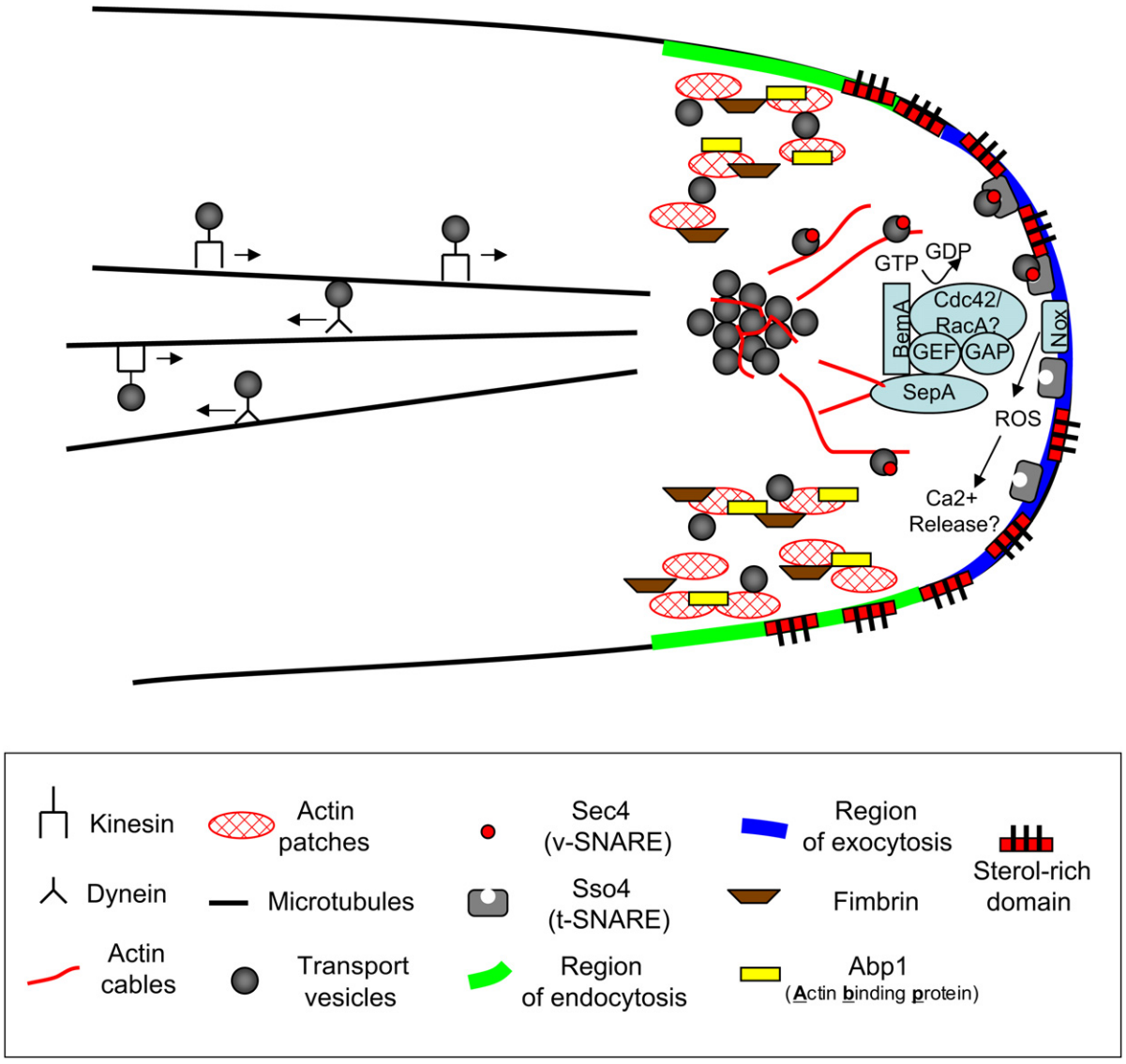

Fig. 2 – Molecular model of hyphal growth in *Aspergillus nidulans*. Vesicles are delivered from the Golgi-like organelles to the apical vesicle cluster (i.e. Spitzenkörper) along microtubules. Vesicular transport on microtubules is powered by motor proteins in the kinesin (anterograde direction) and dynein (retrograde direction) families. From the apical vesicle cluster, the vesicles are transported along actin cables to the plasma membrane. The actin cables at the hyphal tip are nucleated by the formin SepA, which may be activated by small GTPases Cdc42 and/or RacA. Vesicle fusion with the membrane is mediated by t-SNARE and v-SNARE proteins. The hyphal tips of several fungi contain sterol-rich membrane domains. Although the protein content of sterol-rich domains is unclear, they likely represent signaling complexes that contribute to the molecular mechanisms to hyphal growth. The extreme apex of hyphal tips undergoes extensive exocytosis, whereas flanking regions undergo endocytosis to recycle membrane components. Endocytosis at the hyphal tip is dependent upon actin patches, actin binding protein, and fimbrin.

a GPCR involved in glucose detection could also mark the eventual polarization site. At this time, there is no experimental evidence that supports this idea, though heterotrimeric G proteins do regulate the orientation of hyphal growth and control lateral branch formation in other filamentous fungi.

Although the preceding models implicate specific landmarks (i.e., bud site selection proteins, GPCRs) in the selection of new polarity axes, studies of polarity establishment in *S. cerevisiae* suggest that these markers are not needed *per se*. Notably, yeast cells can still switch from isotropic expansion to polarized growth despite the absence of all known landmarks. Under these circumstances, polarity establishment becomes reliant upon a set of positive and negative feedback loops that reinforce initially stochastic fluctuations in local Cdc42 levels until they exceed a given threshold at a random site (Wedlich-Soldner *et al.* 2003). Key elements of these feedback loops include filamentous actin and the modular scaffold

protein Bem1, which act in a complementary manner to promote localized vesicle exocytosis towards the presumptive polarized site, whereas endocytosis enables retrieval of ''polarity factors'' from other sites (Park and Bi 2007). A similar spontaneous polarization mechanism could conceivably operate during spore germination and/or branch formation in *A. nidulans*. For example, current evidence implies that the polarity axis that directs formation of the first germ tube from swollen spores is randomly selected. One the other hand, the second germ tube almost always emerges from the pole opposite the first (i.e., the bipolar germination pattern), which would be consistent with the idea that a specific marking system only comes into play once the first polarity axis is specified (Momany *et al.* 1999). Candidate landmarks for this system could include cortical markers or GPCRs, though the possible role of the mitotic spindle and its resident proteins should perhaps be considered as well.

Surprisingly few functions are known to be required for polarity establishment in *A. nidulans*. It is generally thought that the actin cytoskeleton and vesicle trafficking machinery (i.e., the morphogenetic machinery) are needed to establish a polarity axis. In the latter case, the phenotypes of mutants such as *copA* and *hypA*, which affect proteins required for normal organization of the Golgi apparatus, support this view (Breakspear *et al.* 2007; Shi *et al.* 2004; Whittaker *et al.* 1999). By contrast, there is no direct evidence that demonstrates actin filaments are required for polarity establishment in *A. nidulans*. Mutations that block formation of a sub-class of actin filaments (i.e., mutations affecting the formin SepA) only delay polarity establishment (Harris *et al.* 1997; Sharpless and Harris 2002). Deletions of genes that encode actin and key regulators such as α-actinin and Bud6 are lethal, but it is not known whether this reflects a failure to establish polarity (Virag and Harris, 2006a; Wang *et al.* 2009). Treatment with cytochalasin A dramatically affects polarity maintenance (see below), but its effects on polarity establishment have not been reported. Although functional actin filaments would seem to be an obvious requirement for the localized delivery of regulatory factors (i.e., landmark proteins?) and components needed for cell wall deposition to the polarization site, it is not inconceivable that cytoplasmic microtubules provide a back-up mechanism that enables polarity establishment in their absence. The observation that microtubules become essential for polarity establishment in the absence of SepA provides some evidence for this idea (Virag *et al.* 2007).

A theme that has emerged from genetic screens for polarity mutants in *A. nidulans* is the importance of post-translational modification of proteins to the process of polarity establishment. The observation that a temperature sensitive (Ts) mutation affecting the N-myristoyltransferase SwoF prevents the establishment of a polarity axis implies that at least one protein requires this modification to perform its morphogenetic function (Shaw *et al.* 2002). Because of their known requirement for lipid modification, obvious candidates include GTPases such as RasA, Cdc42, and Rac1, which are each involved in some aspect of polarity establishment in *A. nidulans*. However, a bioinformatics approach identified several additional potential targets (i.e., the 'myristoylome'), of which the most interesting are the Arf GTPases. A combination of genetic and biochemical evidence suggests that ArfA and ArfB are indeed targets of SwoF. The lethality of an *arfA* gene disruption precluded analysis of its role in polarity establishment, which nevertheless seems likely given its localization to endomembranes and its presumed role in vesicle transport (Lee and Shaw 2008). On the other hand, genetic analysis of an insertion mutation in *arfB* documented clear defects in both the establishment and maintenance of polarity axes, and further showed that these are likely caused by reduced endocytosis (Lee *et al.* 2008).

Nuclear division is generally not viewed as a strict requirement for successful establishment of a polarity axis during spore germination in *A. nidulans*, primarily because most never-in-mitosis (*nim*) and blocked-in-mitosis (*bim*) mutants are able to form germ tubes, albeit after a delay in some cases (Harris, 1999). However, three mitotic mutants, *nimL*, *nimM*, and *nimN*, fail to establish polarity under all conditions tested (Harris, 1999). Notably, these mutants each exhibit sensitivity to the DNA replication inhibitor hydroxyurea (HU), and moreover, exposure of wildtype conidiospores to HU also prevents polarity establishment (Harris, 1999). These observations raise the possibility that once DNA replication is initiated, it must be completed for polarity establishment to occur. This effect does not appear to be due to the action of DNA replication or DNA damage checkpoints (S. Harris, unpublished). Instead, it is intriguing to consider the possibility that passage through a specific point in S phase of the cell cycle is required for the establishment of a polarity axis. This might be conceptually similar to ''new end take-off'' (NETO) in *S. pombe*, which is a point during S phase that must be passed before the new polarity axis is established to enable bipolar growth (reviewed by Martin and Chang 2005).

6. Maintenance of a polarity axis

Once a polarity axis has been established, it must be stabilized in order for a germ tube or branch to emerge and form a mature hypha that grows by apical extension. Indeed, it is this ability to maintain a polarity axis for a considerable distance that defines filamentous fungi such as *A. nidulans*. Both forward and reverse genetic approaches have resulted in the identification and characterization of numerous genes required for polarity maintenance in *A. nidulans*. Important functions revealed by these studies include protein O-glycosylation, sphingolipid biosynthesis and organization, the Spitzenkorper, and vesicle trafficking.

In addition to revealing the importance of post-translational modification to polarity establishment, genetic screens for Ts morphological mutants also showed that they have a role in maintaining polarity axes. In particular, results from temperature shift experiments suggest that the swoA mutant is able to establish polarity when grown at the restrictive temperature, but cannot maintain the signals required to sustain polar growth (Momany *et al.* 1999). The *swoA* phenotype was shown to be complemented by the *pmtA* O-mannosyltransferase gene (Shaw and Momany 2002). Accordingly, it seems likely that one or more yet-to-be identified surface protein(s) that contribute to polar growth are modified by O-glycosylation in a manner that affects their function.

The first evidence of the importance of sphingolipids in polarity maintenance came from the characterization of serine palmitoyltransferase (SPT) function in *A. nidulans*. SPT catalyzes the first committed step in sphingolipid biosynthesis, and is thereby required for the formation of all sphingolipid derivatives (i.e., sphingoid bases, ceramides, etc.). Mutational or chemical (i.e., myriocin) inactivation of SPT prevented polarity establishment without adversely affecting growth or nuclear division. It was also found that the absence of sphingolipids terminates existing polarity axes and leads to profuse branching of the hyphal tip (Cheng *et al.* 2001). This study highlighted the key role of compounds such as sphingoid bases and ceramides in multiple aspects of polarized hyphal morphogenesis. Subsequent studies have further analyzed the respective roles of these two compounds. BasA, which is a homologue of *S. cerevisiae* Sur2, is a sphinganine hydroxylase responsible for the synthesis of sphingoid bases (Haak *et al.* 1997). Deletion of *basA* causes severe defects in polarity establishment and maintenance (Li *et al.* 2006). LagA and BarA are two distinct ceramide synthases whose combined function is required for the maintenance of polarity axes, but not their establishment (Li *et al.* 2006). Notably, BarA appears to generate a pool of glucosylceramides that promote localization of the formin SepA at hyphal tips (Rittenour *et al.*, unpublished). Taken together, these studies suggest that the surface of hyphal tips might consist of a patchwork of lipid domains that differ in ceramide composition and mediate recruitment of different complexes that stabilize polarity axes (Virag and Harris 2006b). Sphingoid bases may have an additional set of functions, presumably involved in lipid signaling, that separately promote polarity establishment.

The Spitzenkorper (SPK) is a phase-dark structure present at the extreme apex of fungal hyphae that has been shown to have an intimate role in promoting efficient polar growth (Girbardt 1957). The concept of the SPK as a vesicle trafficking center and the modeling of its function have been previously described (Bartnicki-Garcia *et al.* 1989). The polarisome is a seemingly distinct structure at the hyphal tip that regulates formin-based assembly of actin filaments. In *A. nidulans*, localization of the formin SepA suggests that the polarisome exists as a surface crescent at the hyphal tip, whereas the SPK sits just behind the tip and appears as a spot (Sharpless and Harris 2002). Further refinement of hyphal tip organization has emerged from a recent study that describes the ''tip growth apparatus'' of *A. nidulans* (Taheri-Talesh *et al.* 2008). Results from this study suggest that the SPK and polarisome are components of a dynamic apparatus that localizes to the tip and mediates the delivery of exocytic vesicles to the apex. This apparatus consists of an apical actin cluster embedded within a larger cluster of vesicles that are presumably delivered by kinesin-dependent transport on cytoplasmic microtubules. Within the apparatus, vesicles are likely transferred from microtubules to actin filaments that are nucleated by SepA, followed by transport to a discrete exocytic zone at the extreme apex. Although it has yet to be demonstrated, it seems possible that the polarisome might play a role in formation of the SPK.

Results from several recent studies have highlighted the importance of endocytosis in the maintenance of polarity axes in *A. nidulans*. Whereas exocytosis relies on filamentous actin cables for delivery of vesicles to the apex, endocytosis has been shown to rely on branched actin patches for internalization of vesicles from a distinct cylindrical region located just behind the apex, but still within the ''tip growth apparatus'' (Araujo-Bazan *et al.* 2008; Taheri-Talesh *et al.* 2008; Upadhyay and Shaw 2008). A number of conserved endocytic marker proteins (i.e., AbpA, SlaB, FimA) have been shown to interact with and to stabilize these actin patches. Mutations that eliminate these proteins cause severe defects in polarity maintenance, and in some cases, polarity establishment as well (Araujo-Bazan et al. 2008; Taheri-Talesh *et al.* 2008; Upadhyay and Shaw 2008). These observations demonstrate that the presence of an endocytic zone flanking the apex is just as critical for polarity maintenance as is vesicle exocytosis. It is quite likely that plasma membrane components and important cell surface proteins are recycled *via* endocytosis within this zone as hyphae expand. It is tempting to speculate that the septins, which have a known role in the compartmentalization of distinct cell surface domains (Barral *et al.* 2000), might play a role in demarcating the endocytic and exocytic zones within the ''tip growth apparatus''. Furthermore, the localization and characterization of MesA, a predicted cell surface protein initially identified on the basis of genetic interaction with SepA, suggests that it could facilitate organization of the endocytic zone (Pearson *et al.* 2004).

An elegant series of experiments have described a microtubule-dependent regulatory complex that contributes to the maintenance of polarity axes in *A. nidulans* by stabilizing the position of the position of SPK within hyphal tips. In particular, deletion of the kinesin KipA perturbs the position and size of the SPK, as well as the distribution of microtubules. Whereas the plus ends of microtubules converge at one point in the tips of wildtype hyphae, they often end in two or more points in the tips of *kipA* mutants, suggesting that *kipA* delivers proteins that contribute to the organization of microtubules at hyphal tips and stabilize the SPK. Indeed, KipA is required for the proper localization of cortical marker TeaR, which is a putative prenylated membrane protein that interacts with and is required for proper localization of TeaA (Takeshita *et al.* 2008). TeaA is also required for proper convergence of microtubules at the hyphal tip and for SPK stabilization, though it does not appear to depend upon KipA for its localization. In addition, TeaA interacts with and co-localizes with the formin SepA (Takeshita *et al.* 2008). These observations outline a pathway by which a cortical marker directs the organization of both microtubules and actin filaments, and thereby stabilizes the position of the SPK. It will be interesting to determine what role different lipid domains might play in the localization of TeaR at the hyphal tip.

7. Septum formation

A. nidulans hyphae are partitioned by septa *via* a process that shares similarity with cytokinesis in animal cells (Harris *et al.* 1994; Harris, 2001). This includes the formation and constriction of a cytokinetic actin ring (CAR) in a manner that is coordinated with the completion of nuclear division (Momany and Hamer, 1997). Deposition of the septum occurs concomitant with constriction of the CAR, which in all likelihood provides

a landmark for recruitment of the vesicle trafficking machinery as well as chitin synthetases. Notably, septa do not form a complete barrier between hyphal cells, as a pore (i.e., the septal pore; Tenney et al. 2000) remains that presumably facilitates intercellular communication and nutrient translocation within a hypha. Woronin bodies positioned near the pore provide a mechanism for sealing hyphal cells should they experience osmotic or other forms of stress capable of causing lysis. Septum formation shares certain functions in common with hyphal tip growth (e.g., formin-mediated actin nucleation, localized chitin synthesis), and the two processes can even occur simultaneously in the hyphal tip cell (Harris, 1997). At the same time, there are important distinctions, of which the most important might be tight temporal and spatial coordination with nuclear division (Fig 3).

The multinucleate nature of hyphal cells in *A. nidulans* implies that unlike uninucleate yeast cells, not every nuclear division is associated with cytokinesis. Indeed, it was established very early on that multiple rounds of nuclear division precede formation of the first septum in germinating conidiospores (Clutterbuck, 1970; Harris et al. 1994). This appears to reflect the operation of a size control mechanism that regulates activation of the cyclin-dependent kinase NimX (Wolkow et al. 1996; Kraus and Harris, 2001). Once the size threshold has been exceeded, each subsequent round of nuclear division in the hyphal tip cell is followed by the formation of one or more septa within its basal half (Clutterbuck 1970). The positioning of each septum is largely guided by mitotic nuclei (Wolkow et al. 1996); there is no evidence yet for the existence of cortical markers that specify septation sites independent of nuclei (though the recently characterized TeaC represents an attractive possibility; Higashitsuji et al. 2009). Evidence suggests that as in animal cells, the mitotic spindle generates a signal that is relayed to the cortex and triggers assembly of the CAR. However, because hyphae are not uninucleate, this cannot occur for every spindle. Whether this means that only certain cortical regions are competent to receive the mitotic signal, or, randomly specified sites are able to suppress signal reception in flanking regions, remain important ideas for future investigation. In addition, the basis for suppression of septum formation in the apical region of hyphal tip cells, which can be subverted by activation of developmental programs (Sewall 1994), is not known.

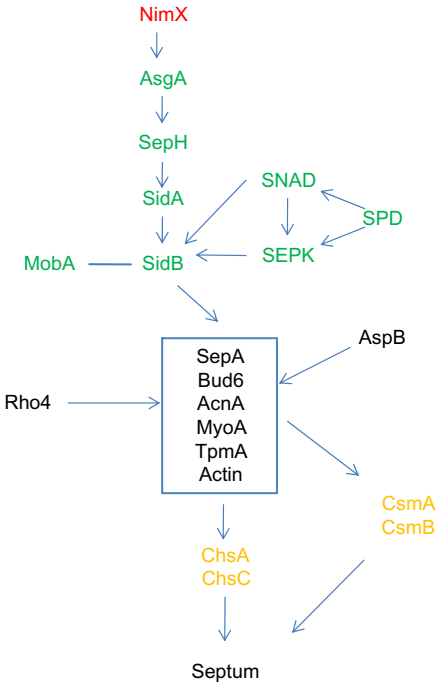

Fig. 3 – Pathways underlying septum formation. Gene products involved in cell cycle regulation (red), the SIN (green), CAR assembly and function (black), and chitin synthesis (brown) are indicated. NimX is proposed to work in conjunction with mitotic signals and a possible cortical landmark to activate the SIN *via* AsgA and SepH. SnaD and SepK anchor the SIN to spindle pole bodies (SPB). The SIN is proposed to regulate the assembly and constriction of the CAR, which in turn likely serves as a landmark to direct deposition of the septum. See text for details.

The septation initiation network (SIN) is a well-characterized signaling pathway that regulates assembly and constriction of the CAR in *S. pombe* (Simanis 2003). The analogous pathway in *S. cerevisiae*, the mitotic exit network (MEN), controls the exit from mitosis in addition to formation of the CAR (Simanis 2003). The first SIN component characterized in *A. nidulans* is SepH, which is a homologue of *S. pombe* Cdc7 that is essential for CAR assembly but not for any apparent feature of mitosis (Bruno et al. 2001). Accordingly, as in *S. pombe*, SIN function appears to be restricted to septation in *A. nidulans*. Recent studies have characterized additional components of the *A. nidulans* SIN and confirmed their role in regulating the assembly and constriction of the CAR (Kim et al. 2006). Surprisingly, the scaffolds that anchor SIN components to the spindle pole bodies (SPBs) are not required for septation in *A. nidulans* (Kim et al. 2009). Moreover, neither the terminal SIN kinase SidB nor its associated regulator MobA needs to associate with the SPB prior to their recruitment to the septation site (Kim et al. 2009). Therefore, unlike *S. pombe*, where SPB localization represents a key step in activation of the SIN (Simanis 2003), the *A. nidulans* SIN is likely activated in the cytoplasm. It remains to be determined whether a Tem1/Spg1-like GTPase activates the *A. nidulans* SIN as in either yeast, and if the localization of this GTPase changes during passage through mitosis. In addition, the identity of the relevant SIN target(s) required for CAR assembly and constriction has yet to be discovered.

The CAR is assembled at the septation site and undergoes constriction simultaneously with centripetal deposition of the septum. In *S. pombe*, the anillin-like protein Mid1 plays a pivotal role in the spatial and temporal coordination of CAR assembly with nuclear division (Chang et al. 1996; Glotzer 2005). However, there is no obvious Mid1 homologue in *A. nidulans*, and the only anillin-like protein, Bud4, appears to function at a later stage of septation (Si and Harris, unpublished). Instead, by analogy to the filamentous fungus *Neurospora crassa*, it seems likely that a Rho GTPase module may act downstream of nuclear signals to direct CAR assembly. In *N. crassa*, Rho-4 is necessary for CAR assembly and its inappropriate hyper-activation triggers the formation of spurious CARs (Rasmussen and Glass 2005). Although the nature of the nuclear signals that might lead to activation of Rho-4 are

not known, the SIN network represents an obvious and attractive candidate. Several components of the *A. nidulans* CAR have been identified and characterized, including the formin SepA (Sharpless and Harris 2002), the tropomyosin TpmA (Pearson 2004), the α-actinin AcnA (Wang *et al.* 2009), the myosin I MyoA (McGoldrick *et al.* 1995), the formin-associated protein Bud6 (Virag and Harris 2006a), and multiple chitin synthases (see below). The order in which these components are recruited, and the dynamics of their interactions within the CAR, have not yet been investigated. For example, it would be interesting to determine whether they initially form multiple small nodes that coalesce into a ring as observed in *S. pombe* (Pollard 2008). In addition, it will be important to understand how these proteins are recruited to the CAR when many of them function concurrently at the hyphal tip to build a different set of actin polymers. The possible role of localized translation in mediating the formation of spatially distinct pools of these proteins should be considered.

The septins are a conserved family of proteins with well-established roles in yeast cytokinesis (Longtine *et al.* 1996). *A. nidulans* possesses at least five septins (Momany *et al.* 2001), one of which, AspB, has been characterized in detail (Westfall and Momany 2002). AspB initially localizes as a ring that co-localizes with the CAR at septation sites. Notably, formation of this ring is dependent upon the SIN pathway as well as the presence of the CAR. The AspB ring subsequently splits into two rings that flank the septum. It is tempting to speculate that these rings may define a membrane compartment that facilitates the targeting of chitin synthases and other components needed for deposition of the septum. Finally, the basal AspB ring (relative to the hyphal tip) disappears, whereas the apical ring persists following the completion of septation. This observation leads to the intriguing suggestion that the latter ring might serve as a directional marker in hyphal cells (Westfall and Momany 2002). Functional characterization of a Ts *aspB* mutant revealed that the apparent absence of AspB does not block septum formation *per se*, but does lead to the formation of faint abnormally thin septa. It will be important to determine whether the other *A. nidulans* septins exhibit the same function and localization patterns as AspB.

Chitin synthesis represents the final step in septum formation, and requires the activity of chitin synthases, which are membrane-associated enzymes that catalyze the polymerization of N-acetylglucosamine. *A. nidulans* possesses eight distinct chitin synthases, including at least one member from each of the seven classes of this enzyme known to exist in fungi. Of these eight chitin synthases, ChsA, ChsC, CsmA and CsmB have each been implicated in septum formation. ChsA and ChsC appear to function in a redundant manner during septation, as inactivation of both chitin synthases (but neither alone) leads to defects in the ultrastructure of the septum as well as its aberrant placement (Ichinomiya *et al.* 2005). Results from localization studies are consistent with the notion that ChsA and ChsC associate with the CAR as it constricts, though they do not strictly co-localize with each other (Ichinomiya *et al.* 2005). CsmA and CsmB are novel chitin synthases that possess an N-terminal myosin motor-like domain implicated in interactions with actin filaments (Takeshita *et al.* 2005; Takeshita *et al.* 2006). Both enzymes localize to septa in a pattern that suggests they associate with the CAR. Nevertheless, although their localization is indistinguishable from each other, there is no evidence that they physically interact. Instead, it has been proposed that they belong to distinct classes of exocytic vesicles that localize to septa (Takeshita *et al.* 2006). The combined inactivation of CsmA and CsmB does not prevent septum formation, but might compromise proper formation of the septal pore (Takeshita *et al.* 2006). According to this model, ChsA and ChsC are primarily responsible for synthesis of the septum, whereas CsmA and CsmB have a more specific function in regulating chitin deposition around the septal pore (Takeshita *et al.* 2006; Horiuchi 2008).

8. Perspectives

Due to their highly polarized mode of growth and their importance to the fungal lifestyle, hyphae have long attracted the interest of fungal researchers. With the increasing availability of sophisticated post-genomics tools and resources, new insights into the mechanisms underlying different aspects of hyphal morphogenesis are emerging with much greater frequency. Many of these advances have been achieved using filamentous fungi other than *A. nidulans*, including *Candida albicans*, *Ashbya gossypii*, and *N. crassa*. Indeed, because different fungi possess different attributes that make them useful for the study of hyphal morphogenesis, the best chance for making real progress towards understanding processes such as polarity establishment, polarity maintenance, and septum formation is to exploit as diverse a set of fungi as is practical. Nevertheless, *A. nidulans* should continue to serve at the vanguard of these efforts. For example, the regularity of the duplication cycle in *A. nidulans* should make it relatively easier to characterize the mechanisms that coordinate the aforementioned processes with growth and nuclear division.

Although there are a myriad of interesting questions pertaining to hyphal morphogenesis that warrant deeper investigation, a somewhat biased sample is presented below. Notably, the answers to many of these questions will not necessarily emerge from studies that use the yeasts *S. cerevisiae* and *S. pombe* as a guiding model. In many cases, it might be more fruitful to consider observations made using migrating animal cells or neurons as a source for relevant ideas. Some of the more important questions include:

1. What is the composition and dynamics of the SPK? Given the importance of the SPK to polarized hyphal growth, a detailed description of its components, their functions, and their interactions is needed. In addition, the functional relationship of complexes such as the polarisome and exocyst to the SPK remains a mystery.
2. How is the polarity axis first established in germinating spores? Is there a set of specific landmark proteins that await identification, or is this an example of spontaneous polarization?
3. What roles do mRNA transport and localized translation play in the spatial coordination of hyphal morphogenesis? For example, do these processes generate distinct spatially segregated pools of SepA that are used for hyphal extension and septum formation?

4. How is septum formation spatially and temporally coordinated with nuclear division? Does the SIN specify when and where the CAR is assembled, and how is assembly at other sites prevented?

It should not be long before attempts to answer these and other questions yield exciting new insights that significantly advance our understanding of hyphal morphogenesis in *A. nidulans*. Because many related Aspergilli impact humans as pathogens and producers of useful compounds, these insights should also have immense practical value.

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