Apical Control of Conidiation in *Aspergillus nidulans*.

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Keywords: filamentous fungi, polar growth, asexual reproduction, conidiation, upstream developmental activation, central developmental pathway

Abstract.

The infection cycle of filamentous fungi consists of two main stages: invasion (growth) and dispersion (development). After the deposition of a spore on a host, germination, polar extension and branching of vegetative cells called hyphae allow a fast and efficient invasion. Under suboptimal conditions, genetic reprogramming of hyphae results in the generation of asexual spores, allowing dissemination to new hosts and the beginning of a new infection cycle. In the model filamentous fungus *Aspergillus nidulans*, asexual development or conidiation is induced by the upstream developmental activation (UDA) pathway. UDA proteins transduce signals from the tip, the polarity-site of hyphae, to nuclei, where developmental programs are transcriptionally activated. The present review summarizes the current knowledge on this tip-to-nucleus communication mechanism, emphasizing its dependence on hyphal polarity. Future approaches to the topic will also be suggested, as stimulating elements contributing to the understanding of how apical signals are coupled with the transcriptional control of development and pathogenesis in filamentous fungi.

1. Introduction: Life-cycle characteristics behind the impact of filamentous fungi.

Filamentous fungi are widely used in industry and medicine as a source of enzymes, antibiotics or immunosuppressants (Gutierrez-Correa et al. 2012). However, they constitute one of the most threatening groups of fungal pathogens, severely affecting animal and plant species as well as the production and quality of grain and fruits (Bebber et al. 2013; Fisher et al. 2012). Filamentous fungi grow fast and efficiently on a variety of substrates and their genomes also encode information for disseminating and responding to environmental perturbations (Adams et al. 1998).

The cell-type that best represents these traits is the vegetative hypha. After its deposition on a substrate and a period of isotropic growth, a spore from the model fungus *Aspergillus nidulans* establishes a polarity site and germinates (Figure 1A). Spore germination is subjected to environmental determinants such as light, conidial density or the presence of 8-carbon oxylipins (Braga et al. 2015; Herrero-Garcia et al. 2011; Rohrig et al. 2013). Spore germination and germ-tube extension generate syncytial (multinucleated) and cylindrical cells with a slightly tapered apex. Hyphae are continuously polarized and apical extension is mediated by the addition of new plasmatic membrane and cell-wall materials that are transported from distal regions to the subapex on microtubules and then to the apex on actin tracks (Riquelme 2013; Takeshita et al. 2014). Hyphal growth and branching generate a complex network of interconnected cells called mycelium, which constitutes the main structure of the invasive phase (Figure 1A). A combination of external (air, light or stress) and internal (metabolites) cues causes in *A. nidulans* the induction of asexual development (Etxebeste et al. 2010; Rodriguez-Urra et al. 2012), resulting in the production of structures called conidiophores (Figure 1B; see below). Each conidiophore bears thousands of asexual spores or conidia, metabolically latent mitospores characterized by

low water content, arrested metabolism and the presence of mycotoxins (Adams et al. 1998). Conidia production is a key step of fungal infection because it permits dispersal and the rapid propagation of mycoses (Gregory 1966). The numerous mitotic divisions required for conidia production have also been linked to an increased mutation supply and, thus, the emergence of strains resistant to fungicides (Zhang et al. 2015). *Aspergillus nidulans* in particular and filamentous fungi in general are also used as model organisms for the study of the mechanisms that control cell reprogramming and specialization in eukaryotes. Due to the relevance of asexual reproduction as a developmental program and its importance in the dissemination of mycoses, this review will summarize the knowledge on the signal transduction mechanism that genetically reprograms *A. nidulans* hyphae from the invasive (vegetative) to the dispersion (conidiation) mode.

2. A combination of growth and budding events drives conidiophore development in *Aspergillus nidulans*.

Apparently, vegetative growth and conidiation are sequential and mutually exclusive processes separated in time by the reception and transduction of the induction signal. However, a detailed analysis of conidiation shows that its control is inexorably dependent on polar growth. During conidiophore development apical growth events are combined with budding processes (Mims et al. 1988) (Figure 1B). A thick-walled cell or foot-cell is initially generated at hyphal compartments distal from the growing tip. The foot-cell constitutes the mainstay of conidiophores. A branch called stalk emerges from the foot-cell and elongates through apical extension until its tip begins to swell, forming the vesicle. After a series of nuclear divisions, a massive multipolar budding at the dome of the vesicle generates metulae, uninucleated sterigmata which grow apically until they bud into phialides. This cell-type gives rise to long chains of conidia. The

generation of as many as sixty metulae from a single vesicle, budding of each one into two phialides and the production of up to a hundred conidia by each phialide permits the synthesis of more than 10.000 conidia per conidiophore, multiplicating the probability of a successful dissemination.

The metula is probably the conidiophore cell-type that best represents the requirement of polarity during conidiophore formation. Although they normally bud into phialides, a reversion of the conditions that initially induced conidiophore development aborts metula-to-phialide transition, resulting in a miss-scheduled apical lengthening and the production of hyphae-like structures (Etxebeste et al. 2009). This observation demonstrated that the activation of conidiophore development is reversible and that the process is completed if the conditions that induced it prevail. The mechanism that executes budding or hyphal-like extension of metulae is completely unknown and revisiting *A. nidulans* mutants failing in metulae emergence, elongation or budding, such as *abaA* or *hymA* (Karos and Fischer 1996; Sewall et al. 1990) could provide key information. Some aberrant-metulae phenotypes correspond to mutations in genes coding for known regulators of polarity such as the Ras-GAP protein GapA, the polarisome component BemA or the GTPase RacA (Harispe et al. 2008; Leeder and Turner 2008; Virag et al. 2007). An analysis of the interaction partners of these proteins in vegetative hyphae and metulae or the comparison of the transcriptional/proteomic profiles between hyphae and dissected metulae could lead to the identification of transcripts/proteins differentially expressed and participating in the decision of budding metulae into phialides. The adaptation to *A. nidulans* of advanced proteomic and transcriptomic procedures such as those developed by Shi and colleagues or Teichert and coworkers can be useful in this approach (Shi et al. 2015; Teichert et al. 2012).

3. Apical control of conidiation and tip-to-nucleus communication in vegetative hyphae.

The morphological transformations described above are regulated at the molecular level by two pathways. To control the synthesis of the conidiophore cell-types, genes from the central developmental pathway (CDP) establish multilayer regulatory mechanisms (Adams et al. 1998; Park and Yu 2012). *brlA* is the first CDP gene, coding for a C2H2 TF and illustrating the complex genetic and molecular control required for building such a specific structure as the conidiophore (Figure 1B). Several transcription factors (TF) bind *brlA* promoter and the presence of an upstream open reading frame (uORF) leads to two possible transcripts, *brlAα* and *brlAβ*, each one being required at different developmental stages (Fischer and Kues 2006; Han et al. 1993). Feedback regulatory loops on *brlA* mediated by CDP TFs have also been described, refining the expression and activity of BrlA isoforms and informing about the completion of the process (Aguirre 1993; Ni and Yu 2007).

Since conidiation proceeds when environmental conditions inducing it predominate, a mechanism must guarantee that those signals are correctly transduced to *brlA* promoter. Upstream developmental activators (UDAs) perform this role and, thus, loss-of-function mutations in UDA genes yield undifferentiated masses of vegetative hyphae forming cotton-like colonies designated as *fluffy* (Cortese et al. 2011; Etxebeste et al. 2010; Wieser et al. 1994; Wieser et al. 1997) (Figure 1B and 1C). This aconidial phenotype is caused by a low and delayed expression of *brlA*. The application of current molecular and cellular biology techniques has furthered the establishment of a temporal framework for important UDA proteins and the assignment of specific subcellular compartments to their activity. In addition, it has shown that those UDAs take advantage of the polar growth machinery to perform their signaling and regulatory roles

from the very beginning of the vegetative stage (Etxebeste et al. 2009; Herrero-Garcia et al. 2015).

FlbB is a bZIP (basic leucine zipper)-type UDA TF essential for the induction of conidiation and constitutes the first known example of a TF locating at the tip of filamentous fungal hyphae (Etxebeste et al. 2008). Once polarity is established, FlbB accumulates at the tip of germlings, apically to the region of endocytosis and independently to the cell-cycle phase (Etxebeste et al. 2009; Perez-de-Nanclares-Arregi and Etxebeste 2014). The presence of FlbB also at the tip of growing metulae demonstrates its tight relationship with polarity. In mature vegetative hyphae, FlbB is detected in nuclei, with the highest concentration in the apical nucleus and steadily decreasing quantities in successive nuclei (Etxebeste et al. 2008). The selective green-to-red photo-conversion of the apical pool of an FlbB::Dendra2 chimera (Etxebeste and Takeshita 2015; Perez-de-Nanclares-Arregi and Etxebeste 2014) and the subsequent detection of red fluorescence in nuclei demonstrated a tip-to-nucleus migration of FlbB (Herrero-Garcia et al. 2015). Therefore, apical localization is a pre-requisite for FlbB to become transcriptionally competent and efficiently induce *brlA* expression in nuclei (Momany 2015). Otherwise, conidiation is inhibited.

Different elements are required to target FlbB to its initial destination: the hyphal apex. Actin cytoskeleton plays an essential role in the transport from the subapex to the apex (Herrero-Garcia et al. 2015). The myosin molecular motor or additional proteins assisting this transport are completely unknown and proteomic approaches should be used in the future for their identification (number 1 in Figure 2). How FlbB reaches the subapex is an additional intriguing question since the addition of benomyl, a microtubule (MT)-destabilizing drug, to the growth medium does not inhibit its apical accumulation (Figure 2). MT-based anterograde transport of vesicles is the

main mechanism for feeding the subapex with plasmatic membrane and cell-wall materials, which are subsequently transported on actin filaments to the plasmatic membrane at the apex (Riquelme 2013). It could be hypothesized that FlbB could travel on a specific subpopulation of MTs less affected by benomyl addition, or that an FlbB subpopulation synthesized close to the tip could join directly actin patches (Manck et al. 2015). However, the latter possibility would imply the transport of the *flbB* mRNA to a region close to the tip and it has been described that polarized transport of mRNAs in filamentous fungi and their localized or on-the-move translation are associated with endosome movement, which depends on MTs (Jansen et al. 2014).

Apical accumulation of FlbB depends on the bZIP transcriptional regulatory domain and the most C-terminal region (Herrero-Garcia et al. 2015). A fully conserved cysteine residue within the C-terminus apparently contributes to the acquisition of the three-dimensional structure required for anterograde transport. The presence within the central region of four conserved cysteine residues suggests that the C-terminal cysteine could determine the spatial conformation of FlbB through the formation of disulfide bonds with them, as occurs with Pap1, a TF that signals oxidative stress in *Schizosaccharomyces pombe* (Castillo et al. 2002). This possibility requires further analyses.

Dimerization domains within bZIPs mediate the formation of complexes with other bZIP- or non-bZIP proteins, regulating its subcellular localization and activity (Schutze et al. 2008). The dimerization domain of FlbB is sufficient to establish an interaction with a second UDA protein, FlbE (Garzia et al. 2009; Herrero-Garcia et al. 2015; Wieser et al. 1994). The absence of FlbE activity inhibits the apical accumulation of FlbB, dispersing it in the cytoplasm. Compared to the subapical accumulation observed when actin cables are destabilized, these results strongly suggest that FlbE not

only stabilizes FlbB at the tip but assist it in the anterograde transport to the apex (Herrero-Garcia et al. 2015) (Figure 2). Bioinformatic analyses on FlbE and its orthologs (Cortese et al. 2011) revealed the presence of conserved regions predictably involved in protein-protein interactions, post-translational modifications, targeting to subcellular compartments and signal recognition (our unpublished results). The determination of the role of these domains in FlbB/E transport and localization, their interaction, as well as the ability to induce conidiation will provide key information on how filamentous fungi locate proteins involved in signal transduction at the tip.

Although speculative, it is tempting to hypothesize with the reception of a signal at the apex, which would cause the modification and release of FlbB (number 2 in Figure 2). Proteins mediating the tip-to-nucleus migration of FlbB are completely unknown and proteomic approaches should be used for their identification (number 3 in Figure 2; see also next section). Nuclear accumulation of FlbB requires a bipartite nuclear localization signal (NLS) located close to the bZIP domain (Herrero-Garcia et al. 2015). Once in the nucleus, FlbB firstly induces the expression of a second UDA TF known as FlbD (Garzia et al. 2010). FlbD activity is essential for the activation of *brlA* expression and also for the completion of specific stages of sexual development (Arratia-Quijada et al. 2012; Garzia et al. 2010). Secondly, FlbB and FlbD bind to target sequences located in a region of 300nts within *brlA* promoter (number 4 in Figure 2) and the loss of FlbB-binding in the absence of FlbD activity suggests that both TFs cooperate in the transcriptional regulation of *brlA* expression (Garzia et al. 2010). However, the exact number and nature of target sequences within *brlA* promoter have not been determined yet. It is worth mentioning that these protein-DNA binding assays were performed using vegetative samples (absence of conidiation). This means that, under non-inducing conditions, UDAs are able to bind *brlA* promoter but that the

configuration of this transcriptional complex cannot trigger conidiation. Consequently, a dynamic functional relationship between FlbB and FlbD, modifications at the chromatin level and the requirement of additional proteins for *brlA* induction have been proposed (Canovas et al. 2014; Kwon et al. 2010).

The absence of the transcriptional activity of FlbB influences additional cellular processes, such as the expression of secondary metabolism genes or the modulation of the timing between sexual and asexual reproductive cycles (Oiartzabal-Arano et al. 2015). Multiple studies have demonstrated that the balance between sexual and asexual development is controlled at different levels and through diverse mechanisms, such as the VeA complex (light reception and secondary metabolism), NsdD (repressor of *brlA* and activator of sexual devevelopment), OsaA and UrdA (activators of *brlA* and repressors of sex, acting downstream of VeA) or Ppo proteins (control of development by fatty-acid derivatives) (Alkahyyat et al. 2015; Bayram et al. 2010; Bayram and Braus 2012; Calvo 2008; Lee et al. 2014; Oiartzabal-Arano et al. 2015; Tsitsigiannis et al. 2005). In conclusion, filamentous fungi invest multiple genetic resources in a continuous crosstalk between developmental programs, inhibiting one of the pathways when the other is induced.

4. Concluding remarks and future prospects.

The induction of conidiation in *Aspergillus nidulans* requires a specific subcellular distribution of two of its regulators, FlbB and FlbE. Most probably, the underlying signaling and transcriptional regulatory mechanism will be sensitive to any perturbation altering their localization or affecting the directional movement of FlbB between the tip and nuclei. Two lines of research should be considered for the future. On the one hand, a deeper understanding of intracellular transport processes is demanding to decipher how signaling molecules such as FlbB are located at the tip, or

how the bZIP TF migrates to nuclei. Theoretically, any genetic defect causing a reduction in the apical accumulation of FlbB will inhibit its transcriptional activity and the ability to produce asexual spores. We hypothesize two groups of proteins participating in such mechanisms: those with a general role in the transport of cargoes (such as cytoskeletal or motor proteins) and those specific players coupling FlbB to the corresponding transport pathway (as seems to be FlbE). Mutations in proteins belonging to the first group are supposed to cause pleiotropic defects while mutations in proteins from the second group should show a decrease in conidia production without affecting polar extension (Etxebeste et al. 2009; Garzia et al. 2009). Screening of aconidial mutants in which apical FlbB was delocalized would permit the identification of genes/proteins from any of these two groups. However, pleiotropic (if not lethal) phenotypes can be anticipated for mutants from the first group and also false positives caused by mutations in UDA genes, which decrease FlbB levels in vegetative hyphae (Garzia et al. 2009; Garzia et al. 2010; Herrero-Garcia et al. 2015). A proteomic analysis is also an interesting, and probably more straightforward, procedure to identify FlbB interactors assisting its anterograde or retrograde transport.

On the other hand, a second major objective would be the analysis of the functional relationship between FlbB and FlbD. How these factors are spatially and temporally coordinated with the substantial number of TFs binding *brlA* promoter must also be clarified. For example, specific target sequences for FlbB and FlbD at *brlA* promoter should be determined as well as any modification in the regulatory mechanism caused by the binding of additional TFs or co-regulators (Spitz and Furlong 2012). A hypothetic interaction between FlbB and FlbD (preceding or following DNA binding) should also be considered. The UDA pathway may well serve as a model to discover novel aspects of transcriptional regulation mediated by bZIP and cMyb TFs.

The completion of those tasks might further, through a mechanistic comparison between *A. nidulans* and filamentous fungal pathogens, the identification of new molecular targets for the impairment of asexual development or the design of inhibitors for the chemical signals inducing it.

5. Acknowledgements.

Work at the UPV/EHU was funded by the UPV/EHU (grant EHUA15/08) and the Basque Government (grant IT599-13). Work at CIB-CSIC was funded by MINECO (BFU2012-33142). E.P-A and E.O-A hold predoctoral fellowships from UPV/EHU.

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Figure legends.

Figure 1: **Vegetative growth and asexual development in *Aspergillus nidulans***. A) Colony formation. After an initial phase of isotropic growth, a polarity-site is selected within a spore. This generates the germ-tube, which grows apically through the addition of plasma membrane and cell-wall materials at the apex. Branching generates new polarity-sites, expanding hyphae in different directions. Blue, red and green circles represent proteins mediating exocitosis and endocitosis at the tip. Grey and maroon lines represent actin and tubulin cytoskeleton, respectively. Grey arrows indicate growth direction. The picture below represents a colony of *Aspergillus nidulans*. Peripheral hyphae (young hyphae; green circle) avoid the contact with each other and grow outwards. Hyphae in the central region (old hyphae; red circle) show an increased branching rate and do not avoid contact. Conidiophore development is also induced in the central region and is promoted by specific signals. B) Genetic control and morphological transformations leading to conidiophore formation. The UDA pathway transduces environmental signals to *brlA*, inducing the CDP pathway, which controls the formation of the vesicle, metulae, phialides and finally conidia (yellow and green color for young and mature conidia, respectively). Grey arrows indicate growth direction. F, S, V, M, P and C indicate foot-cell, stalk, vesicle, metulae, phialides and conidia, respectively. C) The aconidial *flb* (*fluffy*, low-*brlA*) phenotype. Left: Phenotype of wild-type, Δ*flbB*, Δ*flbE* and Δ*flbD* strains in solid minimal medium after 72 hours of culture at 37 ºC. Green color of the wild-type colony indicates the production of conidia. The cottony appearance and white color of mutant colonies indicate the absence of asexual development. Scale bar = 2 cm. Right: Northern-blot showing the expression level of *brlA* in wild-type, Δ*flbB* and Δ*flbE* strains 0, 6, 12, 24 and 48 hours

after the induction of conidiophore development. rRNA was used as loading control. Reproduced from (Garzia et al. 2009) with permission.

Figure 2: FlbB dynamics and transcriptional activity in vegetative hyphae. Numbers indicate the analyses suggested in the main text: 1) and 3) identification and characterization of proteins that mediate anterograde and retrograde migration of FlbB; 2) identification of a hypothetic post-translational modification of FlbB related to an unknown signal; and 4) elucidation of the transcriptional regulatory mechanism mediated by FlbB and FlbD at the promoter of *brlA*.

Figure 1

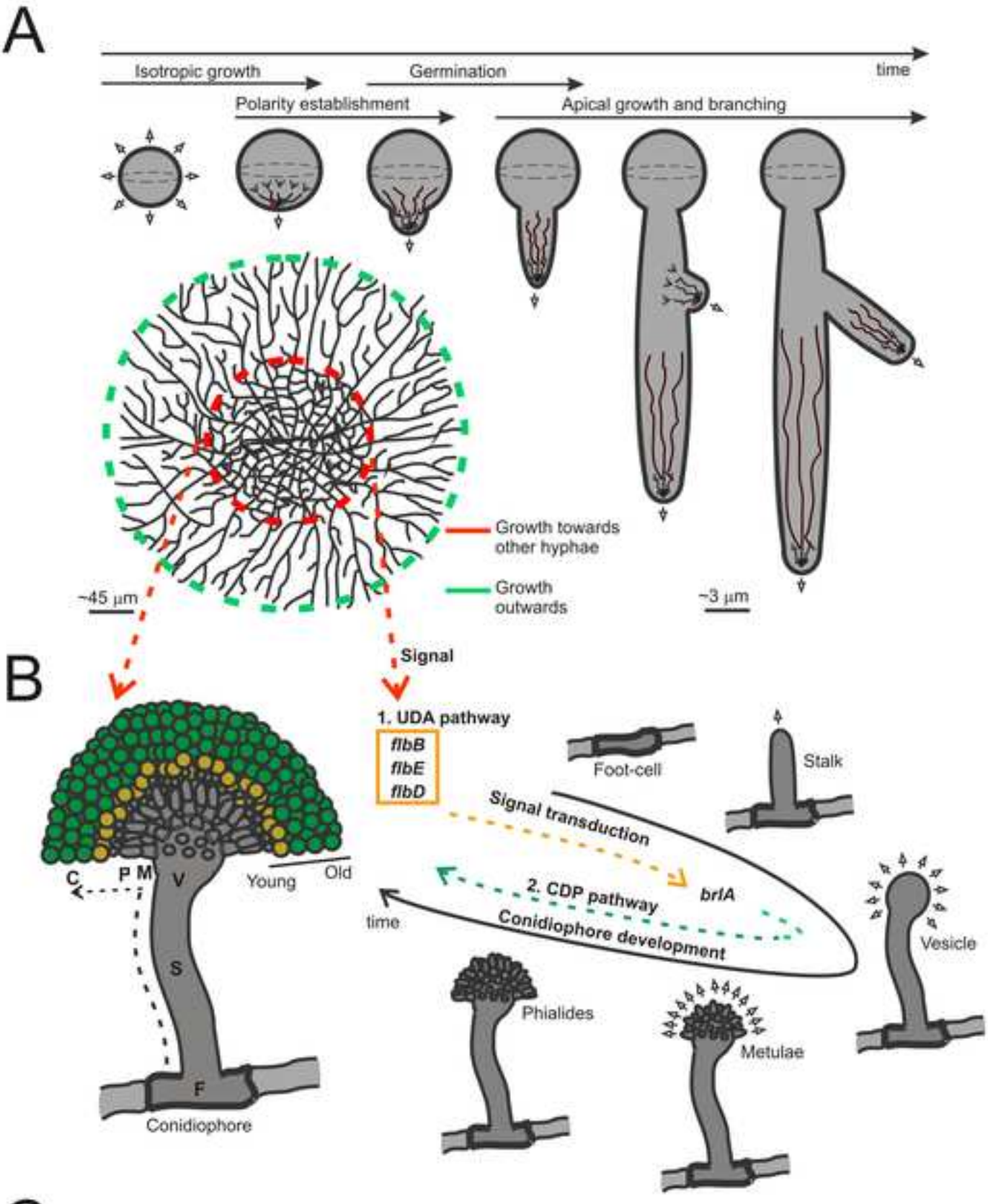

Figure 2

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