NEURONS SHOW THE PATH: TIP-TO-NUCLEUS COMMUNICATION IN FILAMENTOUS FUNGAL DEVELOPMENT AND PATHOGENESIS.

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Running title: Polarity site-to-nucleus communication in neurons and hyphae

One-sentence summary: This comprehensive review compares polarity site-to-nucleus signaling mechanisms of neurons and hyphae, and highlights the importance of long-distance communication in the control of fungal development, stress response and pathogenicity.

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Abstract.

Multiple fungal species penetrate substrates and accomplish host invasion through the fast, permanent and unidirectional extension of filamentous cells known as hyphae. Polar growth of hyphae results, however, in a significant increase in the distance between the polarity site, which also receives the earliest information about ambient conditions, and nuclei, where adaptive responses are executed. Recent studies demonstrate that these long distances are overcome by signal transduction pathways which convey sensory information from the polarity site to nuclei, controlling development and pathogenesis. The present review compares the striking connections of the mechanisms for long-distance communication in hyphae with those from neurons, and discusses the importance of their study in order to understand invasion and dissemination processes of filamentous fungi, and design strategies for developmental control in the future.

Introduction: A need for long-distance communication in polarly growing cells.

Specific cell-types program gene expression in order to extend exclusively in one direction, a property known as polar growth (Sanati and Geitmann 2013). Polar extension can be transient, ranging from short-term polarization during budding in *Saccharomyces cerevisiae* to long-term in neurons during axon guidance, or permanent as in vegetative hyphae of filamentous fungi (Thompson 2013). Nevertheless, prolonged polar growth also imposes remarkable cellular restrictions. The main drawback is a significant increase in the distance between the polarity site, the first region of the cell in penetrating a substrate and prospecting the new environment (Dudanova and Klein 2013; Harris 2009), and the nucleus, where genetic programs are controlled. This cellular architecture has forced the development of sophisticated mechanisms for long-distance communication between the polarity site and the nucleus (Saito and Cavalli 2015). In neurons these mechanisms must overcome distances that range from micrometers to over a meter in large mammals (Rishal, Kam, Perry et al. 2012). In hyphae, the distance between the polarity site, called the tip, and the closest nucleus is in the micrometer range, as for example, an average of 22.0 ± 2.0 μm in hyphae of the corn pathogen *Ustilago maydis* (Bielska, Higuchi, Schuster et al. 2014), 12 μm in hyphae of the model sordariomycete *Neurospora crassa* (Ramos-Garcia, Roberson, Freitag et al. 2009) or 11.0 ± 2.8 μm in hyphae of the model ascomycete *Aspergillus nidulans* (our unpublished results; n = 52; Figure 1). Those distances cannot be overcome simply by diffusion and energy-requiring mechanisms have been developed.

Recent evidence strongly suggests that neurons and hyphae not only share multiple players mediating polar extension, but also general characteristics of the

information pathways that connect polarity sites with nuclei. In hyphae, those mechanisms control key processes such as development, stress response and pathogenesis. The present review focuses on the comparison of growth and polarity site-to-nucleus communication mechanisms in these two cell-types. Despite the obvious differences associated to their evolutionary distance, there are outstanding similarities that raise a provocative question: can neurons serve as a model for the study of tip-to-nucleus communication in hyphae?

Hyphae and neurons: Polar growth serves different functions.

Polar extension enables the generation of different structures and the fulfillment of diverse functions, such as the connection of different regions within a tissue, organ or an organism, acquisition and distribution of nutrients, structural roles, penetration of a host or the delivery of enzymes or chemicals (Sanati and Geitmann 2013). Neurons have developed a highly polarized shape to mediate communication, with structurally and functionally different processes called axons and dendrites, which arise from a mononuclear cell-body or soma (box 1; Figure 2). In filamentous fungi, the main goal of hyphae is to colonize a substrate. With this aim they form a supra-structure known as mycelium. As in plants or metazoans, cells within a mycelium are interconnected and organized in a network (Ugalde and Rodriguez-Urra 2014). But how is a mycelium shaped and, mainly, how does polar growth enable substrate colonization?

The infection/colonization cycle begins with the deposition of a spore on a substrate (Figure 3A). Under appropriate environmental conditions, a polarity site is established within the spore and a germ-tube emerges (Momany 2002). Some fungal pathogens generate initial invasion structures from germ-tubes, such as the appressorium of *Magnaporthe oryzae* in rice or *U. maydis* in corn (Castanheira,

Mielnichuk, and Perez-Martin 2014; Wilson and Talbot 2009). The appressorium attaches tightly to the surface of the substrate and generates a penetration peg that enters the host using turgor pressure. This structure elongates by the addition of new plasma membrane and cell-wall materials to the growing apex (see mechanism below), giving rise to cylinders with a slightly tapered apex: vegetative hyphae. There is a remarkable variability in hyphal organization and extension rates among filamentous fungal species. Usually, hyphae are multinucleated structures with either a highly ordered and almost regular distribution of nuclei, such as in *A. nidulans*, or a random distribution of nuclei as in *N. crassa* (Takeshita, Manck, Grun et al. 2014). In some filamentous fungi such as *U. maydis* hyphae are mononuclear. Filamentous fungi also form septa (Figure 3B), initially open but subsequently closed rings that separate cells within a hypha (Bleichrodt, Hulsman, Wosten et al. 2015). Apical (from the tip) or lateral (from subapical or distal regions) branch formation increases the surface area of the colony (Harris 2008; Riquelme and Bartnicki-Garcia 2004). Branching and fusion of compartments from different hyphae through a process called anastomosis (Figure 3C) generate the mycelium (Roca, Read, and Wheals 2005).

As occurs in neurons (see below), hyphal growth direction can be modified in response to external cues (Figure 3D). The ability to modify hyphal orientation constitutes a key feature of fungal pathogens (Brand and Gow 2012). For example, chemotropism, the growth in response to chemical signals, drives fungus-plant interactions (Turra and Di Pietro 2015). Turrá and colleagues have described that the activity of peroxidases secreted by tomato plants act as chemoattractants for the pathogen *Fusarium oxysporum* (Turra, El Ghalid, Rossi et al. 2015), which is complemented with the secretion by the fungus of plant alkalinizing peptides that increase infection (Masachis, Segorbe, Turrá et al. 2016). *N. crassa* shows

109 thigmotrophism, the ability to respond to a topographical stimulus by altering its axis of
110 growth (Stephenson, Gow, Davidson et al. 2014). Brand and colleagues described the
111 relationship among galvanotropism, the directional growth of an organism in response
112 to an electrical stimulus, and Ca^{+2} in *Candida albicans* hyphae (Brand, Morrison, Milne
113 et al. 2014). Besides the ability to modify growth direction in response to external
114 signals, hyphae retain the potential to reprogram gene expression and develop into
115 asexual and sexual reproductive structures (Fischer and Kües 2006; Pöggeler,
116 Nowrousian, and Kück 2006). Sexual reproduction is linked with long-term survival
117 and genetic exchange while asexual spores are the main mechanism for dissemination of
118 mycoses caused by filamentous fungi (Adams, Wieser, and Yu 1998; Fischer and Kües
119 2006; Todd, Davis, and Hynes 2007b; Todd, Davis, and Hynes 2007a). The deposition
120 of those spores on a new host initiates a new infection/colonization cycle (Figure 3A).

121 **Different functions but mimicked mechanisms: growth-cone and tip extension in**
122 **neurons and hyphae.**

123 Despite the radically divergent functions of neurons and hyphae, both cell-types
124 share a strikingly similar distribution of cytoskeletal and motor proteins, causing an
125 equivalent directionality of vesicle trafficking. The main players mediating exo- and
126 endocytosis at the polarity site not only enable cell extension but generate an
127 asymmetric accumulation of signaling proteins there. This section will compare the
128 morphology of neuronal and hyphal polarity sites as well as the sophisticated molecular
129 mechanisms controlling their extension and dynamics.

130 *Orchestrating growth-cone extension and guidance*.

131 A common characteristic of all elongating axons looking for their targets is the
132 presence at the tip of a dynamic structure controlling extension: the growth-cone

(Lowery and Van Vactor 2009) (Figure 4A). Growth-cones continuously protrude and withdraw finger-like filopodia and broad lamellipodia from the actin filament-rich peripheral region or P-domain (Gomez and Letourneau 2014). These protrusions bear membrane receptors at their tips and thus can sample the environment for the presence of guidance cues. Filopodia and lamellipodia stabilization after binding to the extracellular matrix (Figure 4B) induces F-actin polymerization, which is assisted by actin binding proteins or Abps (Dent, Gupton, and Gertler 2011; Gomez and Letourneau 2014) and results in lamellipodia and filopodia extending the leading edge of the growth-cone (Lowery and Van Vactor 2009) (Figure 4C, number 1). Then, actin clears from the corridor between the adhesion (P) and the central (C) domains, increasing the space between them, also called transition or T-zone (Figure 4C, number 2). Assisted by additional actin structures, actin bundles and actomyosin contractile structures commonly referred to as actin arcs, microtubules (MTs) from the C-domain invade the T-zone, advancing the new C-domain (Schaefer, Schoonderwoert, Ji et al. 2008) (Figure 4D, number 3). Finally, MTs at the growth-cone neck are compacted, stabilizing a new segment of the axon shaft (Figure 4E, number 4).

Reciprocal interactions between actin and tubulin filaments are key for axon specification, guidance and elongation (Dent and Gertler 2003). Besides the role of actin arcs within the C-domain in enabling the advance of MTs into the T-domain, C-domain MTs generate extensions that enter filopodia and interact with F-actin bundles (Lowery and Van Vactor 2009; Tanaka and Kirschner 1991; Tanaka, Ho, and Kirschner 1995). Rho family GTPases regulate the crosstalk between both cytoskeletons (Conde and Caceres 2009) and the interactions are stabilized by actin/MT crosslinking proteins (Dent, Gupton, and Gertler 2011).

Motor proteins mediate the transport of exocytic and endocytic vesicles along actin or MT filaments (Cosker and Segal 2014). Axonal MTs are all oriented with the plus ends pointing to the growth cone (van den Berg and Hoogenraad 2012). Consequently, kinesins transport vesicles toward axon terminals while dynein mediates retrograde movement of axonal cargoes. There are more than 45 distinct kinesin genes, showing a high selectivity with regard to cargo, while only a single dynein gene product, Dnhc1, shows retrograde transport activity (Vale 2003). The interaction of Dnhc1 with different intermediate and light chain gene products allows the formation of motor complexes with different cargo selectivity (Cosker and Segal 2014). Overall, anterograde transport occurs firstly along axonal MTs and when the kinesin motor is detached from the MT, myosin V on the cargo engages F-actin and enables the short-range transport along the P-domain of growth cones (Bridgman 2004; Evans and Bridgman 1995; van den Berg and Hoogenraad 2012).

Exocytosis is mediated preferentially by SNARE proteins, which can be present in vesicles (v-SNARE) and/or target plasma membrane (t-SNARE) (Kasai, Takahashi, and Tokumaru 2012), and the exocyst complex, which tethers exocytic vesicles beneath the plasma membrane before SNARE-mediated fusion (Dupraz, Grassi, Bernis et al. 2009; Fujita, Koinuma, Yasuda et al. 2013). Clathrin-mediated endocytosis (vesicle size of approximately 100nm) and macropinocytosis (larger vesicles, 0.5-5.0 μm in diameter) are the two main endocytic pathways described in neuronal growth cones (Tojima and Kamiguchi 2015).

Functional cargos of exocytic and endocytic vesicles can be trophic factors, neurotransmitters, receptors for trophic factors and guidance cues, cell adhesion molecules or extracellular proteinases (Tojima and Kamiguchi 2015). The anterograde and retrograde transport of those vesicles is mediated by Rab GTPases. Specific types

of Rabs mediate trafficking of exocytic vesicles, thus promoting axon outgrowth (Nakazawa, Sada, Toriyama et al. 2012; Villarroel-Campos, Gastaldi, Conde et al. 2014). Other subpopulations, such as Rab21 or Rab5, control the incorporation of endocytosed materials into early endosomes (EE) and their transference to late endosomes (marked by Rab7) and lysosomes, where are degraded. Alternatively, materials are recycled to trans-Golgi networks and re-inserted into the plasma membrane for reuse (Burd and Cullen 2014; Villarroel-Campos, Gastaldi, Conde et al. 2014).

Tojima and Kamiguchi proposed a minimalistic model to explain the role of exo and endocytosis in axon guidance (Tojima and Kamiguchi 2015). The presence of an extracellular attractive cue as a gradient would promote exocytosis at the side of the growth-cone in contact with the highest concentration of the cue, driving attractive turning. On the contrary, a gradient of an extracellular repulsive cue would promote endocytosis at the growth-cone side with the highest concentration of the cue, inhibiting motility on this side and resulting in repulsive turning.

Optimization of vesicle traffic: exo- and endocytosis in hyphal tip elongation.

Tip extension requires polarization of the machinery controlling growth, with a highly specific distribution and dynamics of each element at apical and subapical compartments (Figure 5). In contrast to animal cells, including neurons, hyphae contain a cell wall composed of polysaccharides (glucans and chitin) and glycoproteins (Osherov and Yarden 2010). Thus, plasma membrane and cell wall materials as well as the enzymes required for their polymerization and processing must be transported to the active growing region of a hypha: the apex of the tip. Furthermore, cell-wall composition is dynamic and is modified when, as a consequence of growth, apical

206 regions become subapical (Riquelme 2013). Also a variety of compounds and proteins
207 have been described to be secreted at the tip of hyphae. Among these are effector
208 proteins weakening host defenses and mediating pathogenesis, or proteins involved in
209 the synthesis of secondary metabolites, chemical compounds conferring a variety of
210 survival functions such as protection from stress or signaling of development (Keller
211 2015; Lim, Ames, Walsh et al. 2014; Rafiqi, Ellis, Ludowici et al. 2012).

212 As in neurons, MT and actin cytoskeletons are differentially located within a
213 hypha (Figure 5). In the region close to the tip MTs orient plus ends in the growth
214 direction, although the opposite orientation can also be observed in MT subpopulations
215 of some filamentous fungal species (Egan, McClintock, and Reck-Peterson 2012).
216 Growing plus ends of MTs reach and usually converge at the subapex, although some of
217 them can reach the apex (Takeshita, Manck, Grun et al. 2014). This subpopulation of
218 MTs has been proposed to mediate the transport of cell-end markers to the hyphal apex,
219 determining growth directionality and purportedly enabling actin filament formation
220 (Ishitsuka, Savage, Li et al. 2015; Takeshita, Higashitsuji, Konzack et al. 2008). MTs
221 are not strictly required for polarized growth but agents altering their stability cause a
222 significant decrease in the growth pace (Horio and Oakley 2005).

223 A set of approximately 10 kinesins controls transport of cargos towards plus
224 ends of MTs (Schoch, Aist, Yoder et al. 2003; Zekert and Fischer 2009), a significantly
225 lower number compared to the more than 45 kinesins in human cells (see above). This
226 difference probably reflects the lower complexity level of a fungal cell. After being
227 recruited to MT plus ends, the single dynein motor complex controls transport of cargos
228 towards minus ends, which coincides with a basipetal transport from the tip to distal
229 regions. The identification of constituents of the dynein complex, the characterization of

their dynamics and the mechanism for cargo loading have been intensely studied in filamentous fungal models during the last twenty five years and have been exhaustively covered in recent research papers and reviews (Bielska, Schuster, Roger et al. 2014; Cianfrocco, DeSantis, Leschziner et al. 2015; Xiang, Qiu, Yao et al. 2015; Yao, Arst, Wang et al. 2015; Zhang, Qiu, Arst et al. 2014).

Regulated by a set of Abps, actin can form three types of macromolecular structures within hyphae: rings for septum formation, actin patches and actin filaments (Berepiki, Lichius, and Read 2011; Lichius, Berepiki, and Read 2011). Actin patches accumulate at the subapical region of the hyphal tip, commonly known as subapical endocytic ring or dynein loading zone, and co-localize with the endocytic machinery (Araujo-Bazan, Penalva, and Espeso 2008; Taheri-Talesh, Horio, Araujo-Bazan et al. 2008; Upadhyay and Shaw 2008) (Figure 5). The elusive actin cables nucleate from formin SepA and pave the way for the short, myosin V-dependent anterograde trafficking of vesicles and cargos between the subapex and the apex (Schultzhaus, Quintanilla, Hilton et al. 2016; Sharpless and Harris 2002; Taheri-Talesh, Xiong, and Oakley 2012). It has been suggested recently that MTs are captured at hyphal tips and pulled along actin filaments through the microtubule guidance protein MigA, which interacts with myosin V and probably enables trafficking towards the apex (Manck, Ishitsuka, Herrero et al. 2015).

Cargos processed and matured within the endoplasmic reticulum (ER)-Golgi network, which is also polarized in hyphae (Markina-Inarrairaegui, Pantazopoulou, Espeso et al. 2013; Pantazopoulou and Penalva 2011; Pinar, Pantazopoulou, Arst, Jr. et al. 2013; Pinar, Arst, Jr., Pantazopoulou et al. 2015), are internalized in exocytic carriers that transit on MTs until they are purportedly transferred to actin filaments

(Pantazopoulou, Pinar, Xiang et al. 2014). The fusion of exocytic vesicles with the membrane at the apex occurs at an enormous rate and is spatially and temporally controlled by a pleomorphic structure interleaved with actin filaments and known as *Spitzenkörper* (Spk) (Riquelme and Sanchez-Leon 2014), which literally means "apical body". The Spk receives exocytic vesicles of different size (Hohmann-Marriott, Uchida, van de Meene et al. 2006; Verdin, Bartnicki-Garcia, and Riquelme 2009) and, apparently, different lipid composition (Schultzhaus, Yan, and Shaw 2015), and synchronizes their delivery to and fusion with the plasma membrane (Figure 5). An increasing amount of information on the sequence of molecular events enabling apical loading of membrane and cell-wall materials has been made available during the last years and involves the participation of multiple proteins and protein complexes such as Rho and RabGTPases, v- and t-SNARE-s as well as polarisome and exocyst complexes (see references within the review by (Schultzhaus and Shaw 2015)).

Besides actin patches, endocytosis of materials at the subapical ring requires the activity of the myosin I protein MyoA (McGoldrick, Gruver, and May 1995; Yamashita and May 1998). It has been suggested that endocytosis polarizes exocytosis in yeast cells (Jose, Tollis, Nair et al. 2013) and the same seems to hold true for filamentous fungi, with the endocytic collar probably causing bundling and constriction of MTs and organelles at the hyphal subapex (Markina-Inarrairaegui, Pantazopoulou, Espeso et al. 2013; Takeshita, Manck, Grun et al. 2014). The existence of clathrin-dependent and independent endocytosis mechanisms has been discussed in filamentous fungi and various proteins purportedly involved in endocytosis have been detected at the subapical ring (Araujo-Bazan, Penalva, and Espeso 2008; Epp, Nazarova, Regan et al. 2013; Schultzhaus and Shaw 2015). However, a deep characterization of the mechanisms that mediate endocytosis in hyphae requires further investigation.

Endocytosed materials can follow two paths. Proteins such as the synaptobrevin SynA, a v-SNARE, join again the exocytic pathway (Taheri-Talesh, Horio, Araujo-Bazan et al. 2008), thus coupling exocytosis and endocytosis and maintaining the high exocytosis rate required for sustaining of hyphal extension (Penalva 2010; Upadhyay and Shaw 2008). Most of the materials are incorporated into EEs, which show long-distance, MT-dependent bidirectional motility in hyphae and ride on kinesin-3 and the dynein complex (Penalva 2010). The GTPase RabA/Rab5 is commonly used as a marker for EEs (Abenza, Pantazopoulou, Rodriguez et al. 2009; Fuchs, Hause, Schuchardt et al. 2006), which mature into late endosomes, marked by RabS/Rab7 (Abenza, Galindo, Pinar et al. 2012), and vacuoles. Hyphae take great advantage of the anterograde movement of EEs, since they serve as platforms for the asymmetric localization and on-the-move translation of mRNAs (Haag, Steuten, and Feldbrugge 2015; Jansen, Niessing, Baumann et al. 2014). Recent works have shown an EE-based intracellular movement of peroxisomes and ER, suggesting that they also mediate the transport and distribution of specific organelles within hyphae (Guimaraes, Schuster, Bielska et al. 2015; Salogiannis, Egan, and Reck-Peterson 2016).

Transmitting fresh information from polarity sites to nuclei.

One of the life functions is the ability to interact with the environment, responding and adapting to its changes. An efficient adaptation requires, however, a fast and accurate transduction of external information, a process that in neurons and hyphae is challenged by the long distances between polarity sites and nuclei. As a result of the polarized exocytosis processes reviewed above, signaling proteins reach polarity sites, enabling the retrograde flow of information.

Long-distance signaling to the neuronal nucleus.

303 Vesicle trafficking generates an exclusive transcriptomic and proteomic
304 microenvironment within growth cones and synapses, with a great deal of mRNAs and
305 proteins being asymmetrically accumulated and/or translated there (Jung, Gkogkas,
306 Sonenberg et al. 2014; Maday, Twelvetrees, Moughamian et al. 2014). Many of these
307 mRNAs and proteins participate in the long-distance communication that links signal
308 reception and the nuclear control of cellular processes. This includes not only the
309 control of axon elongation and guidance but also injury signaling and the induction of
310 repair mechanisms (Rishal and Fainzilber 2014; Saito and Cavalli 2015).

311 Calcium waves are supposed to constitute the main and fastest way for
312 conveying distant signals to the nucleus, inducing an immediate transcriptional
313 regulatory response (Adams and Dudek 2005; Bading 2013). Interestingly, in the case
314 of injury-signaling, propagation of calcium waves towards the cell body correlates with
315 regenerative growth while propagation towards nerve terminals seems to correlate with
316 axon degeneration (Cho, Sloutsky, Naegle et al. 2013; Villegas, Martinez, Lillo et al.
317 2014). Furthermore, signaling through calcium waves is complemented by a slower and
318 more sustained macromolecular trafficking to the nucleus (Panayotis, Karpova, Kreutz
319 et al. 2015) (Figure 6). Two main types of signal transduction pathways have been
320 described: a) kinase-dependent cascades or b) the straight migration of locally translated
321 transcription factors (TFs) to the nucleus (Panayotis, Karpova, Kreutz et al. 2015;
322 Rishal and Fainzilber 2014). For example, the mitogen-activated protein kinase ERK
323 mediates spatial and temporal integration of synaptic signals (Karpova, Mikhaylova,
324 Bera et al. 2013; Zhai, Ark, Parra-Bueno et al. 2013). The transduction of those signals
325 requires the assembly and MT-based transport of a protein module composed of a
326 phosphorylated form of ERK, the dynein motor complex, isoforms of karyopherins α
327 and β, the NMDA-receptor synaptonuclear signaling and neuronal migration factor

328 Jacob and auxiliary proteins such as internexin or vimentin (see the review by
329 (Panayotis, Karpova, Kreutz et al. 2015)). Upon arrival into the nucleus, the signal
330 transported by the kinase module is transmitted to CREB (cAMP response element-
331 binding), a bZIP-type transcription factor controlling multiple cellular processes.
332 Depending on the phosphorylation state of Jacob upon arrival in the nucleus cell
333 survival or cell death can be activated. Additional examples of kinases involved in long-
334 distance signaling to the neuronal nucleus are p38, JNK (JUN amino-terminal) or DLK
335 (dual leucine zipper) kinases (Rishal and Fainzilber 2014).

336 Of note is the role played by importins-α and -β in the long-distance retrograde
337 transport of cargos (Hanz, Perlson, Willis et al. 2003; Thompson, Otis, Chen et al.
338 2004). This demonstrates that karyopherin activity in eukaryotes is not exclusively
339 limited to the nuclear periphery. According to the general model, importin-β is the
340 transporter while importin-α acts as a cargo adaptor (Fried and Kutay 2003). In neurons,
341 importin-β mRNA constitutes an additional example of an asymmetrically accumulated
342 transcript (Figure 6). The reception of specific cues induces its translation and the
343 formation of the signaling complex (Hanz, Perlson, Willis et al. 2003; Perry, Doron-
344 Mandel, Iavnilovitch et al. 2012). Recent reports have demonstrated the asymmetric
345 accumulation of transcriptional regulators and co-regulators or their local synthesis in
346 synapses or growth-cones (Ben-Yaakov, Dagan, Segal-Ruder et al. 2012; Cox, Hengst,
347 Gurskaya et al. 2008; Ivanova, Dirks, Montenegro-Venegas et al. 2015; Ji and Jaffrey
348 2012), constituting additional cargos for locally assembled importin complexes. The
349 importance of the TF-based axon-to-nucleus signaling mechanism will be in all
350 probability higher because multiple mRNAs coding for TFs and co-regulators are
351 differentially accumulated in axons (Ji and Jaffrey 2014).

352 *Uncovering a sensor role for the hyphal tip: tip-to-nucleus signaling and control*
353 *of cellular responses.*

354 Increasing evidence suggests that, besides sustaining polar extension, tips of
355 hyphae prospect the new environment they are colonizing, conveying the information to
356 nuclei. In a noticeable correspondence with neurons, the already known tip-to-nucleus
357 communication mechanisms are mediated by kinases/phosphatases or TFs which
358 retrogradely migrate to nuclei and control, among others, development, stress response
359 and pathogenesis (Figure 7).

360 Steinberg´s group described that EEs moved retrogradely in hyphae of the corn
361 pathogen *U. maydis* during plant infection and that the impairment of this movement
362 inhibited fungal effector production and plant infection (Bielska, Higuchi, Schuster et
363 al. 2014) (Figure 7A). Taking kinase-based long-distance communication mechanisms
364 from neurons as a reference, the authors identified Kpp4/Ubc4 (Muller, Weinzierl,
365 Brachmann et al. 2003), the ortholog of human MEK1, and Crk1 (Garrido and Perez-
366 Martin 2003), with no predicted human orthologs, as kinases occasionally or
367 permanently moving along hyphae and accumulating in the nucleus. Surprisingly, the
368 homologue of ERK1 and ERK2 (which mediated spatial and temporal integration of
369 dendritic signals in neurons; see above), Kpp2/Ubc2, did not move under these culture
370 conditions. Crk1::GFP localized to rapidly moving Rab5-positive EEs but,
371 unexpectedly, the null *crk1* mutant showed an increased effector production compared
372 to the reference strain. Consequently, the authors proposed that Crk1 is a repressor of
373 effector production and implicitly suggested that additional players should move to
374 nuclei to act as inducers.

Bayram and colleagues elucidated the composition and dynamics of a second kinase-dependent tip-to-nucleus signaling module (Bayram, Bayram, Ahmed et al. 2012). *A. nidulans* Ste7/MkkB, Ste11/SteC and Fus3/MpkB are, respectively, a MAP3K, a MAP2K and a MAPK that form a complex attached to the membrane through Ste50/SteD (Bayram, Bayram, Ahmed et al. 2012; Paoletti, Seymour, Alcocer et al. 2007; Wei, Requena, and Fischer 2003). All module components were detected at the tip of hyphae and, interestingly, could migrate simultaneously to the nuclear periphery. Only Fus3/MpkB was able to accumulate in nuclei, where it controlled the regulatory activity of VeA, a light-dependent TF balancing sexual and asexual developmental cycles as well as secondary metabolism (Bayram, Braus, Fischer et al. 2010; Calvo and Cary 2015; Rodriguez-Romero, Hedtke, Kastner et al. 2010), and Ste12/SteA, a TF required for sexual reproduction (Vallim, Miller, and Miller 2000) (Figure 7B). Thus, this kinase module couples apical signals with the nuclear control of development and secondary metabolism.

Calmodulin (CaM) is a calcium-binding messenger protein that under high calcium concentrations binds four Ca^{+2} atoms, inducing its interaction with downstream effectors (Clapham 2007; Kursula 2014). One of those effectors is the protein phosphatase complex calcineurin (Guerini 1997) (CN), which in fungi dephosphorylates Crz transcription factors (Cyert 2003; Thewes 2014). In filamentous fungi such as *A. nidulans* and *A. fumigatus*, both CaM and the catalytic subunit of CN, CnaA, localize to the tip of hyphae (Chen, Song, Cao et al. 2010; Juvvadi, Fortwendel, Pinchai et al. 2008; Juvvadi, Fortwendel, Rogg et al. 2011), while the Crz homologue CrzA moves bi-directionally between the cytoplasm and the nucleoplasm to regulate target genes under different salt or pH stress conditions (Hernandez-Ortiz and Espeso 2013; Soriani, Malavazi, Savoldi et al. 2010). By using a CrzA mutant form lacking the calcineurin-

400 binding domain, Hernández-Ortiz and Espeso delayed the pace of the nuclear import of
401 CrzA (under review). This caused a non-synchronous and transient nuclear
402 accumulation of CrzA, with apical nuclei being filled first with the TF. Overall, these
403 observations strongly suggest that the signals conveyed to nuclei through the CaM-CN-
404 CrzA pathway were originated at the tip (Figure 7C).

405 Asexual development in *A. nidulans* is induced by a set of regulators including
406 FlbB, which constitutes the first known example of a TF localizing at the tip of hyphae
407 (Etxebeste, Ni, Garzia et al. 2008). Nuclear localization is limited to apical nuclei, with
408 the highest concentration in the most apical nucleus and steadily decreasing quantities
409 in successive nuclei. A recent report clarified the relationship between apical and
410 nuclear pools of FlbB and the directionality of its movement (Figure 7D). First, FlbB
411 transport to and accumulation at the tip are mediated by actin filaments and a small
412 protein known as FlbE, which is also required for developmental induction (Garzia,
413 Etxebeste, Herrero-Garcia et al. 2009; Herrero-Garcia, Perez-de-Nanclares-Arregi,
414 Cortese et al. 2015). Photo-convertible tagging of FlbB with Dendra2 (Perez-de-
415 Nanclares-Arregi and Etxebeste 2014) showed that it migrates from the tip to nuclei and
416 the authors showed that the apical localization is a pre-requisite to become
417 transcriptionally competent and induce asexual reproduction in nuclei (Herrero-Garcia,
418 Perez-de-Nanclares-Arregi, Cortese et al. 2015; Momany 2015). FlbB controls asexual
419 development jointly with a transcription factor of the cMYB family known as FlbD,
420 establishing a model for bZIP-cMYB interactions regulating eukaryotic development
421 (Garzia, Etxebeste, Herrero-Garcia et al. 2010; Tahirov, Sato, Ichikawa-Iwata et al.
422 2002). FlbB also participates in the repression of sexual development (Oiartzabal-
423 Arano, Garzia, Gorostidi et al. 2015).

Although a direct link with any of the signaling mechanisms described above has not been established yet, importin-α and –β homologues KapA and KapB move bi-directionally between the tip and nuclei of *A. nidulans* hyphae (Etxebeste, Villarino, Markina-Inarrairaegui et al. 2013), suggesting an important role for the nuclear transport machinery in communicating these two regions. This observation may establish, however, a clear difference compared to neurons, where importin-β is locally translated far from the nucleus, triggering the assembly of the signaling complex.

Neurons show the path. Conclusions and Future prospects.

The integration of fast calcium signals with a slower macromolecular transport in neurons may offer a range of mechanisms that leads to a more consistent output and the improvement of neuronal functions (Panayotis, Karpova, Kreutz et al. 2015). Long-distance transport of macromolecules undeniably plays key roles in axon guidance and neuronal regeneration, and has been proposed that it also senses axonal length (Albus, Rishal, and Fainzilber 2013; Ibañez 2007; Panayotis, Karpova, Kreutz et al. 2015). Consequently, the study of these mechanisms has furthered a better understanding of the molecular basis of severe neuronal diseases (Saito and Cavalli 2015), paving the way for the design of strategies for their prevention or treatment. Due to the correspondence in the organization of the cytoskeleton and dynamics of molecular motors, hyphae have served as a valuable model for the study of neuronal processes and diseases. For example, the characterization of the protein NudF from the fungus *Aspergillus nidulans*, which is required for initiating dynein-driven motility, allowed the identification of its human homolog Lis1 and contributed to the understanding of lissencephaly, a neurological disease (Egan, Tan, and Reck-Peterson 2012; Morris, Efimov, and Xiang 1998). Additional studies described the functional relationship and the role in dynein-

448 mediated transport of *A. nidulans* FtsA, HookA and FhipA (Yao, Wang, and Xiang
449 2014; Zhang, Qiu, Arst et al. 2014), the orthologs of the human FTS/Hook/FHIP
450 complex proteins (Xu, Sowa, Chen et al. 2008). *A. nidulans* also served as a model for
451 the establishment of a link between dynein-mediated transport of early endosomes and
452 VezA, a vezatin-like protein (Yao, Arst, Wang et al. 2015). Vezatin was previously
453 known to be involved in neuronal functions but had never been linked to MT-based
454 transport (Yao, Arst, Wang et al. 2015). Highly accessible and reproducible laboratory
455 techniques, which allow the generation of knock-out or random and site-directed
456 mutants easily, and the avoidance of most ethical issues associated with the generation
457 of genetically modified human cell-lines should also be considered as potential benefits
458 of using hyphae as models for the study of intracellular transport mechanisms in
459 neurons.

460 The current situation in the study of tip-to-nucleus communication in hyphae is
461 clearly behind research in neurons. Due to the extreme and permanent polarization as
462 well as the centrality of polar extension in host colonization, the tip of hyphae has
463 attracted the attention of multiple research groups as the hyphal region exclusively
464 dedicated to the maintenance of growth. Although several questions remain to be
465 answered, multiple characteristics have been elucidated during the last years,
466 remarkably improving our understanding of the mechanisms that allow such a fast
467 apical growth pace. While the sensor role of growth-cones and synapses is obvious, this
468 possibility has been underestimated in hyphae. In 2009 the Spk was defined for the first
469 time as a signaling hub for the control of fungal development (Harris 2009). The
470 examples described in this review allow a refinement of this definition, including its
471 role in the control of both developmental cycles in filamentous fungi (sexual and
472 asexual), adaptation to stress conditions and effector production during pathogenesis.

The information available suggests that the mechanistic basis of those polarity site-to-nucleus communication pathways is partially conserved in neurons and hyphae, with retrogradely migrating kinases/phosphatases and transcription factors as key players in both systems. In this scenario, the information available in neurons might serve as an interesting reference for the design of future experiments in hyphae and could importantly contribute to the elucidation of the mechanisms that control the dynamics and activity of apical signaling proteins. Although long-distance communication in neurons and hyphae has been adapted to two radically different lifestyles and, consequently, include species-specific cargos, such as FlbB in hyphae, we believe that the knowledge derived from research in neurons could make major contributions at three levels. Firstly, in the determination of how signaling proteins are accumulated at the tip or are retrogradely transported to nuclei, if they are anterogradely transported as mRNAs (see below) or proteins, the possibility of their on-the-move translation or the hypothetic existence of adaptors enabling the attachment to exo- or endocytic carriers. Secondly, in the identification of the cues which trigger retrograde signaling as well as translational or post-translational effects that directly or indirectly cause the release of signaling proteins from the tip. Finally, in the investigation of the transcriptional regulatory mechanisms induced by these pathways, including the determination of targets at promoter regions, chromatin modifications or the participation of co-regulators or pioneer transcription factors.

Currently, it is difficult to assess the genuine significance of tip-to-nucleus communication in hyphae but the biological and applied impact could be remarkable. On the one hand, fundamental stages of the filamentous fungal life-cycle are controlled by those mechanisms and thus their study could make major contributions to the understanding of how filamentous fungi respond and adapt to changing environments,

undermine host defenses and disseminate to new niches. Preliminary results suggest that new examples of tip-to-nucleus communication mechanisms could arise in the future. For example, approximately 40 mRNAs have been identified to be transported to *C. albicans* hyphal tips by the RNA trafficking machinery (also called SHE machinery), six of them coding for TFs or coactivators (Elson, Noble, Solis et al. 2009). The application of laser capture microdissection coupled to transcriptomic/proteomic analyses of tips of hyphae grown under different conditions could contribute to the identification of new apical signaling proteins, despite the fact that sample volume will probably be a rate-limiting step in this experimental design. On the other hand, the narrow evolutionary distribution of some of the proteins conveying apical signals to nuclei and their important roles in the control of development could enable their assessment as potential therapeutic targets, opening an avenue for the design of advanced strategies for the containment of mycoses caused by filamentous fungi.

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Conflict of interest.

The authors declare no conflict of interest.

Box 1. Neuronal polarization and axon specification.

Neurons are considered the core components of the nervous system and form ensembles that are differentially activated to allow neural circuit functions (Yuste 2015). The axon transmits signals to dendrites of other neurons by the release of neurotransmitters (Figure 2A). Dendrites normally arise from the other side of the cell body, extending and branching to form dendritic spines and giving rise to the dendritic tree. Dendrites contain neurotransmitter receptors to receive signals that axons from other neurons release to the synaptic cleft, the gap between pre- and postsynaptic cells (Lopez-Munoz, Boya, and Alamo 2006).

The establishment of neuronal polarity requires a chain of events that include axon and dendrite specification, axon elongation and axon guidance (Polleux and Snider 2010). Axon elongation and the path followed until the neuron contacts the target is oriented by guidance cues, chemotrophic signals that act as diffusible attractants and repellants, instructing the axon which direction to grow (Gallo and Letourneau 2004; Tamariz and Varela-Echavarria 2015). Dissociated rodent hippocampal neurons served as a basic *in vitro* model for the study of neuronal polarization (Craig and Banker 1994; Dotti, Sullivan, and Banker 1988). Basically, morphological changes were divided into five stages (Figure 2B): 1) Shortly after plating, neurons retracted their processes, beginning their development from round spheres that spread filopodia. 2) Between days 0.5 and 1.5, cultured neurons form minor neurites, which alternate growth and retraction stages. 3) After 1.5-3 days of culture, one of these equivalent minor neurites grows rapidly to become an axon. 4) After 4-7 days of culture, the remaining minor neurites have developed into dendrites and 5) after more than 7 days of culture, the axon and dendrites are functionally polarized and dendritic spines are formed. *In vivo* neuronal

development has different properties depending on the cell type and developmental stage (Takano, Xu, Funahashi et al. 2015). Some neuron types inherit their polarity, with apical and basal processes that eventually develop into a dendrite and an axon, respectively (Barnes and Polleux 2009) (Figure 2C), while others establish polarity during migration and differentiation (Noctor, Martinez-Cerdeno, Ivic et al. 2004; Solecki, Govek, Tomoda et al. 2006). Axons can also branch, increasing the synaptic capacity and neuronal surface area (Winkle, McClain, Valtschanoff et al. 2014).

554 **Figure legends.**

555 **Figure 1: Tip and nuclei in hyphae.** DIC and fluorescence microscopy images of a
556 growing hypha of the model filamentous fungus *Aspergillus nidulans*. The position of
557 the tip (T; arrowhead), which controls hyphal extension, and nuclei (N; black arrows)
558 are marked using a constitutively expressed GFP chimera of the transcription factor
559 FlbB (Etxebeste, Ni, Garzia et al. 2008). The white arrow indicates growth direction and
560 the white dotted arrow marks the distance between the tip and the most apical nucleus.
561 Scale bar = 5 μm.

562 **Figure 2 (in Box 1). Neuronal polarization and axon specification.** A) General
563 structure of a neuron. Dendrites and dendritic spines, the cell-body containing a nucleus
564 and the axon (axon shaft and growth-cone) are represented. B) and C) Stages of
565 neuronal polarization for *in vitro* and *in vivo* (inherited polarity) models, respectively.
566 Modified from (Takano, Xu, Funahashi et al. 2015).

567 **Figure 3. Life-cycle of filamentous fungi and formation of the mycelium**. A) The
568 life-cycle of two filamentous fungal models, *Aspergillus nidulans* and *Magnaporthe*
569 *oryzae*, is summarized as an example. The invasive phase begins with the germination
570 of a spore (yellow background). Some filamentous fungal species generate an
571 appresorium and a penetration peg (gray background). Hyphae extend at the tip and
572 form branches, which fuse to generate a network of interconnected cells: the mycelium.
573 Changes in environmental conditions induce the generation of structures bearing
574 asexual spores (conidiophores and conidia), the main vehicle for fungal dispersion (light
575 green). The asexual phase is followed by the production of sexual structures (perithecia
576 and cleistothecia, respectively) and sexual spores (ascospores), which are related to
577 long-term survival and genetic exchange. B) Drawing of a septum, which separates cells

within a hypha. C) Representation of hyphal branching and anastomosis (hyphal fusion) processes. D) Some external signals guiding hyphal orientation (chemical stimulus or chemotropism, topographical stimulus or thigmotropism, electrical stimulus or galvanotropism, and others).

Figure 4. Mechanism for growth-cone extension and axon guidance. A) Molecular organization within the growth-cone and its turning in response to guidance cues. P- (gray), T- (purple) and C- (blue) regions are indicated, together with MTs, actin filaments, arcs and bundles, molecular motors and exocytic/endocytic vesicles. The growth-cone extends towards attractive cues, avoiding repulsive signals. Modified from (Lowery and Van Vactor 2009). B) Binding of filopodia and lamellipodia to the extracellular matrix. C) Advance of filopodia and widening of the T-zone (due to actin polymerization). D) Advance of the C-domain due to the invasion of the T-zone by MTs from the C-region. E) MTs at the growth-cone neck are compacted, stabilizing a new segment of the axon shaft.

Figure 5. Molecular organization at the hyphal tip. The region between the hyphal apex and the closest nucleus is represented. Organelles and molecular complexes included are indicated below the picture. Red arrows indicate the sense of the molecular transport. Recycled materials or vesicles originated at the endoplasmic reticulum are transported on MT tracks by kinesins, via Golgi apparatus, to the subapex. There, secretory vesicles are purportedly transferred to actin cables. Myosins mediate the transport of vesicles to the apex and the *Spitzenkörper* synchronizes their delivery and fusion with the plasma membrane. Actin patches and actin-binding proteins at the endocytic collar allow the internalization of materials. Then, dynein retrogradely

transports EEs on MT-tracks. Finally, EEs mature into late endosomes and vacuoles or are re-incorporated into the secretory pathway via recycling endosomes.

Figure 6. Simplified model for the macromolecular communication between neuronal polarity sites and the nucleus. Upon stimulation, importin-β mRNA is translated, triggering the assembly of the dynein signaling complex that will mediate the retrograde transport and nuclear import of the kinase ERK. There, the signal will be transmitted to the transcription factor CREB, which can induce cell-viability or cell-death depending on the phosphorilation state of the signaling complex. After injury, specific mRNAs coding for transcription factors are translated at the axon or at the polarity site. The importin-α/β heterodimer mediates again the assembly of a signaling complex which retrogradely transports the transcription factor to the nucleus, where it induces regeneration.

Figure 7. Tip-to-nucleus communication in hyphae. Simplified models representing the four mechanisms known to convey apical signals to hyphal nuclei: A) EE-mediated kinase migration for the control of effector production in *U. maydis* (Bielska, Higuchi, Schuster et al. 2014), B) MkkB/SteC/MpkB/SteD kinase module migration and control of secondary metabolite synthesis and sexual development in *A. nidulans* (Bayram, Bayram, Ahmed et al. 2012), C) Control of the nucleo-cytoplasmic dynamics of the TF CrzA by the CaM/CN system and the regulation of the cellular response to salt or pH stress conditions in *A. nidulans* (Hernández-Ortiz and Espeso, under review), and D) induction of asexual development by FlbB, an *A. nidulans* TF that migrates from the hyphal tip to nuclei (Herrero-Garcia, Perez-de-Nanclares-Arregi, Cortese et al. 2015).

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974

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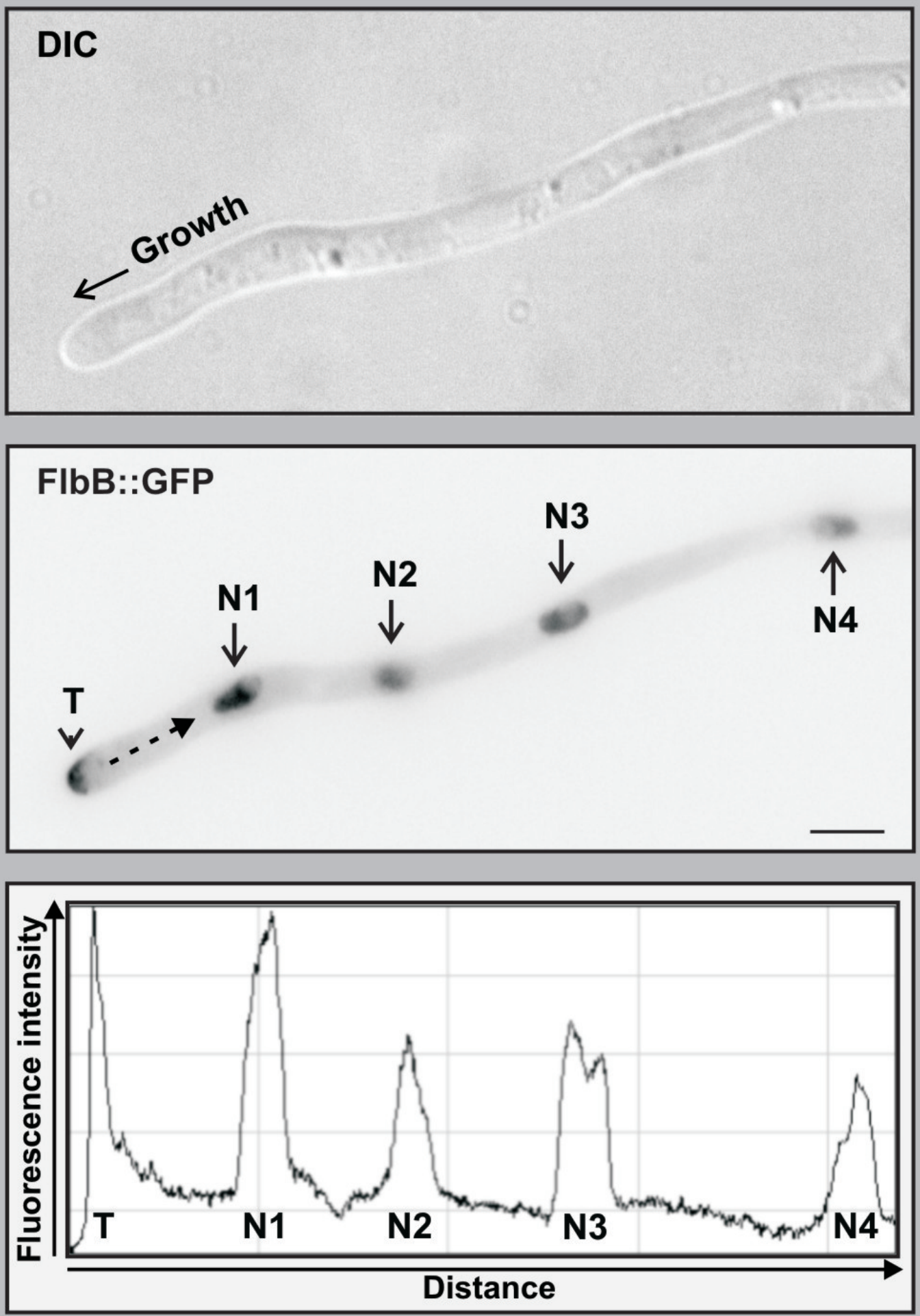
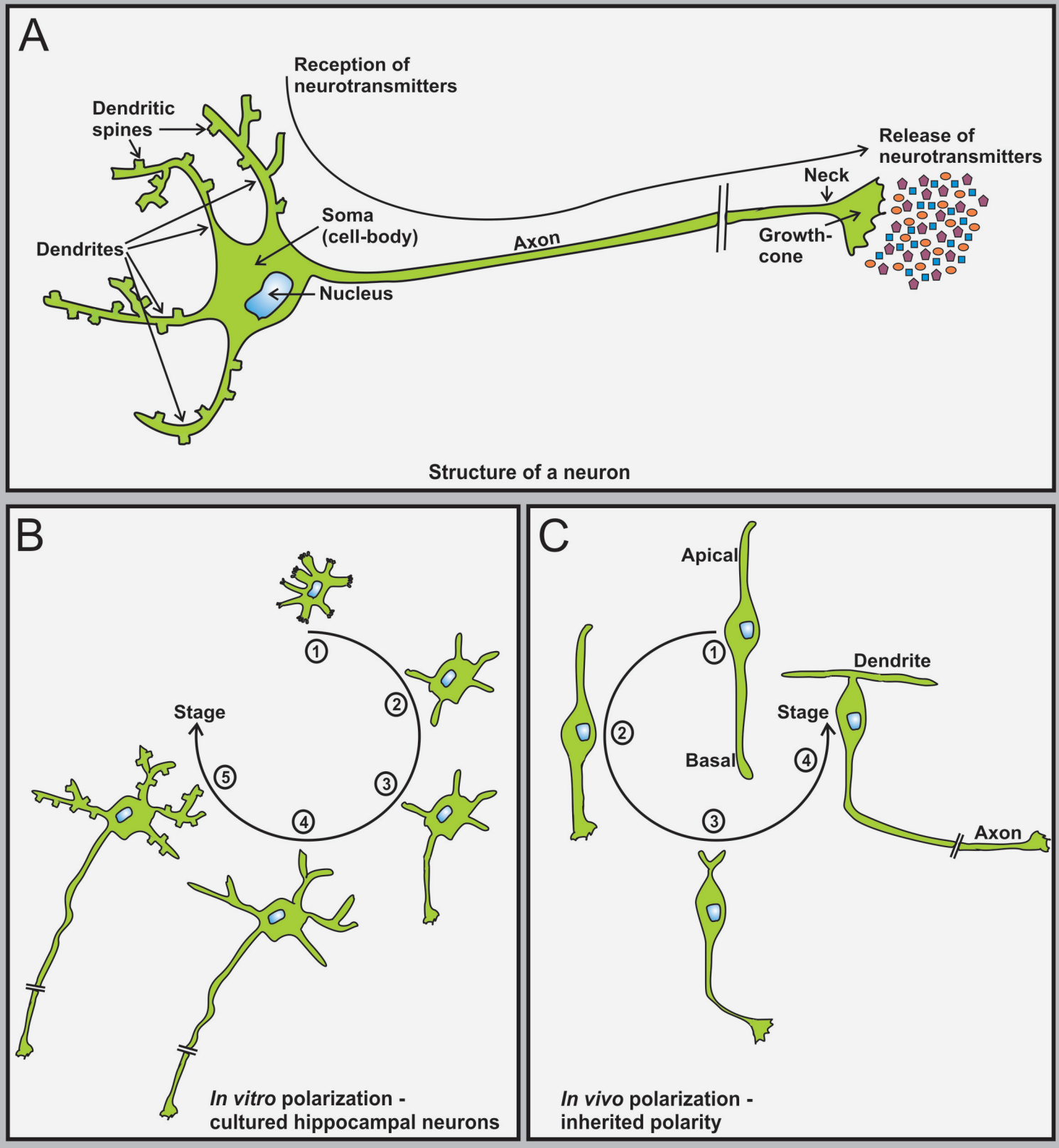
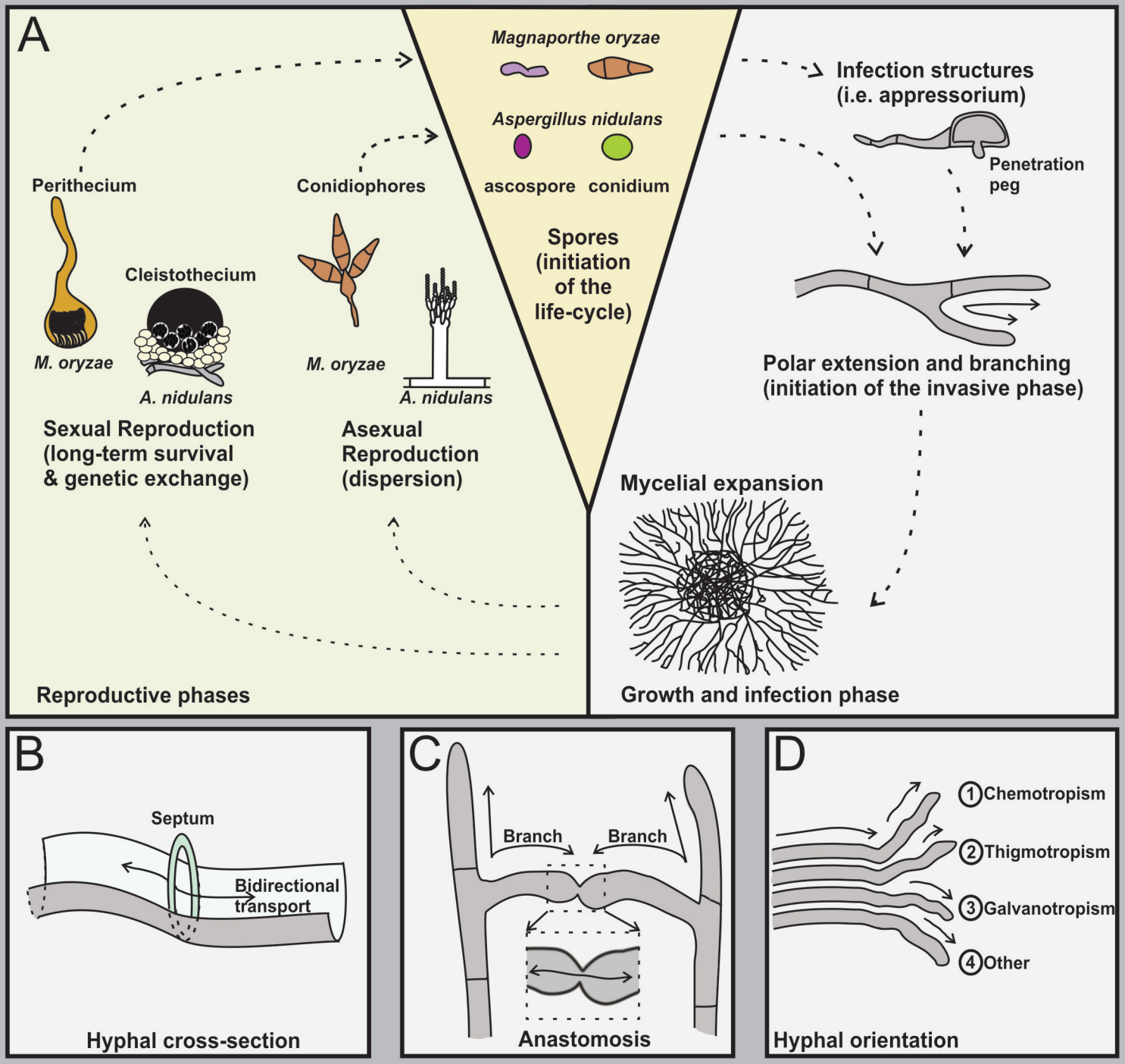
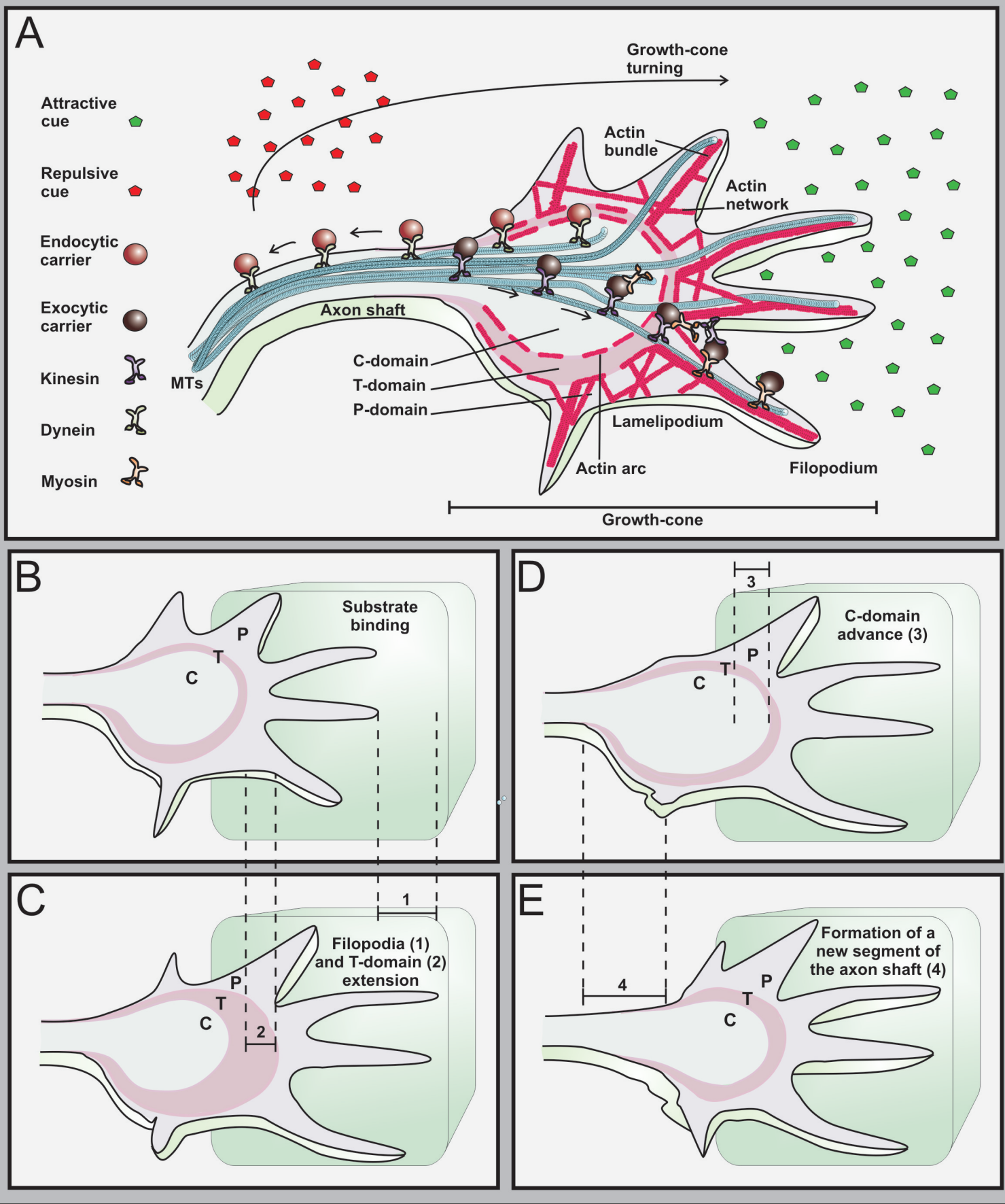
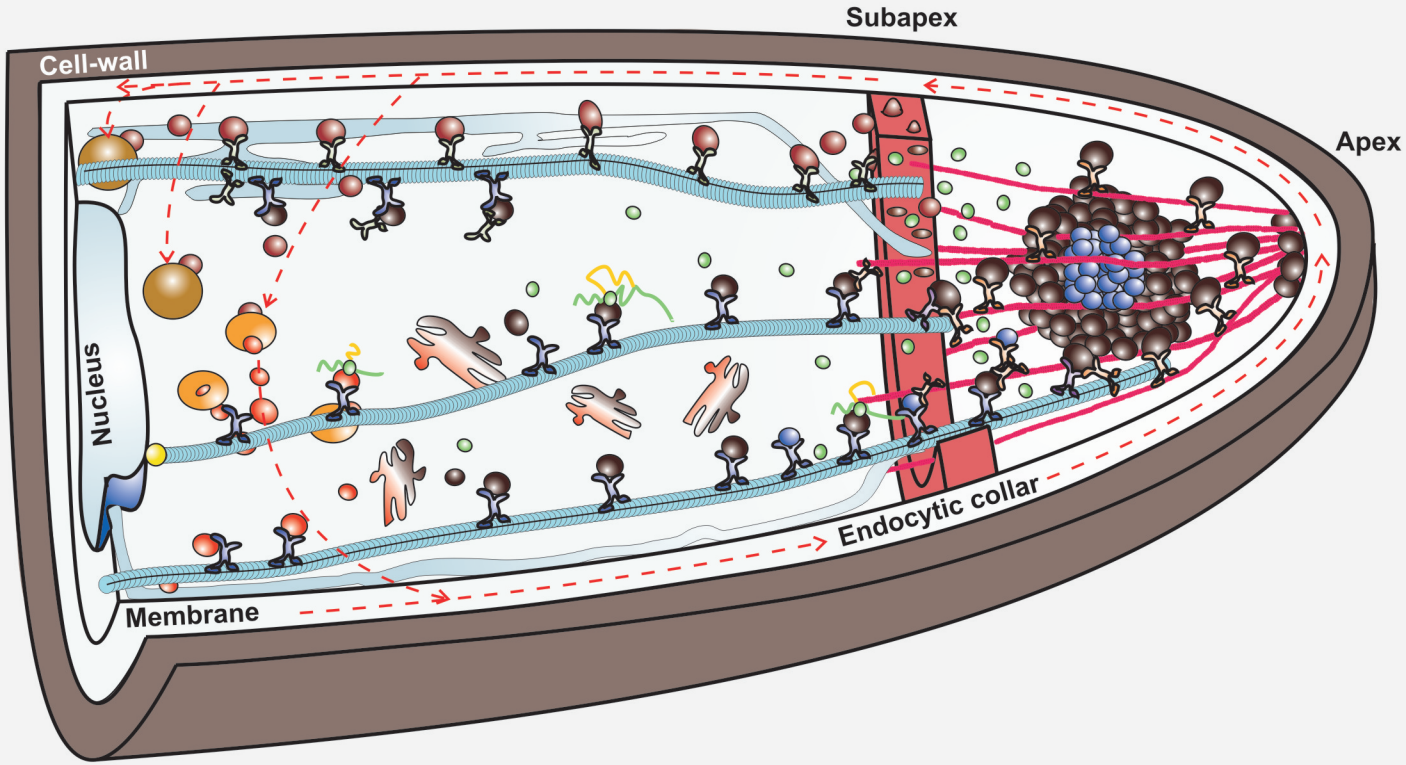

DIC
Growth
FlbB::GFP
T
N1
N2
N3
N4
Fluorescence intensity
T
N1
N2
N3
N4
Distance


A
Reception of neurotransmitters
Dendritic spines
Release of neurotransmitters
Neck
Soma (cell-body)
Axon
Dendrites
Growth-cone
Nucleus
Structure of a neuron
B
Stage
1
2
3
4
5
In vitro polarization - cultured hippocampal neurons
C
Apical
1
Dendrite
Stage
2
Basal
4
3
Axon
In vivo polarization - inherited polarity


A
Magnaporthe oryzae
Aspergillus nidulans
ascospore
conidium
Spores (initiation of the life-cycle)
Perithecium
Cleistothecium
M. oryzae
A. nidulans
Conidiophores
M. oryzae
A. nidulans
Sexual Reproduction (long-term survival & genetic exchange)
Asexual Reproduction (dispersion)
Reproductive phases
Infection structures (i.e. appressorium)
Penetration peg
Polar extension and branching (initiation of the invasive phase)
Mycelial expansion
Growth and infection phase
B
Septum
Bidirectional transport
Hyphal cross-section
C
Branch
Branch
Anastomosis
D
1 Chemotropism
2 Thigmotropism
3 Galvanotropism
4 Other
Hyphal orientation


A
Growth-cone turning
Attractive cue
Repulsive cue
Endocytic carrier
Exocytic carrier
Kinesin
Dynein
Myosin
Actin bundle
Actin network
Axon shaft
MTs
C-domain
T-domain
P-domain
Lamelipodium
Actin arc
Filopodium
Growth-cone
B
Substrate binding
P
T
C
C
Filopodia (1) and T-domain (2) extension
1
2
D
3
C-domain advance (3)
E
4
Formation of a new segment of the axon shaft (4)



MT

MT-organizing center

Actin filament

Kinesin

Dynein

Myosin

Spitzenkörper

Endoplasmit Reticulum

Golgi-directed ER and recycled vesicle

Golgi Equivalent

Early Endosome

Recycling Endosome

Late Endosome

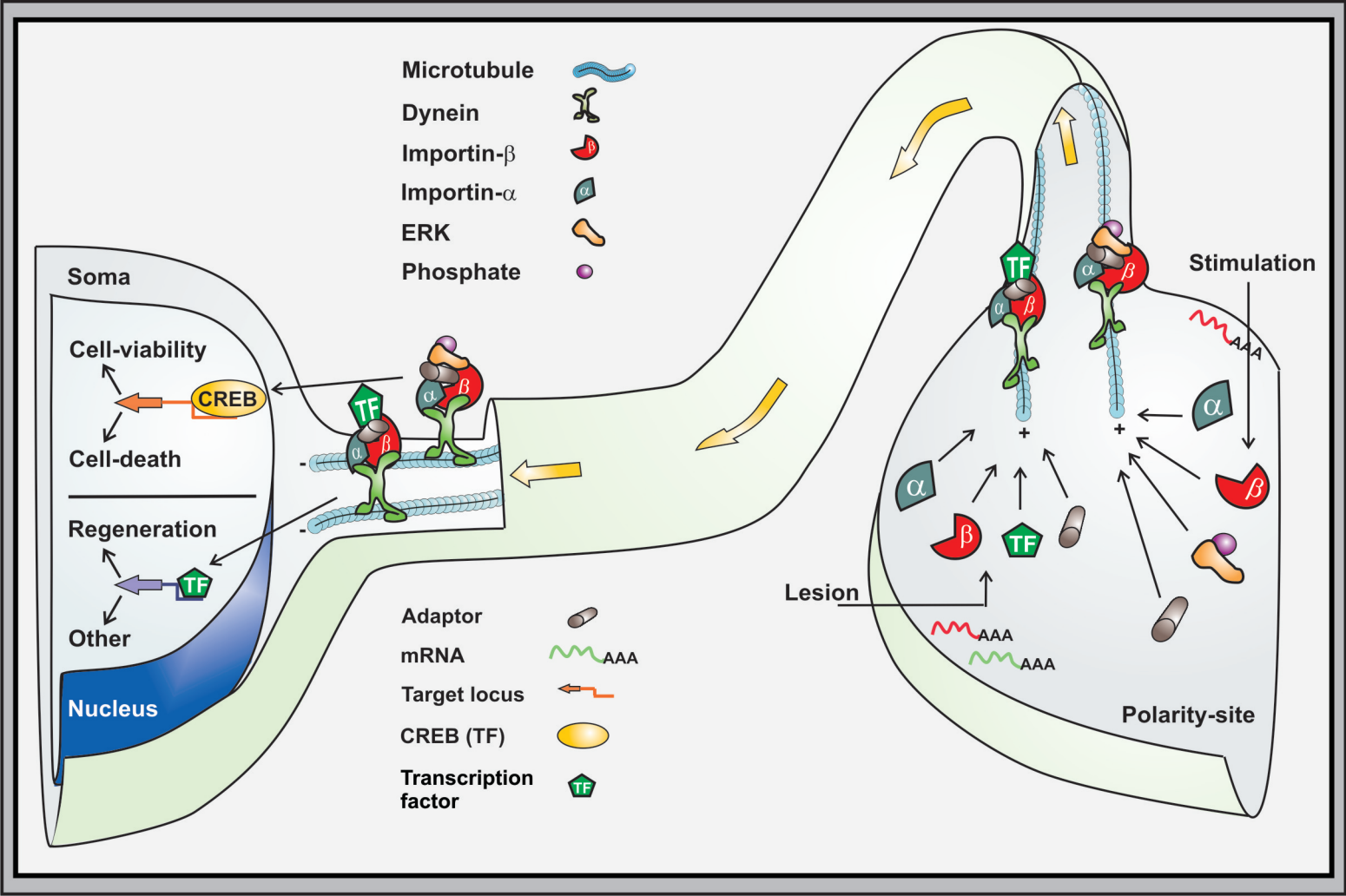
Ribosome

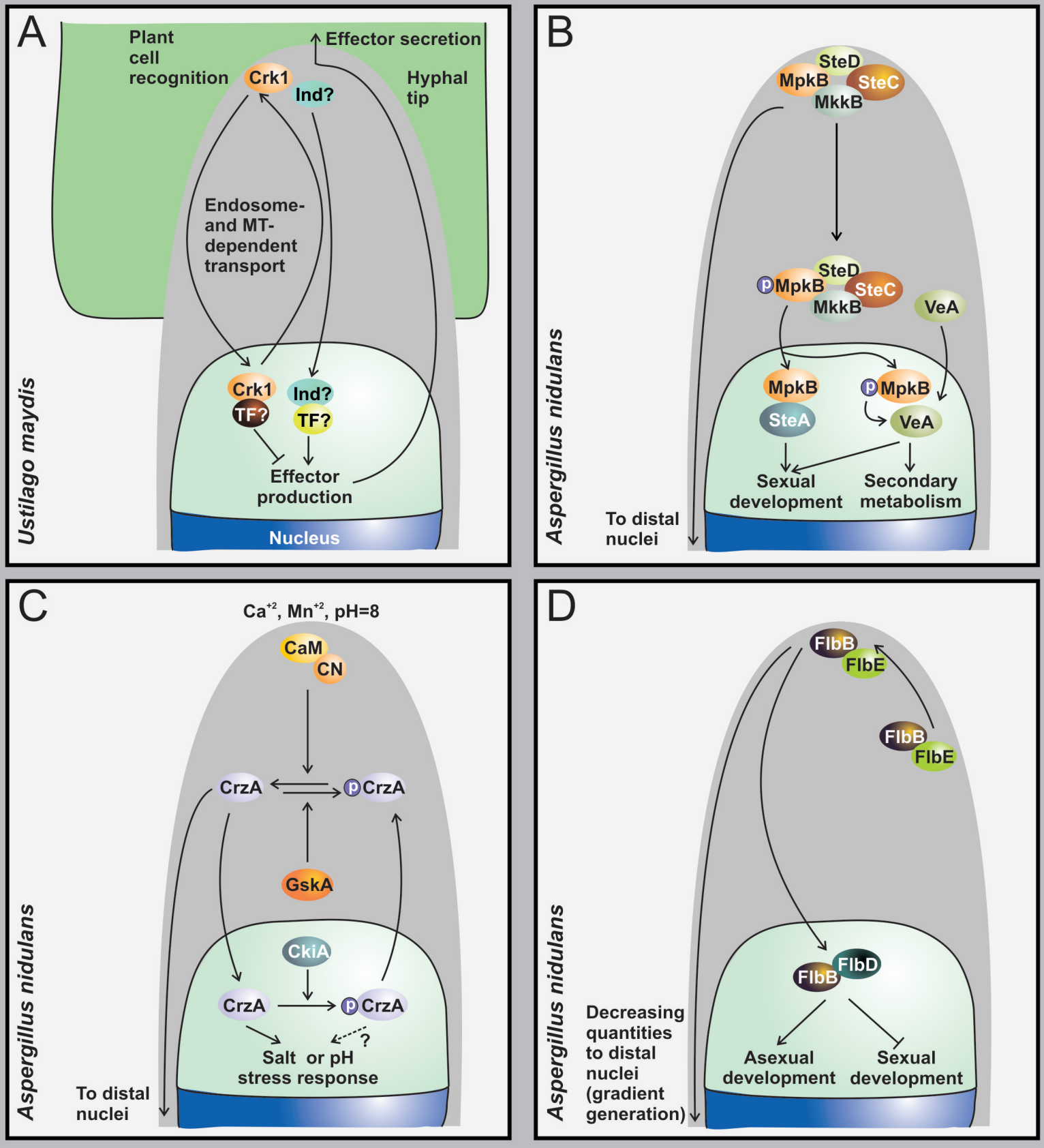
mRNA

Secretory macrovesicle

Secretory microvesicle

Sense of material flow


Microtubule
Dynein
Importin-β
Importin-α
ERK
Phosphate
Soma
Cell-viability
CREB
Cell-death
Regeneration
TF
Other
Nucleus
Adaptor
mRNA
AAA
Target locus
CREB (TF)
Transcription factor
TF
Stimulation
AAA
Lesion
AAA
AAA
Polarity-site


A
Plant cell recognition
Crk1
Ind?
Effector secretion
Hyphal tip
Endosome- and MT-dependent transport
Crk1
TF?
Ind?
TF?
Effector production
Nucleus
Ustilago maydis
B
SteD
MpkB
SteC
MkkB
SteD
PMpkB
SteC
MkkB
VeA
MpkB
SteA
PMpkB
VeA
Sexual development
Secondary metabolism
To distal nuclei
Aspergillus nidulans
C
Ca+2, Mn+2, pH=8
CaM
CN
CrzA
PCrzA
GskA
CkiA
CrzA
PCrzA
?
Salt or pH stress response
To distal nuclei
Aspergillus nidulans
D
FlbB
FlbE
FlbB
FlbE
FlbB
FlbD
Asexual development
Sexual development
Decreasing quantities to distal nuclei (gradient generation)
Aspergillus nidulans