IDENTIFICATION OF A NOVEL LINK BETWEEN THE MOTOR PROTEINS DYNEIN AND KINESIN-1

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Tarik El Mellouki, Candidate for the Doctor of Philosophy Degree
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ABSTRACT

The motor proteins dynein and kinesin are fascinating biological machines which, like vehicles in a city, move various cellular cargoes along cytoskeletal microtubules (MT). In filamentous fungi, these motors are important for the hyphal growth, which is characterized by localized extension at the tip of the apical hyphal cell. This type of growth requires the movement of a large number of vesicles from and to the hyphal tip in order to support the continuous addition of new plasma membrane and cell wall. Recent studies in filamentous fungi, have shown that early endosomes (EEs) are specifically recognized and moved by dynein from the MT plus-ends at the hyphal tips to more distal cellular compartments. Interestingly, the targeting of dynein to MT plus-ends, is dependent on kinesin-1, and its disruption lead to an aberrant accumulation of EEs at hyphal tips and a reduction in mycelial expansion. While great advances have been made in our understanding of dynein interaction with its specific cargoes, the mechanism of its interaction with kinesin-1 have remained unknown. In an effort to expand our understanding of dynein physical and regulatory interactions, we employed genetic, molecular, and fluorescence microscopy techniques to isolate and analyze mutants affected in hyphal growth and in the localization of cytoplasmic dynein in the model organism *Neurosporacrassa*.

Here we report the identification of a novel protein, which is required for the physical interaction between dynein and kinesin-1 during their journey to the hyphal tip. Interestingly, this protein is only detected in the genomes of the filamentous Ascomycota species but have the ability to physically interact with Drosophila kinesin-1, DmKHC.

APPROVAL PAGE

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ABBREVIATIONS

µm	Micrometer
Å	Angström (measurement unit equivalent to 10^{-10} meter)
AAA	ATPase associated with various cellular activities
ADP	Adenosine Di-Phosphate
AHC	Apical Hyphal Cell
ATP	Adenosine Tri-Phosphate
ATPase	Adenosinetriphosphatase
aa	Amino Acid
Arp	Actin-related protein
CC	Coiled-Coil
cot-1^{ts}	Colonial temperature sensitive
C-terminus	Carboxy-terminus
Da	Dalton (atomic mass unit)
DAPI	4',6-diamidino-2-phenylindole
DHC	Dynein heavy chain
DIC	Dynein intermediate chain
DLC	Dynein light chain
DLIC	Dynein light intermediate chain
DNA	Desoxyribonucleic acid
FGSC	Fungal Genetics Stock Center
GFP	Green fluorescent protein

KIF	Kinesin superfamily
Kin	Kinesin; ex. Kin-1
KLC	Kinesin Light Chain
kDa	Kilodalton (10^3 Daltons)
LC8	Light Chain 8
LIS1	Lissencephaly 1
Ma	Million years ago
MDa	Megadalton (10^6 daltons)
mCherry	Monomeric Red Fluorescent Protein
mRNA	Messenger ribonucleic acid
MT	Microtubule
MTOC	Microtubule organizing center
NEZ	Nuclear Exclusion Zone
Nkin	Neurospora conventional kinesin
nm	Nanometer
N-terminus	Amino-terminus
nud	Nuclear distribution mutant; ex. nudA
ro	Ropymutant; ex. ro-1
Rb	Roadblock
Spk	Spitzenkörper
Tctex	T-complex testis-expressed protein
WT	Wild-Type

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Chapter 1

INTRODUCTION

Fungi are a highly diverse and widespread group of organisms found in the soil, on decomposing organic matter, in symbiotic associations and as pathogens (Watkinson*et al.*, 2016). Many species of fungi are used in the food and pharmaceutical industries and as biocontrol agents (Watkinson *et al.*, 2016). Although they are morphologically diverse, the majority of known fungi are characterized by their unique mode of propagation, known as hyphal growth. The hyphae (singular hypha) are tube-like cells, typically 5-20 μm in diameter, and are characterized by polarized elongation (Watkinson *et al.*, 2016). Some fungi, such as yeasts, grow as single cells and only use hyphal growth under particular conditions; the fungi which grow predominantly as hyphae are known as filamentous fungi. Representatives of this group, such as *Neurospora crassa*, have been used since the 19th century in the study of the genetics, biochemistry and cell biology of living organisms (Casselton and Zolan, 2000). For example, the experiments conducted by George W. Beadle and Edward L. Tatum, using *Neurospora crassa* mutants, were instrumental in putting forward the one gene-one enzyme hypothesis; an important step in understanding the interplay between genetic information and proteins in living organisms (Beadle and Tatum, 1941; Davis and Perkins, 2002). Despite their historic use as model organisms, we are only starting to appreciate the molecular mechanisms which support hyphal growth. In the 1990s, and with the advancements made in genetic transformation and fluorescence microscopy,

microtubule (MT)-based transport has been recognized as an important component in hyphal growth. In the present dissertation we describe the identification of a novel protein involved in the interaction between the motor proteins dynein and kinesin-1. Before presenting our results, we first give a necessary introduction to the filamentous fungi and their mode of propagation and to MT-based transport and its role in hyphal growth. A glossary, relevant to this work is provided at page 114.

1.1. Hyphal growth

Filamentous fungi are thought to have evolved from aquatic ancestors and adapted a hyphal mode of growth as they colonized terrestrial environments some 500 million years ago (Watkinson *et al.*, 2016; Stajich *et al.*, 2009). While the oldest known fossils of filamentous fungi date back to around 450 million years ago (Ma), molecular clocks, based on mutation rates, estimate their land colonization to over 1000 Ma (Remy *et al.*, 1994; Selosse and Tacon, 1998; Redecker *et al.*, 2000; Heckman *et al.*, 2001). Since they were found as plant symbionts in the fossil record, it is suggested that filamentous fungi have played a key role in the evolution of land plants and to a larger extent, of life on earth (Selosse and Tacon, 1998). In fact, recent studies, have shown that some filamentous fungi can prime the resistance of plants to pathogens and serve as an underground communication network between individual plants (Jung *et al.*, 2012; Babikova *et al.*, 2013). The ability of this group of living organisms to adapt to various biological niches is reflected by its great diversity. Although at first they appeared closest to plants, it is now accepted that they are genetically and molecularly closest to animals. Like plants, they use a rigid cell wall to support cellular morphology, but like animals they are heterotrophs and

lack chloroplasts. Interestingly, their cell wall rarely contains the cellulose found in plants but rather is composed, of glucan and chitin; this latter being found in the exoskeleton of insects.

1.1.1. An overview of fungal taxonomy.

As their unique features and great diversity were recognized, the Fungi got a kingdom of their own and their taxonomy has been ever-changing, especially with the modern use of molecular phylogenetics. Here we adopt one of the most recent collaborative taxonomy works which integrates morphological and molecular characteristics and define six major phyla within the kingdom Fungi: Basidiomycota, Ascomycota, Glomeromycota, Blastocladiomycota, Chytridiomycota and Neocallimastigomycota (Fig. 1; Watkinson *et al.*, 2016; McLaughlin *et al.*, 2009; Hibbett *et al.*, 2007). While some species form pseudo-hyphae and others only adopt hyphal growth in specific environments (such as some yeasts); the majority of taxa are filamentous fungi (Watkinson *et al.*, 2016). The relatively reduced hyphal growth and the production of flagellated spores (zoospores) in the Blastocladiomycota, Chitridiomycota and Neocallimastigomycota separates these groups from the rest of the fungi. The Neocallimastigomycota, previously included within the Chitridiomycota, found in the digestive tracts of certain herbivores are anaerobic fungi and thus lack mitochondria (Watkinson *et al.*, 2016; Hibbett *et al.*, 2007). Because of distinct zoospore structure and genetic phylogeny, the Blastocladiomycota, were also separated as a distinct phylum from the Chitridiomycota (James *et al.*, 2006; Hibbett *et al.*, 2007). On the other hand, the phylum Glomeromycota was separated from the phylum previously called

Zygomycota; in this latter the relationships between the different clades (Mucoromycotina, kickxellomycotina, Zoopagomycotina and Entomophthoromycotina) are still unresolved and are categorized as *incertae sedis* (of uncertain placement). The Glomeromycota includes most of the known plant symbionts, known as Arbuscular Mycorrhizae (AM); which form important mutualistic relationships with around 80% of all vascular plants (Schüßler *et al.*, 2009). Finally, the Ascomycota and Basidiomycota, which contain the majority of fungal species, are grouped together in the sub-kingdom Dikarya. This latter is characterized by species that can maintain vegetative growth while harboring genetically distinct nuclei; a formation known as a heterokaryon and which is a useful tool in the study of genetic interactions (Fig. 1; Davis, 2000). This sub-kingdom contains over 95% of all the known fungal species and can be distinguished from the other phyla by the presence of well-defined septa which separate hyphal cells during vegetative growth (Hibbett *et al.*, 2007; McLaughlin *et al.*, 2009; Stajich *et al.*, 2009; Peterson, 2013). The formation of septa provides an advantage to the Dikarya by providing a mechanism to prevent the "bleeding-through" of hyphae in case of cellular wounding (Plamann, 2009; Jedd and Pieuchot, 2012). The structure of septa is variable and sometimes is used to distinguish between the different clades in the Dikarya; for example, species of the Basidiomycota are known to form clamp connections during septum formation and those of the Ascomycota use a ‘plug’, known as a Woronin body, to regulate cytoplasmic streaming through septal pores (Trinci and Collinge, 1973; Ng *et al.*, 2009; Plamann *et al.*, 2009; Jedd and Pieuchot, 2012). However, the defining characteristic, which separate the Ascomycota from the Basidiomycota, is

the location of spore formation; in the Ascomycota, the spores, called ascospores, are formed within asci (sac-like structures) and in the Basidiomycota, the spores (basidiospores) are formed on club-like structures, the basidia (Watkinson *et al.*, 2016). Additionally, the Dikarya are known to maintain a vesicular structure known as the Spitzenkörper involved in hyphal tip growth. More on this apical body will be said in the next paragraphs.

For a more detailed comparison between the different groups of fungi in general, and of filamentous fungi in particular, please refer to these publications: Watkinson *et al.*, 2016; Peterson, 2013, McLaughlin *et al.*, 2009; Stajich *et al.*, 2009, Hibbett *et al.*, 2007. In order to maintain relevance to the study provided in this dissertation and for a necessary simplification, our reference to the filamentous fungi in the subsequent text will only include the Dikarya, the largest group of filamentous fungi. Fungi which grow as unicellular yeasts or only use filamentous growth under particular circumstances, including the model organisms *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* and the human pathogen *Candida albicans*, will also be excluded from our reference to the filamentous fungi.

1.1.2. The mycelium: Fragile hyphae with mighty powers

In general, the hyphae first emerge from a spore, elongate and form branches which fuse with each other to from a mycelial colony (Fig. 2). This latter expands radially from the center of the colony while maintaining cellular communication between the different parts of the hyphal network and maintain the growth of the mycelium as a single organism (Watkinson *et al.*, 2016). Thus, the colonization of the growth substrate is optimized towards optimal nutritional resources and away from

unfavorable conditions. Once the colony reaches a certain age, asexual spores are produced through mitosis and upon their dispersal can initiate independent colonies. When the hyphae of different mating types interact they can initiate the production of sexual spores through meiosis, and restart a new reproduction cycle. Sexual reproduction and the maintenance of heterokaryons, increase dramatically the genetic diversity of the population and its adaptability to environmental changes. The expansion of the mycelium, which can reach speeds of 5 mm/hour in *N. crassa*, is the result of hyphal tip growth.

1.1.3. Tip growth in filamentous fungi

Filamentous fungi are heterotrophs and rely for their growth on the use of organic matter from the environment. In order to mobilize the nutrients from their growth substrates, they secrete diverse enzymes which can break down the substrate on which they grow. Interestingly, most of the secretion, uptake of nutrients and growth occur at the tip of the apical hyphal cell. The first recorded description of apical growth in the hyphae dates back to 1892 in a publication titled "Das Wachsthum der Pilzhyphen" (The growth of the fungal hyphae) by the German scientist Max Otto Reinhardt (Bartnicki-García, 2015). The hints to this mode of growth came from observations in which subapical regions marked by metallic particles remained behind the apex as the hyphae grew (Watkinson *et al.*, 2016). Studies of the hyphal tip using radiographic methods, were indicative of the intense secretory and cellular activities at the hyphal tips (Zokalar, 1959). In the 1960s, microscopic and biochemical experiments describing the ultrastructure of the apical hyphal cell (AHC) and showing that the addition of cell wall is restricted to the apical dome of the

hyphae provided definitive proof for hyphal tip growth (Butler 1961; Robertson, 1965; Brenner and Carroll, 1968; McClure *et al*., 1968; Bartnicki-García and Lippman, 1969; Girbardt, 1969; Grove and Bracker, 1969).

1.1.4. Microscopic organization of the apical hyphal cell

The AHC is composed of all major organelles of eukaryotic cells: nuclei, Endoplasmic Reticulum (ER), Golgi apparatus, mitochondria and vesicular organelles (reviewed in Fig. 3 and 4; Grove and Bracker, 1969; Howard and Aist, 1980; Howard, 1981). In this cell, the cytoplasm is densely populated with ribosomes, indicative of intense protein synthesis, and with cytoskeletal elements, indicative of active cargo trafficking. The many mitochondria found at the AHC provide the supply of energy required for the multitude of cellular processes maintaining hyphal integrity and continuous growth. Interestingly, the volume of individual mitochondria becomes smaller in hyphal regions that are distal from the apical growth zone (Fig. 3 J-K). The larger volume of mitochondria at the hyphal tip is most likely the result of a more important energetic demand at the hyphal tip *versus* at distal regions. In addition to the mitochondria, the AHC contains multiple nuclei which share the same cytoplasm; in species with large hyphal diameters, such as *N. crassa*, a large number of nuclei can be seen moving in different directions throughout the hyphal cells (Freitag *et al.*, 2004; Ramos-García*et al.*, 2009; Fischer, 1999). In the Ascomycota, the nuclei can be observed crossing the septal pores as they move from cell to cell driven by cytoplasmic streaming (fast movement of cytoplasmic mass; Plamann, 2009). However, the nuclear distribution, especially in the hyphal germlings, is not random and has been shown to involve

MT-dependent motors (more on this below). Interestingly, a zone devoid of nuclei, or nuclear exclusion zone (NEZ), about 12 µm from the tip in *N. crassa*, is maintained throughout growth and can be disturbed if MT-based transport is perturbed (Fig. 5; Freitag *et al.*, 2004; Ramos-García *et al.*, 2009). This zone contains a large number of vesicles, including the characteristic vesicular body of most filamentous fungi, the Spitzenkörper (Fig. 3 and 4; Brunswik, 1924; Girbardt, 1969; Grove and Bracker, 1969; Howard and Aist, 1980; Howard, 1981; reviewed in Riquelme and Sánchez-León, 2014). The Spitzenkörper (Spk), which is German for apical body, was first described in 1924 by Brunswik; it is highly dynamic and is essential for hyphal growth and morphogenesis (Brunswik, 1924; Bartnicki-García *et al.*, 1995; Riquelme *et al.*, 1998; Bartnicki-García, 2015). In fact, it has been shown that when the hyphae cease to grow, the Spk disappears, and when the growth resumes, it reforms (Bartnicki-García *et al.*, 1995). On electron micrographs, the Spk appears composed of two populations of vesicles with diameters of 25-40 nm and 70-100 nm, called microvesicles and macrovesicles, respectively (Fig. 4; Grove and Bracker, 1969; Howard and Aist, 1980; Howard, 1981; Riquelme *et al.*, 2014). While the microvesicles occupy the core of the Spk, the macrovesicles are found in an outer layer (Fig. 3; Grove and Bracker, 1969; Howard and Aist, 1980; Riquelme *et al.*, 2014). The Spk is considered by many studies to be a "vesicle-supply center", receiving vesicles, sorting them and supplying them to the exocytic machinery (Bartnicki-García*et al.*, 1989). Consistent with this model, the ER and Golgi apparatus which generate different secretory vesicles can be observed throughout the hyphae and close to the Spk (Fig. 4). Recent studies, using fluorescent protein

markers, have shown that ER-to-Golgi and Golgi-derived vesicles, carrying the small GTPases Ypt1, Rab11 and Sec4, can cluster at the Spk (Riquelme *et al.*, 2007; Sánchez-León *et al.*, 2015). Additionally, vesicles containing cell wall modifying enzymes which are destined to exocytosis can be localized to the Spk in *N. crassa*, *A. nidulans* and *U. maydis* (Riquelme *et al.*, 2007; Takeshita *et al.*, 2005; Verdin *et al.*, 2009; Weber *et al.*, 2006). Interestingly, in the well-defined Spk of *N. crassa*, these enzymes can occupy different layers; for example, Chitin Synthase (CHS) and Glucan Synthase Complex (GSC), are found at the core and outer layers of the Spk, respectively (Fig. 3 D, H, M and N; Sánchez-León *et al.*, 2011; Verdin *et al.*, 2009). Finally, the components the exocyst complex, conserved in filamentous fungi, yeast and metazoa and which is involved in exocytosis, can be visualized at the apical dome of the hyphae intermediate between the Spk and the plasma membrane (Fig. 3 I; Taheri-Talesh *et al.*, 2008; Riquelme *et al.*, 2014; Guo *et al.*, 2015). In addition to its vesicular components, the Spk contain the cytoskeletal components, actin and tubulin, which organize into actin filaments and MTs (Fig. 4). The latter is important for long distance transport and the former is important for the myosin-based short-distance transport of vesicles and for endocytosis. In fact, endocytosis plays important roles in hyphal growth including the internalization of nutrients and the recycling of plasma membrane and other elements of tip growth (Peñalva, 2010; Shaw, 2011). In proximity to the Spk, is a sub-apical region of the AHC, a ring of fluorescently marked actin filaments that can be observed along the hyphal circumference (Fig. 4 E; Berepiki *et al.*, 2010; Berepiki *et al.*, 2011; Delgado-Alvarez *et al.*, 2010; Echauri-Espinosa *et al*., 2012; Kilaru *et al*., 2015; Schultzhaus *et al*.,

2016). This corresponds to an endocytic zone which generates a large number of vesicles, including early endosomes (EEs), which have to be sorted and transported by MT-dependent motors into the different compartments of the AHC (Peñalva, 2010; Shaw, 2011). In fact, the long-range movement of secretory vesicles, the general organization of the hyphae and the integrity of the Spk all require the MT-dependent transport (Xiang and Plamann, 2003; Riquelme *et al*., 2011). The important role of MTs in the general organization of the AHC has long been recognized and the use of the MT-binding drug Benomyl as a fungicide is a testament to that (Howard and Aist, 1980). With the use of fluorescently tagged MTs, we are able to visualize MTs emanating from the microtubule organizing centers (MTOCs) which are associated with septa and nuclei (Freitag *et al.*, 2004; Ramos-García*et al.*, 2009). These latter can appear pear-shaped (pyriform) as they are pulled throughout the busy hyphal cytoplasm (Fig. 5). MTs at the AHC are in general organized parallel to the growth axis and can be seen converging at the Spk (Freitag *et al.*, 2004). The motor proteins dynein and kinesin have been shown to be important for hyphal growth and morphogenesis (see below). In the next sections of the introduction we will give an overview of MT-based transport in general and its role in hyphal growth in particular.

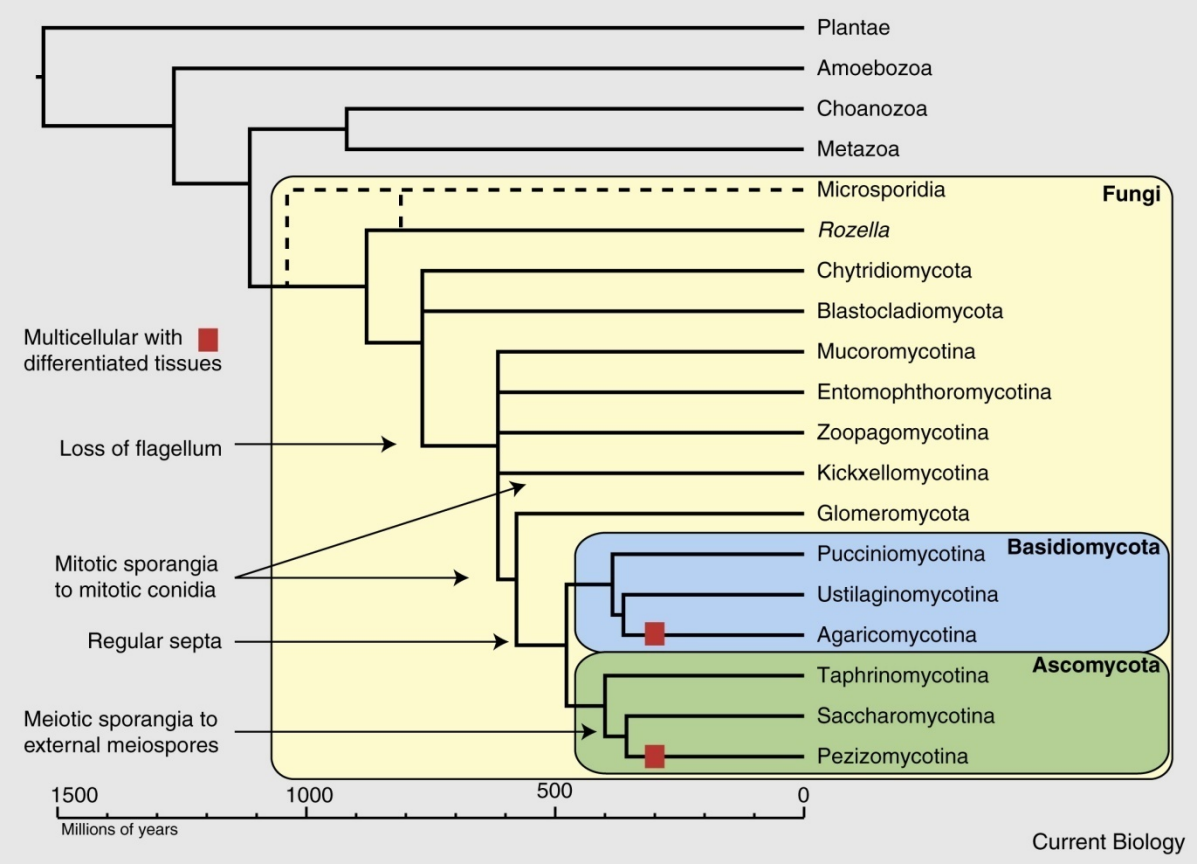


Figure 1. **The fungi.** Arrows depict changes in morphology including the major loss of the flagellum, transition of mitotic sporangia to mitotic conidia, invention of regular septa, and meiotic sporangia to external meiospores. The blocks indicate branches where most membershave multicellular differentiated tissues. The phylogenetic position of the Microsporidia is notconfidently resolved as indicated by the dotted line. Reprinted from Current Biology Vol. 19 No. 18, Stajich *et al.*, The Fungi, R840, Copyright 2009, with permission from Elsevier.

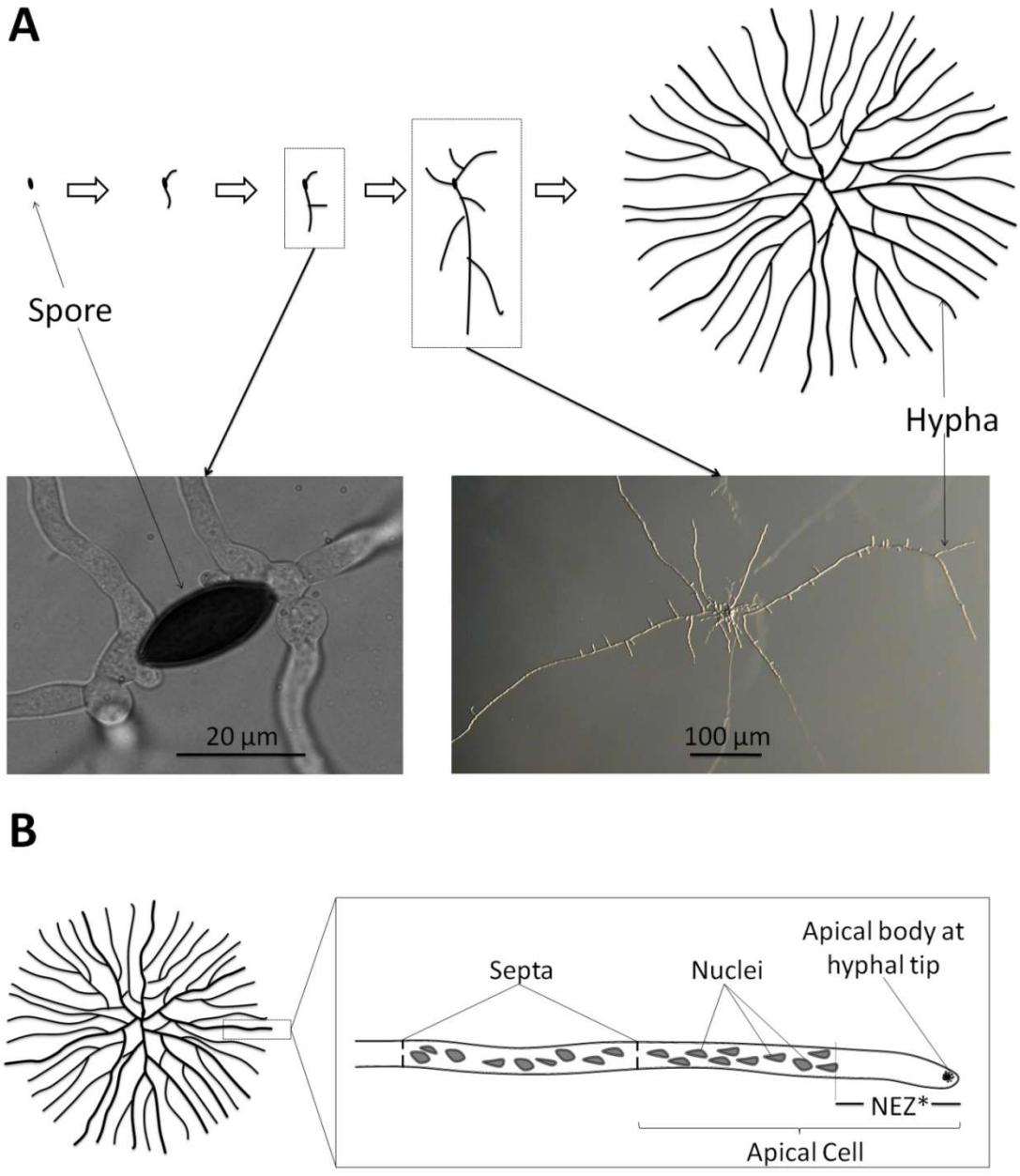
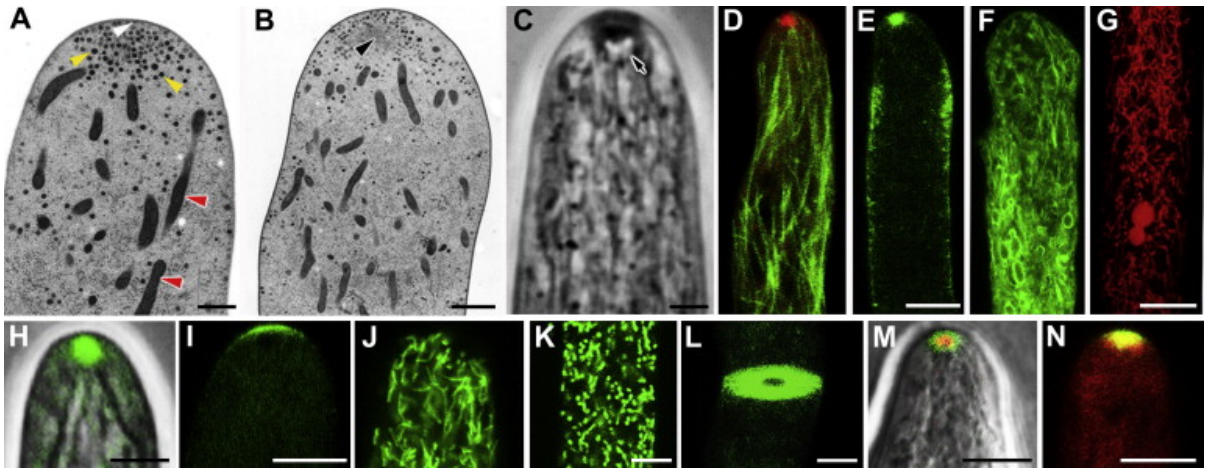


Figure 2. **The hyphal organization of the mycelium.** (A) When the hyphae emerge from a spore (about 20µm in length) they elongate and form branches which then connect in a mycelial network. (B) The hyphae are tube-like cells containing multiple nuclei and separated by septa. The apical hyphal cell is characterized by a nuclear exclusion zone (NEZ) and terminates with a dome-like tip containing an apical body known as the Spitzenkörper.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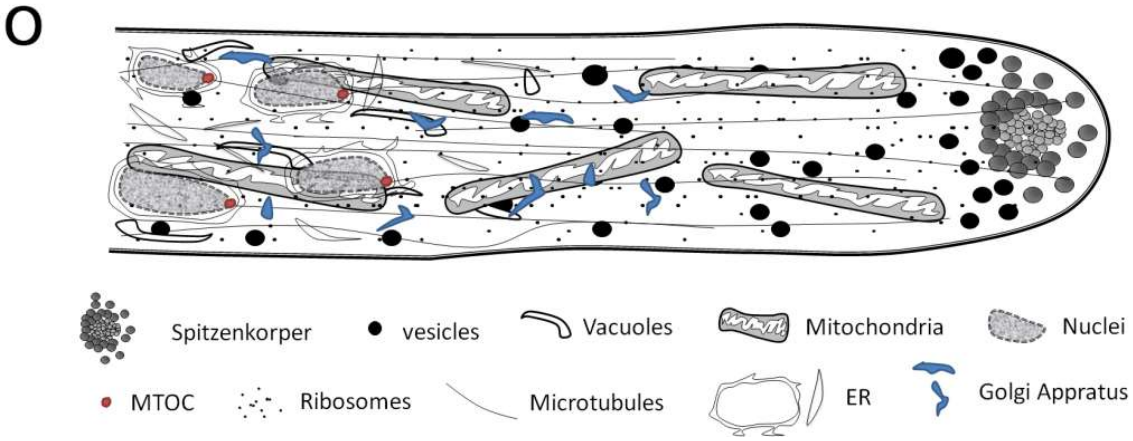

O
Spitzenkorper
vesicles
Vacuoles
Mitochondria
Nuclei
MTOC
Ribosomes
Microtubules
ER
Golgi Appratus

Figure 3. **Structure and ultrastructure of cellular components in the hyphae of the ascomycete *Neurospora crassa*.** (A-B) Transmission electron micrographs (TEM) of the hyphal tip showing the Spitzenkörper (Spk) with macrovesicles (yellow arrowheads) and microvesicles (black arrowheads). Some of the macrovesicles can be seen fusing with the cell surface (white arrowheads). Many mitochondria (Red arrowheads) provide the energy required for the growth-supporting cellular activity. Scale bars are 0.8 µm and 1.4 µm in (A) and (B), respectively. (C) Under Phase-contrast microscopy the Spk appears as a dark apical body, in contrast to phase-light elements (black arrow). Scale bar 1.7 µm. (D) Laser scanning confocal microscopy (LSCM) of a heterokaryon showing microtubules labelled with GFP and CHS-1 labelled with mChFP. (E) LSCM showing Lifeact-GFP at the Spitzenkörper core and the cortical subapex (Delgado-Alvarez *et al.* 2010). Scale bars for D and E, 5 µm. (F) Endoplasmic Reticulum at the hyphal tip of a strain expressing GFP-tagged NCA-1, a protein encoding a CA-transporting ATPase. (G) Vacuoles in the region approximately 300 µm behind the apical tip, as visualized by fusing RFP to CAX, a calcium-H+ exchange protein. Scale bars for F and G, 10 µm. (H) Overlap of phase-contrast and LSCM showing CHS-1-GFP at the Spk core. (I) Exocyst component SEC-6 tagged with GFP by LSCM. (J−K) ARG-4, a mitochondrial enzyme of the arginine metabolic pathway, fused to GFP shows how mitochondria exhibit different structures at the apical tip region (J), and at regions approximately 1mm distal to the tip (K). (L) 3D reconstruction of a completed septum in a strain expressing CHS-1-GFP. (M) Heterokaryon showing GS-1-GFP at the Spitzenring and CHS-1-mChFP at the Spitzenkörper core. (N) Heterokaryon showing the polarisome component SPA-2 tagged with GFP and CHS-1-mChFP. Scale bars for H−N, 5 µm. A-N, Reprinted from Fungal Biology, Vol 115 /edition 2011, Riquelme *et al.*, Architecture and development of the Neurospora crassa hypha - a model cell for polarized growth, Pages 446-474., Copyright (2011), with permission from Elsevier. (O) A general and schematic overview of the organization of the hyphal apical cell.

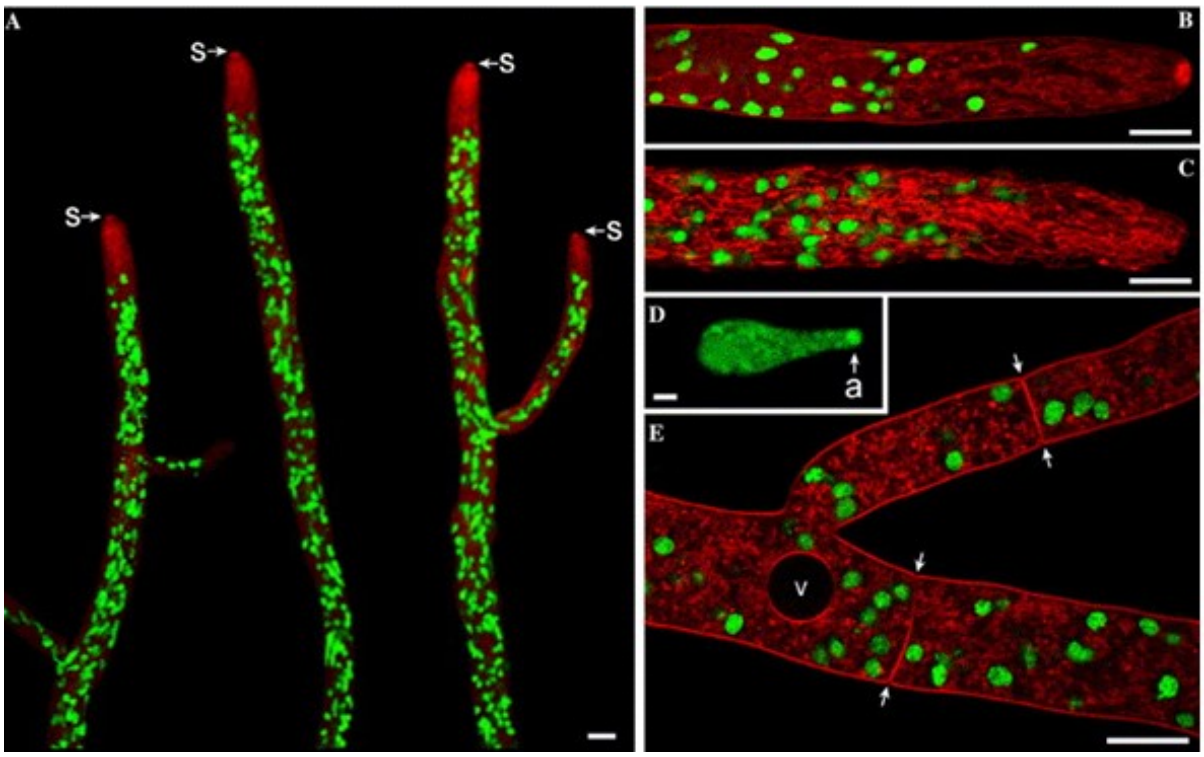


Figure 4. **Nuclear distribution in the hyphae of *N. crassa*.** (A-B) Images showing growing hyphae at the colony periphery (A) with a close-up on the hyphal tip (B). Nuclei are visualized through GFP-tagged histone protein H1 (green) and vesicles are labeled with the membrane-selective dye FM4-64 (red). Note the brightly stained Spitzenkörper (s) within the hyphal tips and a region 20–25µm behind it which is devoid of nuclei Bar = 10 µm. (C) Growing tip showing nuclei (green) and co-labeled with the mitochondrion-selective dye DASPMI (red). Note network of elongated mitochondria. Bar = 10µm. (D) High magnification image of a single pear-shaped nucleus which is moving towards the right in the direction of the hyphal tip. One strong fluorescent focus at the tip of the nucleus (a) localizes to the region predicted to contain the microtubule organizing center (MTOC) and to be in close association with the microtubule to which the moving nucleus is attached. Bar = 1µm. (E) Subapical hyphal region from which a branch is emerging, with nuclei (green) and co-labeled withFM4-64 (red). Note that FM4-64 has stained the plasma membrane, including that on either side of the two septa (arrows), the tonoplast of the vacuole (v) and the membranes of numerous small organelles. Bar = 10µm.Reprinted from Fungal Genetics and Biology, Vol. 10, Issue 10, Freitag M., Hickey P.C., Raju N.B., Selker E.U. and Read N.D., GFP as a tool to analyze the organization, dynamics and function of nuclei and microtubules, Pages 897-910, Copyright (2004), with permission from Elsevier.

1.2. Microtubule-based transport

Microtubule-based transport is of particular importance in cells or organisms which are characterized by polarized growth such as neurons in animals, pollen tubes in plants and hyphae in filamentous fungi. In these systems directed transport of macromolecules, vesicles and organelles by MT-dependent motor proteins is of fundamental role for growth and development (Xiang and Plamann, 2003; Franker and Hoogenraad, 2013). The eukaryotic motor proteins dynein and kinesin, are among the most fascinating biological machines in the living world. Like vehicles in a city, they are involved in cellular trafficking and thus are essential for the achievement of many fundamental cellular processes including the transport of membrane-bound organelles, protein complexes, morns and ribosomes (Scalia and Woehlke, 2003; Vale, 2003). They also play a central role in chromosome segregation during cell division as they are involved in the assembly and function of meiotic/mitotic apparatus (Schliwa and Woehlke, 2003; Cross and McAinsh, 2014).Using their characteristic adenosinetriphosphatase (ATPase) activity, they release energy from adenosine triphosphate (ATP) molecules and use it to power directional movement (Schliwa and Woehlke, 2003). Although structurally different, both dynein and kinesin are composed of multiple subunits and they both use cytoskeletal microtubules (MTs) as "tracks" (Schliwa and Woehlke, 2003).Unlike urban vehicles, they are highly efficient and traffic jams are not tolerated. In fact, deficiencies in dynein or kinesin can lead to growth and development defects or, in most severe cases, to lethality in many organisms including humans. The importance of these motor proteins in humans is highlighted by their involvement in

many pathological conditions which include, Charcot-Marie-Tooth, Lissencephaly, polycistic kidney and neurodegenerative diseases (Qin *et al.* 2001; Vallee *et al.* 2001; Zhao *et al.* 2001;De Vos *et al.* 2008).

1.2.1. A brief history of the discovery of dynein and kinesin

In the middle of the twentieth century, the cellular basis for motility was one of the important questions investigated by biologists. The great advances made in electron microscopy (EM) have yielded detailed descriptions of the microscopic organization of the flagella and cilia, the structures which support the motility of spermatozoids and many unicellular organisms (Afzelius, 1959; Gibbons and Grimstone, 1960). While MTs were found to be at the core of the organization and function of these structures, it was not evident how motility is generated (Afzelius, 1959; Gibbons and Grimstone, 1960). Using biochemical approaches, it was established that the energy extracted from ATP powers this motility and a link between the axoneme (the MT-containing core of flagella and cilia) and ATPase activity was established (Gibbons 1963). In search for this axonemal ATPase, Gibbons and colleagues discovered the first microtubule-associated motor which they named Dynein, after the unit of force *dyne* (Gibbons and Rowe, 1965; for a more detailed account of the discovery of axonemal dynein, please refer to Gibbons, 2012). The analysis of dynein, showed that this motor corresponds to the arms which extend from the MT-doublets and slide over the adjacent MT-doublets during axonemal movement (Ogawa *et al.*, 1977; For a review see: Gibbons, 2012). The early characterization of dynein showed that it is a multi-subunit complex with a megadalton size, one of the biggest protein assemblies ever found. Soon after its discovery, its importance in human

health was emphasized when it was found that a case of immotile spermatozoids was due to the lack of axonemal dynein (Afzelius *et al.*, 1975). The ensuing studies of MTs and MT-associated proteins (MAPs) and the development of live-imaging, revealed that intra-cellular motility of organelles and membrane-bound vesicles is also MT-dependent. The search for the motor(s) powering intracellular motility led to the isolation of kinesin (conventional kinesin or kinesin-1) from Squid axoplasms, bovine brain and Sea urchin eggs (Brady, 1985; Vale *et al.*, 1985a; Scholey *et al.*, 1985). Kinesin was found to move towards the plus-end of MTs and to be responsible for anterograde transport in axons (Vale *et al.*, 1985b; Brady *et al.*, 1990). The analysis of high molecular weight MAPs revealed the existence of a cytoplasmic version of dynein which was then identified in mammalian brain tissue, the nematode *Caenorhabditis elegans* and the amoeba *Dictyostelium* (Paschal *et al.*, 1987a; Lye *et al.*, 1987; Koonce and McIntosh,1990). Early experiments on cytoplasmic dynein quickly revealed that this motor is responsible for the retrograde transport of vesicular cargoes (Paschal *et al.*, 1987b; Schnapp and Reese 1989; Schroer *et al.*, 1989). Using EM and biochemical analysis, scientists established that cytoplasmic dynein is a two headed ATPase motor that is specifically stimulated by MTs (Shpetner *et al.*, 1988). As the research on MT-based transport advanced, the conservation of the structure, function and regulation of MTs and motor proteins became established.

1.2.2. Microtubules

Microtubules, found in all studied eukaryotes, are divided into two major groups based on their cellular distribution, stability and organization. The first group is

known as cytoplasmic MTs; these are the largest constituent of the cytoskeleton and are organized into a network of single tubules which are highly dynamic (Becker *et al.*, 2006). The second group is found in the cilia and flagella and are known as axonemal MTs (Becker *et al.*, 2006). Microtubules have a diameter of ~25 nm and are formed of 10 to 13 protofilaments which result from the polymerization of α/β tubulin heterodimers (Fig. 5 B; Gibbons and Grimstone 1960; De-Thé 1964; Mohri, 1968; Weisenberg, 1968, 1972). Mutations in the tubulin proteins can have severe consequences on the organism and in humans they have been reported to cause brain malformations (Romaniello *et al.*, 2015). The asymmetric α/β tubulin subunit polymerizes in a head-to-tail fashion leading to MTs with a polymerization/depolymerization end, known as the plus-end, and the opposite end, known as the minus-end (Fig. 5 A; Allen and Borisy, 1974). This latter is associated with the microtubule organizing centers (MTOCs; Allen and Borisy, 1974). The MTOCs correspond to the centrosomes in mammals and to the spindle pole body in yeast/fungi, and are known to associate with nuclei, Golgi apparatus and hyphal septa (Kreis, 1990; Lüders and Stearns, 2007). The MT network supports cargo trafficking throughout the cell with kinesin generally directing traffic towards the cell periphery (MT plus-ends) and dynein moving it towards the inside of the cell (Fig. 5 A; Morgensen *et al.*, 1989). Additionally, recent research has unraveled that MTs are not simply supporting tracks but they contain information encoded as tubulin modifications and which might provide direction and regulation to the MT-system (Janke, 2014). A close relationship exists between dynein, kinesin and MTs; these

motors are activated by the presence of MTs and MT dynamics are influenced by these motors.

1.2.3. Dynein

To date, all dynein motors discovered move along MTs in direction of the minus-end and all kinesins, with the exception of the kinesin-11 family, are plus-end directed motors. Here we limit our description to the cytoplasmic dynein (hereafter dynein) and the conventional kinesin (hereafter kinesin-1). For more details on axonemal dynein please refer to reviews by King (2012), Kobayashi and Takeda (2012) and Roberts *et al.* (2013). For more information on the different members of the kinesin superfamily please refer to reviews by Hirokawa *et al.* (2009) and by Verhey and Hammond (2009).

a) The organization of dynein motors.

Dynein motors operate as large multi-protein complexes composed of heavy chains (DHCs), intermediate chains (DICs), light intermediate chains (DLICs) and multiple light chains (DLCs; Fig. 6; Reviewed in Roberts *et al.* 2013). DLCs are encoded by three gene families known as T-complex testis-expressed protein 1 (*Dynlt1*), Roadblock (*Dynlrb1*/*Dynlrb2*) and Light Chain 8 (Pfister *et al*., 2005). Studies of the subunit organization of dynein provided evidence that its different subunits are organized into dimers within the motor complex (Fig. 6; Neely *et al.*, 1999). DHC, the largest of these subunits (~500 KDa; 4300-4600 aa), folds into a motor domain at its C-terminus and forms a flexible tail at the amino-end (Fig. 6). These two, are connected to each other through a linker domain which is important for the conformational changes occurring during ATP hydrolysis (See below; Fig. 6; Roberts

et al. 2013). The tail is required for DHC dimerization and serves as a binding platform for DIC and DLIC (Fig. 6; Gee *et al.*, 1997; Habura *et al.*, 1999; King 2000; Tynan *et al.*, 2000; Meng *et al.*, 2006). The dimerization of DHC is important for ATPase activity, ATP-dependent release from MTs and for motor directionality (Iyadurai *et al.*, 1999; Hook *et al.*, 2005). Moreover, the different subunits of the dynein complex can interact with non-dynein proteins and thus allow for an increase of possible dynein interactions and regulation. In fact, the DIC contains at its C-terminus WD40 (Tryptophan-aspartic acid dipeptide) repeats which mediate interaction with DHC tail, and at its N-terminus, a coiled-coil region which mediates interaction with DLCs and with the dynactin complex (see below; Vaughan and Vallee, 1995; Wilkerson *et al.*, 1995; Ma *et al.*, 1999; Habura *et al.*, 1999; Lo *et al.*, 2001; Mok *et al.*, 2001; King *et al.*, 2002). Interestingly, mammalian cells may carry a variety of DIC isoforms which are generated through alternative splicing and can be further differentiated through postranslational modifications (Dillman and Pfister, 1994; Pfister *et al.*, 1996; Nurminsky *et al.*, 1998; Kuta *et al.*, 2010). The motor domain (also called head domain) contains 6 AAA modules (ATPase Associated with various cellular Activities; Neuwald *et al.*, 1999, Iyer *et al.*, 2004) which are organized into a ring-like structure (Fig. 6; Samsó and Koonce, 2004; Carter *et al.*, 2011; Kon *et al.*, 2011, 2012). The AAAs are characterized by the Walker A or P-loop (Phosphate binding-Loop) and the Walker B motifs which are found in many prokaryotic and eukaryotic proteins (Walker *et al*., 1982; Hanson and Whiteheart, 2005). Walker A, with the signature sequence GXXGXGKT/S (G: Glycine, K: Lysine; T/S: Threonine or Serine and X: any amino acid), is involved in the binding of

phosphate from the nucleotides (*i.e.* ATP) and in the coordination of magnesium ions (Walker *et al*., 1982; Hanson and Whiteheart, 2005). The Walker B motif, with the general sequence hhhhDEXX (h: hydrophobic, D: Aspartate, E: Glutamate and X: any amino acid) is the site of ATP hydrolysis (Gibbons *et al*., 1991; Ogawa, 1991; Koonce *et al*., 1992; Mocz and Gibbons, 1996; Moz *et al*., 1998; Hanson and Whiteheart, 2005). In addition to the Walker motifs, sensor 1, which allow to discriminate between ADP and ATP, and sensor 2, involved in nucleotide binding, are also characteristic regions in AAA modules (Karata *et al*., 1999; Hattendorf and Lindquist, 2002a, 2002b; Hanson and Whiteheart, 2005). While all six AAA modules share sequence and structural similarities, they are functionally distinct. In fact, only AAA1 is known to carry out ATP hydrolysis; AAA2-4 are involved in binding ADP and ATP nucleotides and AAA5 and AAA6 have degenerate walker sequences and do not appear to participate in nucleotide binding (Gibbons *et al*., 1987; 1991; Ogawa, 1991; Koonce *et al*., 1992; Mocz and Gibbons, 1996; Moz *et al*., 1998; Takahashi *et al*., 2004; Kon *et al*., 2004; Cho *et al*., 2008; Carter *et al.*, 2011). Between AAA4 and AAA5, a ~10 nm MT-binding stalk extends and terminates with a globular MT binding domain (MTBD; Fig. 6; Gee *et al.*, 1997; Koonce, 1997; Carter *et al.*, 2008, 2011; Kon *et al.*, 2011, 2012; Nishikawa *et al.*, 2014). The long physical distance between the AAA1 module and the MT-binding stalk, implies a long-range intramolecular communication between the different domains. Indeed, the interaction with MTs has been shown to stimulate the ATPase activity of the motor domain (Paschal and Vallee, 1987; Shpetner *et al*., 1988; Shimizu *et al*., 1989) and the binding of ATP to AAA1 to dissociation from MTs (Kon *et al*., 2005; Imamula *et al*.,

2007). Recently, mutational analysis of the dynein complex has revealed that mutations in different parts of the complex can be alleviated by other mutations in distant sites of the motor (Sivagurunathan *et al*., 2012). The overall conformation of the DHC incurs significant conformational changes as it hydrolyses ATP and moves on MTs.

b) Dynein mechanochemistry.

The generally accepted model of dynein mechanochemical cycle, represent the motor complex as two globular head domains 'walking' on their respective MT binding stalks and 'stepping foot' with their MTBDs (Fig. 7; Carter). In this model, a cycle of 1) ATP binding, 2) ATP hydrolysis to ADP+Pi and 3) ADP release, is accompanied by weak MT-binding, dissociation from MT and strong MT-binding, respectively (Carter *et al*., 2013; Roberts *et al*., 2013). When one of the DHCs binds and hydrolyses ATP a conformational change involving the movement of the linker domain away from the stalk domain dissociate that DHC from the MT (Fig. 7; Imamula *et al*., 2007; Carter *et al*., 2013; Roberts *et al.*, 2013; Imai *et al*., 2015; Bhabha *et al*., 2016). This is called the "pre-power stroke" state and is followed by the release of inorganic phosphate (Pi) and a conformational change of the ADP-bound motor known as the "power stroke". During this state the linker domain returns to the pre-hydrolysis conformation and a strong binding to MTs occurs in a "post-stroke" state called Apo (Fig. 7; Imamula *et al*., 2007; Carter *et al*., 2013; Roberts *et al.*, 2013; Imai *et al*., 2015; Bhabha *et al*., 2016). In a recent study, it has been suggested that the role of AAA3 is to regulate the transmission of the cascade of conformational changes which originate at AAA1 (Bhabha *et al*., 2014; 2016).

While all these conformational changes are occurring a coordination between the two DHCs must occur in order to prevent the motor from falling-off the MT track. However, this can occasionally occur and when it does the dynein partner dynactin can anchor the motor complex to the MTs and thus increase its processivity.

c) Dynein partners and regulators

The study of dynein *in vitro*, has revealed that its long-range movement on MTs is supported by the multi-protein complex dynactin (dynein activator; Schroer and Sheetz, 1991; Gill *et al*., 1991). It has also been shown that Lissencephaly 1 (Lis1) protein is involved in the regulation of dynein (Reiner *et al.*, 1993; Reiner and Sapir, 2013).

Dynactin. It is now well established that dynactin is an essential partner in the function of dynein. Dynactin increases the enzymatic processivity of dynein, i.e. the ability to go through successive ATP hydrolytic cycles without falling off MTs (King and Schroer, 2000). This was initially shown in genetic studies using yeast, filamentous fungi and Drosophila and was confirmed using biochemical studies (Reviewed in Schroer, 2004). Using quick-freeze deep-etch electron microscopy, dynactin appears as an asymmetric complex with a rod-like mass (~10 x 40 nm) out of which extends a 25-50 nm arm (Fig. 8; Schafer *et al*., 1994; Chowdhury *et al*., 2015; Urnavicius *et al*., 2015). The majority of the rod-like mass is composed of the actin-related protein, Arp1, which polymerizes into a structure (4 dimers of Arp1), reminiscent of an actin filament (Bingham and Schroer, 1999). Arp1 filament interacts at one of its ends with the actin-capping protein CapZ and at the other end with the p62 subunit and another actin related protein, Arp11, which is thought to

stop addition of other Arp1 subunits (Eckley *et al*., 1999; Schafer *et al*., 1994). Two additional subunits, p25/p27, which also exist in free form in the cytoplasm, can bind to p62 (Fig. 8; Urnavicius *et al*., 2015). In addition, Dynamitin (p50) and p24/p22 subunits, bind to the complex at the junction between the Arp filament and the dynactin arm (Fig. 8). This latter, corresponds to the largest subunit, $p150^{Glued}$ (150 KDa), of dynactin, with ~1300 aa (Fig. 8; Urnavicius *et al*., 2015). At its N-terminus p150 folds into two MT binding domains: CAP-Gly, for cytoskeleton-associated protein-glycin-rich, and a basic domain, comprised of ~15 basic amino acids (Fig. 8; Riehemann and Sorg, 1993; Culver-Hanlon *et al*., 2006). These two domains have different affinities for MTs and thus can mediate tight interactions through the CAP-Gly domain or weak interactions with MTs, through the basic domain (Culver-Hanlon *et al*., 2006). In addition, to MT-binding, the p150 arm can mediate the association of dynactin with dynein through a ~220 aa flexible coiled-coil (CC) domain which can bind to DIC (King *et al*., 2003). The interaction between dynein and dynactin provides an additional increase in cargo binding possibilities for this fascinating motor complex. In fact, dynamitin has been shown to be involved in recruiting dynein to kinetochores through its interaction with the mitotic-checkpoint protein, Zeste white 10 (ZW10; Starr *et al*., 1998; Varma *et al*., 2006). Moreover, dynactin also interacts through dynamitin with Bicaudal D (BicD) protein (Bullock and Ish-Horowicz, 2001; Hoogenraad *et al*., 2001; Matanis *et al*., 2002). This protein, important for body axis determination in *D. melanogaster*, is involved in the transport of mRNAs during embryonic development and is also involved in dynein-mediated ER-to-Golgi movement of vesicles containing the small GTPase Rab6 (Bullock and

Ish-Horowicz, 2001; Hoogenraad *et al*., 2001; Matanis *et al*., 2002). The N-terminal region of BicD protein has been shown to interact with DIC and its C-terminal portion with dynamitin and thus provide an additional link between dynein and dynactin and between the motor complex and cargo (Hoogenraad *et al*., 2001; Matanis *et al*., 2002; Short *et al*., 2002; Fumoto *et al*., 2006; Wanschers *et al*., 2007; Razafsky *et al*., 2009). Using cryo electron microscopy, these interactions, were recently visualized in a reconstructed Dynein-Dynactin-BicD (DDB) complex in association with MTs (Fig. 9; Chowdhury *et al*., 2015).

Lis1. Lissencephaly, which litterally means "smooth brain", is a human disorder characterized by the absence of normal convolutions in the cerebral cortex and an abnormally small head (microcephaly; Fry *et al*., 2014). It is accompanied by many morphological and physiological malformations and most children with this disorder die within the first decade of their life (Fry *et al*., 2014). Genetic studies have identified many causative agents with different lissencephaly phenotypes. Classical lissencephaly, also known as type 1, is caused by mutations in three genes: *DBX*, (or doublecortin, a MT-associated protein), *TUBA1A* (involved in the biosynthesis of α-tubulin) and the PAFAH1B1 (Fry *et al*., 2014). This latter was the first gene identified as a causative agent of Lissencephaly and was thus called Lis1 (Reiner *et al*., 1993; Fry *et al*., 2014). LIS1 protein is well conserved between metazoa and fungi and is now known to interact with dynein (Xiang *et al*., 1995; Liu *et al*., 2000; Efimov and Morris, 2000; Lei and Warrior, 2000; Lee *et al*., 2003; Smith *et al*., 2000; Wynshaw-Boris and Gambello, 2001; Xiang, 2003). LIS1 is a 45 kDa polypeptide which folds into a C-terminal WD repeat that interacts with the tail domain and the

AAA1 module in dynein and with the dynamitin subunit in dynactin (Tai *et al*., 2002;). This interaction with dynein modulates the enzymatic activity of dynein and regulates the initiation of transport at the MT plus-ends (Mesngon *et al*., 2006; Yamada *et al*., 2008). In filamentous fungi, it can be localized to the MT plus-ends, together with dynein and dynactin (more on this below). As a dynein regulator, Lis1 is also involved in the many cellular functions of dynein, including the organization of the mitotic spindle and the transport of cargo (Xiang, 2003; Moon *et al*., 2014; Callejas-Negrete *et al*., 2015).

d) Dynein cellular functions and their organismal importance

Dynein performs a large number of functions during cell divisions and during the interphase. These functions include, but are not limited to, organelle distribution, centrosomal positionning of Golgi, ER-to-Golgi traffic, lysosomal distribution, endosome transport and recycling, cytoskeleton organization and cell division. Wagner *et al*., 2004; Johansson *et al*., 2007; Rocha *et al*., 2009; Delacrois *et al*., 2003; Heerssen *et al*., 2004; Yudin *et al*., 2008; Rishal and Fainzilber, 2010). During cell division, dynein participates in the assembly of the MT apparatus which segregates chromosomes, known as the the spindle (Heald *et al*., 1996; Merdes *et al*., 1996; Zimmerman and Doxsey, 2000). It also localizes to the kinetochores where it is in charge of moving spindle-assembly checkpoint proteins when chromosomes are properly attached to spindle MT (Foley and Kapoor, 2013). The kinetochore is a protein assembly connecting centromeric DNA to the spindle MTs, and in mammalian cells it has been reported that dynein help maintain this connection during poleward chromosome movement (Yang *et al*., 2007; Varma *et al*., 2008).

Additionally, during cell division, dynein can attach simultaneously to the cell cortex and to MTs and position the spindle in a specific location, such as in the case of asymmetric cell divisions (Kotak and Gönczy, 2013; McNally, 2013). During interphase, dynein, tethered at the cell cortex, can apply a pulling force on the MTs to allow the movement of the cytoskeleton in concert with the migrating edge of neurons or fibroblasts (Tsai *et al*., 2007; Dujardin *et al*., 2003). Moreover, dynein is involved in all well known examples of nuclear migration including the congression of male and female pronuclei during fertilization, the movement of daughter nuclei into the bud of *S. cerevisiae* during cellular reproduction, nuclear positioning to the egg cortex during embryogenesis in *D. melanogaster* and nuclear distribution during hyphal development of filamentous fungi (Robinson *et al*., 1999; Wynshaw-Boris and Gambello, 2001; Dujardin *et al*., 2003; Malone *et al*., 2003; Xiang *et al*., 1994; Shu *et al*., 2004; Tanaka *et al*., 2004; Xiang and Fischer, 2004; Tsai *et al*., 2005). Additionally, dynein and dynactin are important for membrane traffic during endocytosis and related-trafficking and the transport of intermediate filaments (Helfand *et al*., 2001; Shea and Flanagan, 2001; Soldati and Schliwa, 2006). It is obvious from all these cellular functions that dynein mutations which disrupt its proper operation will lead to significant effects on the growth and organization of cell. For a review of dynein disorders please check review by Schliwa and Woehlke (2003) and Jaarsma and Hoogenraad (2005).

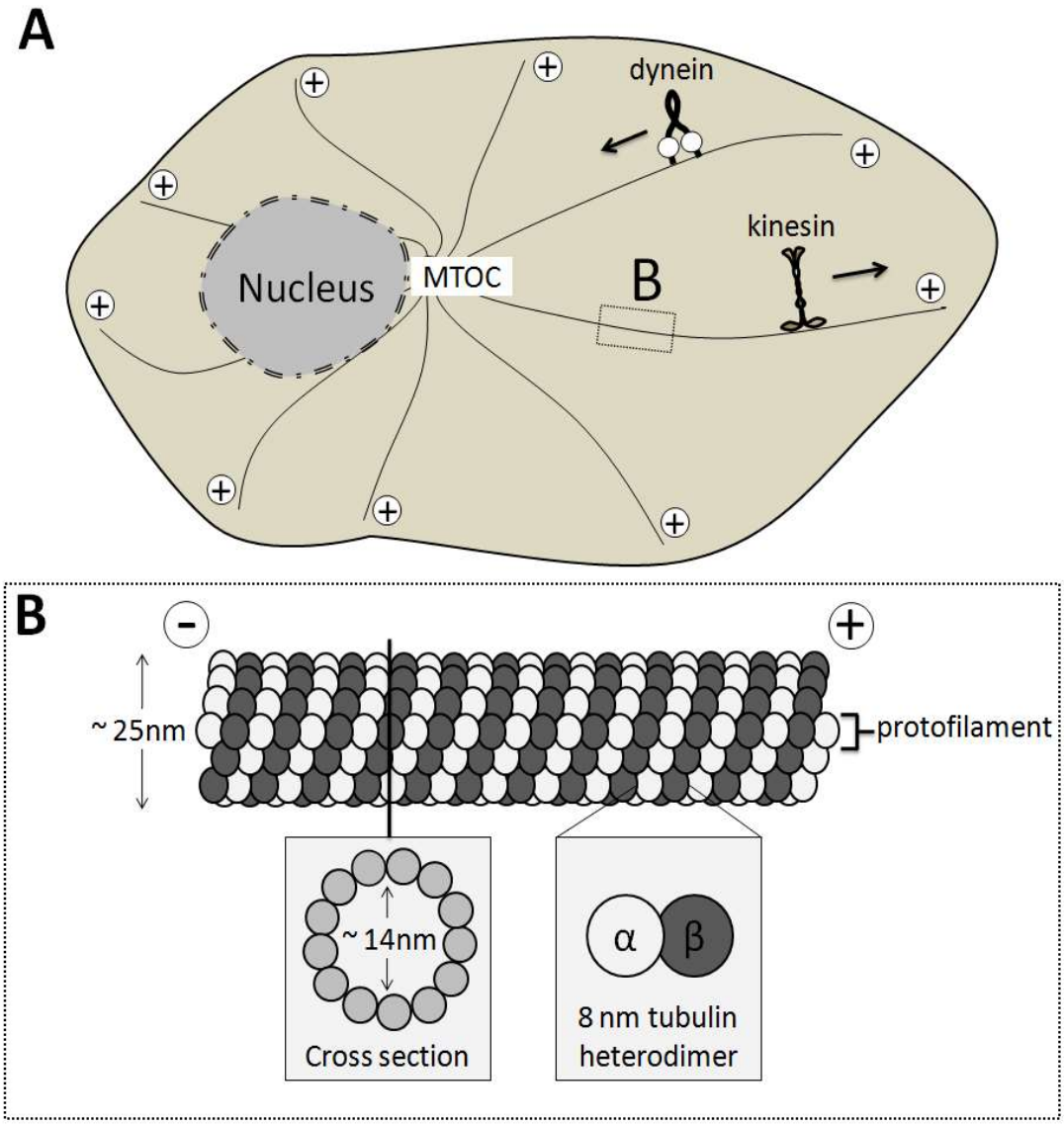


Figure 5. **General organization of the MTs in eukaryotic cells.** (A) MTs originate from the MT organizing center (MTOC) found inside the cell and extend their plus-end (+) towards the cell periphery. The plus-end is the site of polymerization/depolymerization of MTs. The motor protein dynein moves cargoes towards the minus-end/MTOC and kinesin (with the exception of kinesin-11 class) transports cargoes towards the plus-end. (B) MTs are organized into a cylindrical structure composed of 13 protofilaments, with an outer diameter of ~25nm and an internal diameter of ~14nm. Each protofilament is a polymer of the heterodimeric α/β subunit.

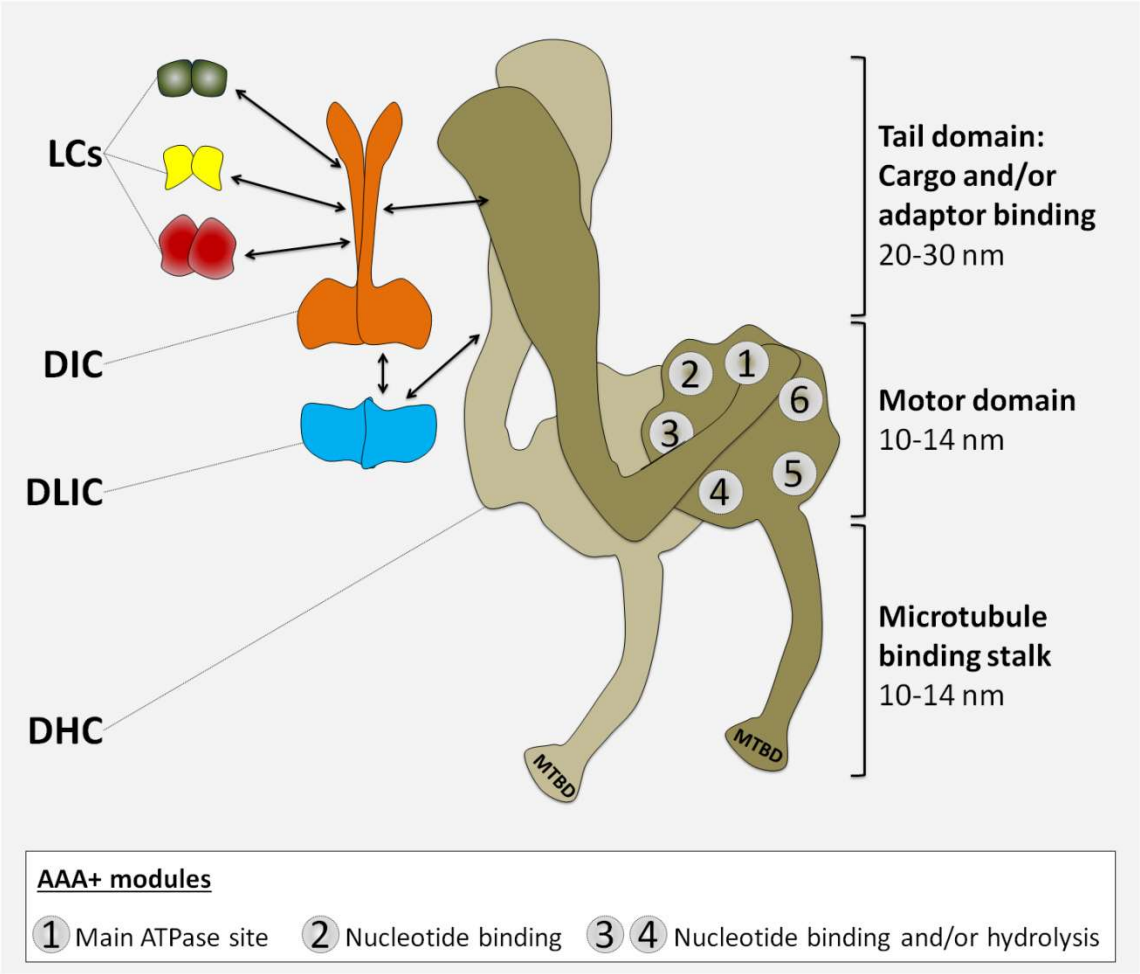


Figure 6. **The overall subunit organization of the dynein motor complex.** The dynein heavy chain (DHC) is the largest subunit of the complex and forms a dimer through its N-terminal tail domain. The motor domain folds as a ring composed of six AAA (ATPase Associated with various cellular Activities) modules. AAA1 is the main ATPase site and AAA 2-4 are involved in ATP/ADP (nucleotide) binding. A microtubule binding stalk extends between AAA 4 and 5 and terminates in a globular microtubule binding domain (MTBD). In addition to dimerization, the tail domain also serves as binding site for Dynein Intermediate chain (DIC) and dynein light intermediate chain (DLIC). Additional dynein light chains (LCs) are recruited to the motor complex through their interactions with DICs.

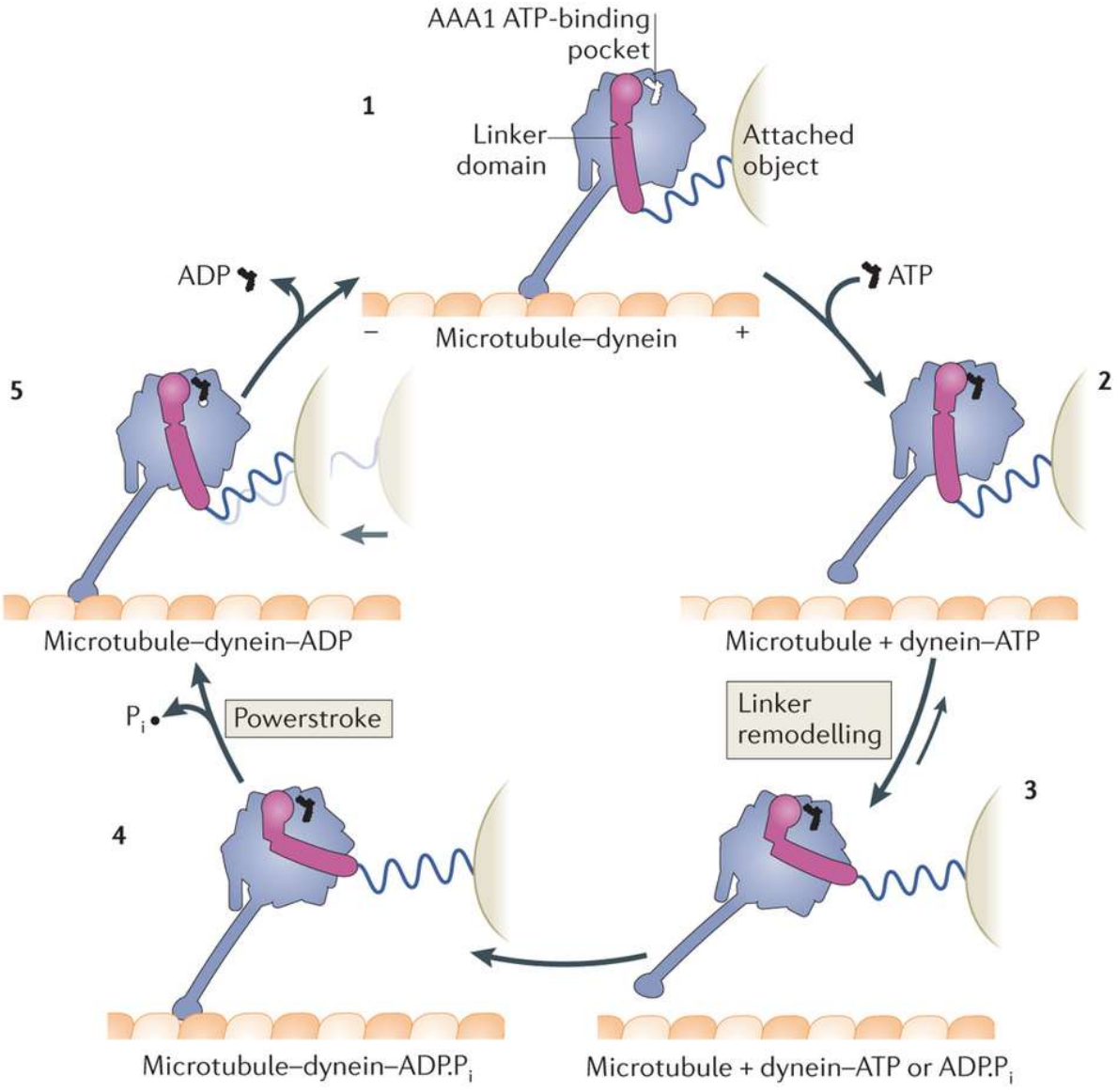

AAA1 ATP-binding pocket
1
Linker domain
Attached object
ADP
ATP
–
Microtubule–dynein
+
5
2
Microtubule–dynein–ADP
Microtubule + dynein–ATP
P_i
Powerstroke
Linker remodelling
3
4
Microtubule–dynein–ADP.P_i
Microtubule + dynein–ATP or ADP.P_i

Figure 7. **Model of the mechanochemical cycle of a cytoplasmic dynein motor.** Plus and minus signs indicate microtubule polarity. The dynein motor domain moves towards the minus end of the microtubule. Force exerted on an attached object is shown schematically by the stretching of a spring, which does not represent a part of the dynein structure. Conceptually, the attached object could represent a partnering motor domain in a cytoplasmic dynein dimer or the cargo microtubule of an axonemal dynein. With no nucleotide bound at the AAA1 ATPase site, the dynein motor domain is tightly bound to the microtubule (1). ATP binding induces the remodeling of the linker domain, which is displaced across the AAA+ ring (3). Remodeling of the linker extends the search range of the microtubule-binding domain along the microtubule. After hydrolysis of ATP to ADP and inorganic phosphate (Pi), the motor domain is thought to engage a new binding site on the microtubule, initially via a weak interaction (4). Strong binding to the microtubule accelerates the release of Pi from AAA1, inducing the linker to revert to its straight form. This transition is speculated to represent the powerstroke: the main step in which force (indicated by the light grey arrow) is transmitted to the attached object (5). Finally, ADP is released from AAA1 and the cycle restarts. Although the cycle is shown starting with dynein having no nucleotide bound at AAA1, it is not meant to imply that this is the longest-lived state. Indeed, in vivo, where the ATP concentration is in the millimolar range, ATP would rapidly bind at AAA1 once ADP is released, and thus this state would be populated only transiently.

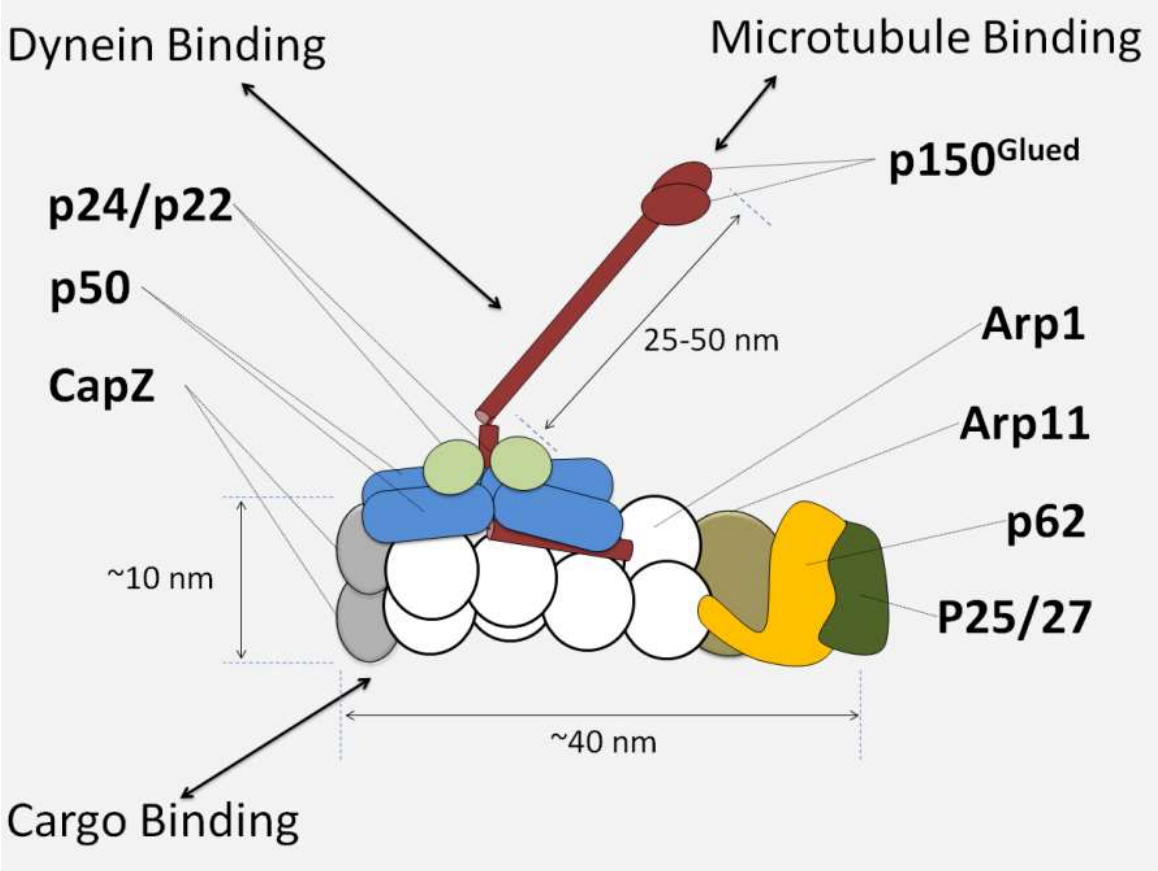


Figure 8. **The subunit organization of the dynactin complex.** Most of the dynactin subunits are contained within an actin-like filament composed of a short polymer of actin-like protein, Arp1. While CapZ protein is found at one end of the filament, Arp11 subunit is found at the other end and together they serve as capping proteins preventing further addition of Arp1 proteins. Arp11 also interacts with p62 subunit which in turn is bound by p25/p27 subunits. This 10 x 40 nm rod-like structure is involved in cargo binding. Out of the Arp1 filament extends a 25-50 nm flexible arm composed of a dimer of the largest subunit of the complex, $p150^{Glued}$. The proper folding of $p150^{Glued}$ depends on its interaction with the rest of the complex. Dynamitin (p50) and p24/22 subunits are involved in anchoring the p150 arm to the rest of the complex. $p150^{Glued}$ can interact with the dynein intermediate chain and with MTs.

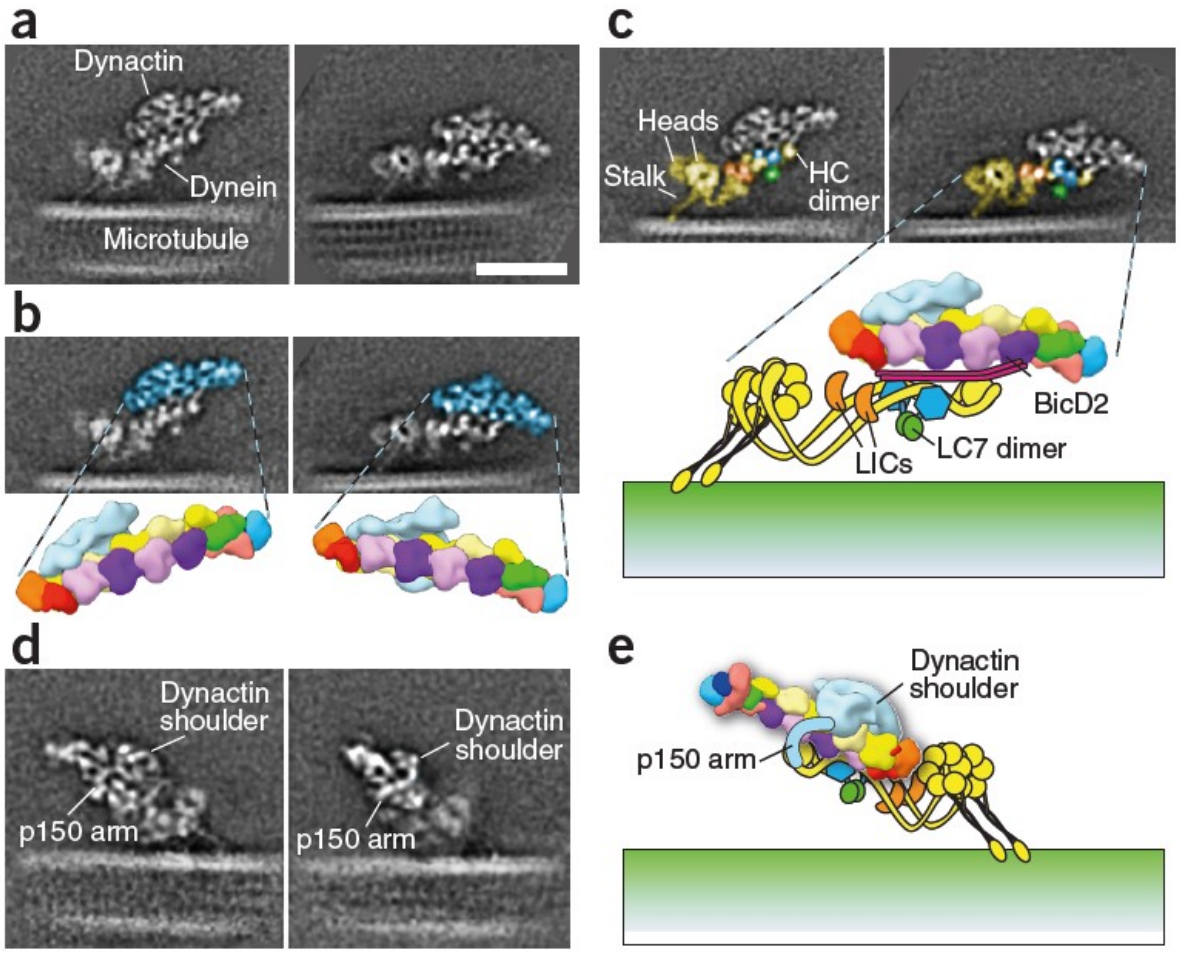


Figure 9. **Organization of the Dynein-Dynactin-Bicaudal (DDB–MT) complex.** (a) Two cryo-EM class averages of MT-bound DDB complexes, attached at slightly different angles relative to the MT. (b) Top, same class averages as in a, with dynactin colored blue. Bottom, low pass–filtered 3D model, oriented to correspond to the class average. (c) Dynein components in the 2D averages, assigned on the basis of information determined from the isolated-dynein class averages. Yellow, DHCs; orange, DLICs; blue, DIC C-terminal WD40 domain; green, putative LC7 dimer. (d) Two class averages of DDB–MT complexes, showing the location of the shoulder and an extension (p150 arm) that wraps around the Arp1 filament and contacts dynein. We propose that this corresponds to the $p150^{Glued}$ coiled coil. (e) A model of the DDB complex in the same orientation as the averages in d, with the path of the putative $p150^{Glued}$ coiled-coil extension (p150 arm) traced in light blue. Scale bar in a, 25 nm; all EM images are at the same scale.

1.2.4. Kinesin

While only one cytoplasmic dynein is known to carry minus-end directed transport, kinesin form a superfamily (also known as KIF) which is organized into a dozen families with over forty kinesin genes found in mammalian genomes (Miki *et al.* 2001). The defining feature of the KIF is the highly conserved motor domain (Hirokawa, 2009). The position of this latter within the protein define three major types: N-kinesins, with a N-terminal motor domain, M-kinesins, with a central motor domain, and C-kinesin, where the motor domain is C-terminal (Hirokawa, 2009). N-kinesin includes most known kinesins which move towards the plus-end of MTs; M-kinesin, with one representative, is known to depolymerize MTs and C-kinesin, with two representatives, are minus-end directed motors (Hirokawa, 2009; Jana *et al*., 2012). The description below of kinesin structure and organization will be limited to the conventional kinesin, kinesin-1.

a) The organization of kinesin-1 motors

Conventional kinesin was the first identified kinesin and remains the best studied plus-end directed motor. When kinesin-1was isolated from neuronal tissue, it was found to be a tetrameric complex with two kinesin heavy chains(KHC) and two kinesin light chains (KLC; Brady, 1985; Vale *et al.*, 1985a; Scholey *et al.*, 1985). However, subsequent isolations and studies of kinesin-1 from other tissues and organisms revealed that kinesin-1 can function as a dimeric complex composed of only KHCs. The association of KHC with KLCs reflects an enrichment of KLCs in mammalian tissues, including the brain, which is relevant to the function of kinesin-1 in these tissues. In fungi, as we will discuss later, KLCs are not conserved (Schoch

et al., 2003). Among the functions of kinesin-1 which does not involve KLC is the transport of mitochondria in neurons which uses an adaptor protein complex (Syntabulin-Milton-Miro complex)to interact with mitochondrial proteins (Cai *et al*., 2005, Glater *et al*., 2006; Cho *et al*., 2007; Wang *et al*., 2009). KHCs are organized into three domains: motor (also called head domain), stalk and tail (Fig. 10). The motor domain, which is very well conserved, is globular in shape and contains ATP and MT binding sites (Hirokawa *et al*., 1982; Aizawa *et al*., 1992; Vale and Fletterick, 1997; Kirchner *et al*., 1999; Schliwa and Woehlke, 2003). Studies suggest that the motor domains of kinesin and of the actin-dependent motor myosin, share a common ancestral origin related to the cell signaling G-proteins(Kull *et al*., 1998). In fact, the motor (roughly amino acids 1-350)contains two motifs called Switch I and Switch II which are found in myosin and G-protein (Kull and Endow, 2002). These 'switches', connecting ATP and MT binding sites to the rest of the motor, undergo conformational changes during ATP binding and hydrolysis which are important for kinesin movement (Sablin *et al*., 1996; Kull and Endow, 2002). In addition to these domains the motor head contains a phosphate-binding loop (P-loop) similar to the ones found in the AAA modules of dynein and in other nucleotide binding proteins (Kull *et al*., 1996; Sablin *et al*., 1996). Mutations in the P-loop can cause kinesin to be in a rigor state characterized by tight binding to MTs. In addition to the catalytic core, the motor domain contains a neck linker which is followed by the stalk domain and which is subject to conformational changes during kinesin movement (see below "kinesin mechanochemistry"; Vale and Fletterick, 1997; Case *et al*., 2000; Schliwa and Woehlke, 2003). The stalk which provides a dynamic link between the motor

and the tail domains contains coiled coil elements which are required for the homodimerization of KHCs (Vale and Fletterick, 1997; Kirchner *et al*., 1999; Schliwa and Woehlke, 2003). Following the stalk domain, is a tail region which has been shown to be required for cargo binding and for motor regulation (Stock *et al*., 1999; Coy *et al*., 1999; Friedman *et al*., 1999; Seiler *et al*., 2000).

b) The mechanochemistry of kinesin

The movement of kinesin along MTs share a similar "walking" behavior to that of dynein described above. However, the divergence between dynein and kinesin-1 motors is reflected by discrepancies in the molecular basis of movement production. Kinesin-1 motors are highly processive and can take hundreds of steps once they are attached to MTs (Howard *et al*., 1989; Block *et al*., 1990). The mechanism of movement of kinesin-1, which we describe here, is the widely accepted and empirically supported model, and is called "hand-over-hand" (Asbury *et al*., 2003; Yildiz *et al*., 2004). When kinesin is dissociated from MTs the two motor domains, one in each KHC, are tightly bound to ADP. When one of the motor domains contacts a MT, ADP is released and the motor domain becomes tightly bound to the MT. In this state, ATP binds the catalytic core of kinesin motor domain and induce a conformational change in the linker domain causing the other motor domain to move forward (in direction of MT plus-end; Fig. 10). This motion brings this latter closer to the MT where it attaches to the next tubulin heterodimer and binds ATP. The now trailing motor domain hydrolyses ATP into ADP-Pi, releases Pi and detaches from the MT. Simultaneously, an ATP-induced conformational change occur in the neck linker of the leading motor domain, bringing forward the ADP-bound motor domain,

which moves from trailing to forward position and attaches to the MT. The cycle of binding and hydrolyzing ATP then repeats causing the whole motor to walk processively along the MT track (Fig. 10). A study using inferometry techniques, have shown that one ATP molecule is consumed per each 8 nm step taken by kinesin-1 (Schnitzer and Block, 1997).

c) Kinesin regulation

In the absence of cargo, kinesin must be regulated to prevent unnecessary consumption of energy and congestion of MT tracks. Autoinhibition of kinesin motors is considered to be a general mechanism for the regulation of kinesin-1, kinesin-2, kinesin-3 and kinesin-7 families (Verhey and Hammond, 2009). When a cargo binds kinesin it leads to the release of autoinhibition.The earliest hints for this type of regulation came from electron microscope observation of kinesin-1 in a folded conformation (Hirokawa *et al*., 1989). Subsequent biochemichal and molecular studies showed that this conformation corresponds to the motor in an inactive state (Hackney *et al*., 1992; Verhey *et al*., 1998; Stock *et al*., 1999; Coy *et al*., 1999; Friedman *et al*., 1999; Seiler *et al*., 2000; Cai *et al*., 2007). The folded conformation of kinesin-1 is possible because the stalk domain contains hinge segments between the coiled-coil motifs. In fact, removal or mutation of these segments lead to a constitutively active kinesin (Coy *et al*., 1999; Friedman *et al*., 1999; Imanishi *et al*., 2006). When kinesin-1 folds the tail domain comes to close proximity with the motor domain and blocks ATPase activity and MT binding.The highly conserved amino acid sequence QIAKPIRP (Q, Glutamine; I, Isoleucine; A, Alanine; K, Lysine; P, Proline; R, Arginine) in the tail domain is in fact required for the autoinhibition of

motor activity (Seiler *et al*., 2000; Hackney *et al*., 2000; Cai *et al*., 2007). Recent biochemical and structural studies have shown that this sequence of kinesin-1 directly contacts the Switch I helix in the motor domain and prevents the release of ADP, thus blocking MT binding(Dietrich *et al*., 2008; Hackney and Stock, 2008; Kaan *et al*. 2011). Interestingly, for the mitotic kinesins, kinesin-5 and kinesin-7, phosphorylation of the tail domain has been shown to play a direct role in the release of the autoinhibition of the motor domain (Cahu *et al*., 2008; Verhey and Hammond, 2009).

d) Kinesin cellular functions and their organismal importance

The large numbers of kinesins which are conserved across eukaryotes is indicative of the many important functions of this fascinating motor. In neurons, where MTs plus-end are directed towards the axon termini, N-kinesins are responsible for the fast anterograde transport of membrane organelles and the slower transport of tubulin and neurofilament proteins (Hirokawa *et al*., 2009). Among the cargoes transported by the kinesin-1 and kinesin-3 are the synaptic precursor vesicles which contain proteins important for the function of the neurotramsmitter-containing synaptic vesicles (Hall and Hedgecock, 1991; Okada *et al*., 1995; Zhao *et al*., 2001; Byrd *et al*., 2001; Toda *et al*., 2008). Mutations affecting the function of KIF1B, a kinesin-3 member, leads to onset the type 2A Charcot-Marie-Tooth disease affecting approximately 1 in 2500 people in the United States (Zhao *et al*., 2001). This disease affects both motor and sensory nerves and causes weakness of the lower leg muscles during adolescence and hand weakness and sensory loss in later age. Other cargoes transported by kinesin in neurons and other cells, include,

mitochondria, early and late endosomes and cytoskeletal elements (Hirokawa *et al*., 2009). Together with dynein, kinesin, is also involved in the ER-Golgi, Golgi-ER and Golgi-plasma membrane traffic (Hirokawa *et al*., 2009). Moreover, kinesin is important for the progression of mitosis (Cross and McAinsh, 2014).

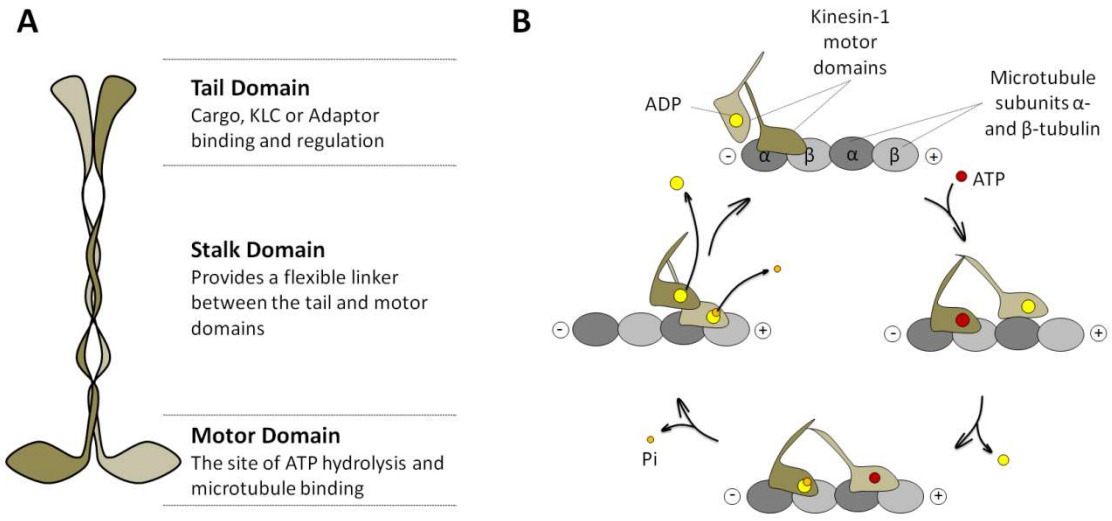


Figure 10. General organization and mechanochemistry of conventional kinesin. (A) Conventional kinesin (kinesin-1) is a homodimer organized into a motor domain, a stalk domain and a tail domain. In filamentous fungi kinesin light chains (KLC) are not conserved. The tail domain contains a regulatory sequence which interact with the motor domain and inhibit its ATPase activity. (B) A schematic description of the "hand-over-hand" model of kinesin movement. Only the motor domains are represented. The motor domain which is bound to ADP is detached from the MT; when it comes in contact with the MT, ADP is released. This leads to tight attachment to the MT which is accompanied by ATP binding. ATP binding and hydrolysis triggers conformational changes which brings forward the trailing head (ADP-bound) which attaches to the MT and binds ATP. The cycle repeats causing the motor heads to move in front of each other as the whole complex moves towards the MT plus-end (see text for more details).

1.3. The role of MT-based transport in hyphal growth

To achieve constant growth, the addition of novel plasma membrane and cell wall at the tip of the hyphae is required. While some of the components of growth are made on site, many of the vesicles providing plasma membrane and building materials are delivered from distal regions. Also important for growth, is the internalization of endocytic vesicles containing nutrients and recycled materials and their distribution to appropriate locations throughout the AHC. Moreover, as the hyphae grow, organelles, such as nuclei and mitochondria must be moved to avoid clustering and hyphal disorganization. A dynamic and well-regulated microtubule-based trafficking, carried by the motor proteins dynein and kinesin, is thus deployed throughout the apical hyphal cell. The development and use of large scale genetic screens, and of fluorescence microscopy, in the ascomycetes *N. crassa* and *A. nidulans* and the basidiomycete *U. maydis*, have allowed a better understanding of the function and regulatory mechanisms of MT-based transport in filamentous fungi. In *N. crassa*, *A. nidulans* and *U. maydis*, MTs are generally organized in such way that their plus-ends, which grow and shrink as needed, are directed towards the hyphal tip, and their minus-ends, associated with microtubule-organizing centers (MTOCs), are found in the distal regions of the apical cell (Han *et al*., 2001, Konzack *et al*., 2005; Schuchardt *et al*., 2005; Mouriño-Pérez *et al*., 2006, 2013). The hyphae of Neurospora can be distinguished from *N. nidulans* and *U. maydis* by their large hyphal diameter and greater number of MTs, which in some studies are suggested to adopt a mixed polarity at hyphal tips. However, it remains true that the majority of MTs plus-ends are directed towards the hyphal tip. The MT network is highly

dynamic and can reorganize during branch formation (Mouriño-Pérez *et al*., 2006). In this system, dynein carries cellular cargoes, such as early endosomes (EEs), from the hyphal tip to specific distal hyphal compartments (a transport qualified as retrograde). On the other hand, different kinesins, including kinesin-1 and kinesin-3, move cargoes towards the hyphal tips (anterograde transport).

1.3.1. Dynein and hyphal growth

Dynein was indirectly discovered in two independent studies; one aiming to understand the role of the COT-1 kinase in *N. crassa* and the second investigating nuclear distribution in *A. nidulans* (Plamann *et al*., 1994; Xiang *et al*., 1994).The first study, identified dynein and dynactin mutations as partial suppressors of the *co*lonial *t*emperature sensitive-1 (*cot-1*)mutant, which is characterized by compact colonial growth at restrictive temperatures, in contrast to the wild-type spreading mycelium of *N. crassa* (Collinge and Trinci, 1974; Steele and Trinci, 1977; Yarden et., 1992; Plamann *et al*., 1994). The *cot-1* gene encodes a serine/threonine protein kinase of the Nuclear Dbf-2-Related (NDR) family which is required for cell differentiation and polar morphogenesis in various organisms (Yarden et., 1992; Hergovich *et al*., 2006). In filamentous fungi, this NDR is required for hyphal elongation and its dysfunction leads to the cessation of tip extension and the absence of conidia (propagules used for asexual reproduction by *N. crassa*; Collinge and Trinci, 1974; Steele and Trinci, 1977; Yarden et., 1992; Plamann *et al*., 1994; Gorovits *et al*., 2000; Dvash *et al*., 2010). The temperature sensitive COT-1 ($COT\text{-}1^{ts}$) protein associates with the plasma membrane under permissive temperatures, and becomes exclusively cytoplasmic under restrictive temperatures (Gorovits *et al*.,

2000). The mutations in dynein and dynactin are thought to increase the residency of COT-1 at the plasma membrane; however how this might occur is unclear. The partial suppression by dynein and dynactin mutations of *cot-1*^{ts}allow hyphal elongation to resume but causes a *ropy* phenotype characterized by curly hyphae (Plamann *et al*., 1994; Bruno *et al*., 1996; Tinsley *et al*., 1996; Minke *et al*., 1999; Seiler *et al*., 1999). *ropy* mutants are also characterized by defects in Spk morphology, MT organization and vacuolar and nuclear distribution (Plamann *et al*., 1994; Bruno *et al*., 1996; Tinsley *et al*., 1996; Minke *et al*., 1999; Seiler *et al*., 1999; Riquelme *et al*., 2000, 2002). In *A. nidulans*, the study of *nu*clear *d*istribution (nud) mutants also led to the identification of the genes encoding dynein and dynactin subunits (Xiang *et al*., 1994, 1995a, 1999; Beckwith *et al*., 1998). The role of dynein in nuclear migration was also demonstrated in *U. maydis* and *Nectria haematocacca* in addition to *S. cerevisiae* and *Ashbyii gossypii* (Eshel *et al*., 1993; Suelmann *et al*., 1997; Inoue *et al*., 1998; Alberti-Segui *et al*., 2001; Straube *et al*., 2001). In addition to nuclear distribution, dynein is important for the motility of membrane vesicles, specifically EEs, and its dysfunction leads to the accumulation of EEs at hyphal tips (Lenz *et al*., 2006; Abenza *et al*., 2009; Zhang *et al*., 2010; Zhang *et al*., 2011; Egan *et al*., 2012). The interaction of dynein with EEs requires dynactin and the conserved Hook protein (HookA and Hok1 in *A. nidulans* and *U. maydis*, respectively; Bielska *et al*., 2014; Zhang *et al*., 2014; Xiang *et al*., 2015). This latter, interacts physically with dynein-dynactin and EEs and its role appears to be conserved between fungal and animal species (Bielska *et al*., 2014; Zhang *et al*., 2014). In recent years, other functions of dynein within the hyphae have been associated with EE motility; this

includes mRNP transport and ribosome distribution (Jansen *et al*., 2014; Higuchi *et al*., 2014). The repertoire of dynein function and cargoes is likely to be extended as the research on MT-based transport continues in filamentous fungi.

1.3.2. Kinesin and hyphal growth

The presence of motor proteins was directly investigated when organelle movement in the hyphae of *N. crassa* was found to be microtubule-dependent (Steinberg & Schliwa, 1993). This led to the MT-based purification and characterization of the conventional kinesin in *N. crassa* (NKIN or KIN-1; Steinberg & Schliwa 1995, 1997).

In addition to NKIN in *N. crassa*, conventional kinesin has been isolated from *A. nidulans* (Ascomycota), *Fusarium* (Ascomycota), *Ustilago maydis*(Basidiomycota) and from the Zygomycete *Syncephalastrum racemosum* (Lehmler *et al.*, 1997; Steinberg, 1997; Wu *et al.* 1998). Interestingly, kinesin light chain, which co-purifies with mammalian kinesin-1, was absent from all kinesin-1 prepared from fungal tissue(Steinberg & Schliwa 1995, 1997; Lehmler *et al.*, 1997; Steinberg, 1997; Wu *et al.* 1998). The analysis of genomic sequences from the Ascomycota and Basidiomycota have shown that there are 10 to 12 kinesins conserved within the filamentous fungi (Schoch *et al.*, 2003; Schuchardt *et al.*, 2005). However, the only kinesins for which a specific function was assigned are kinesin-1, kinesin-2 and kiensin-3. The general organization of kinesin-1 is the same as in metazoa, however some fungal-specific properties have emerged. For instance, the neck domain contains a helix which is suggested to be behind the high speeds of fungal kinesin-1 (Grummt *et al*., 1998; Kallipolitou *et al*., 2001; Song *et al*., 2001). In fact, the speed of kinesin-1 is four- to fivefold higher than its animal counterparts (Steinberg and

Schliwa, 1996; Kirchner *et al*., 1999; Grummt *et al*., 1998). The functions of kinesin motors within the hyphae include vacuole formation, microtubule-microtubule interactions, transport of EEs and possibly other membrane vesicles and transport of class III and V chitin synthase (Steinberg *et al*., 1998; Straube *et al*., 2006; Takeshita *et al*., 2015).Dissection of kinesin-1 from *N. crassa*, yielded important information about the regulation of the motor domain. The cargo-binding domain (CBD) is located roughly between residues 781 and 830 and contains the highly conserved regulatory motif (RIAKPL) between residues 886 and 892(Kirchner *et al*., 1999; Seiler *et al*., 2000). Deletion of CBD or the regulatory sequence leads to a constitutively active NKIN which become highly enriched at hyphal tips (Seiler *et al*., 2000).

1.3.3. The interaction between dynein and kinesin-1and its role in hyphal growth

To maintain retrograde transport, dynein motors must be concentrated at the hyphal tips where they can interact with vesicular cargoes, i.e. EEs. Using fluorescent tags, dynein, dynactin and Lis-1 can be observed at the microtubule plus-ends where they are considered to form cargo "loading zones" (Han *et al.* 2001; Zhang *et al.*, 2003; Lenz *et al.*, 2006). Therefore, any disturbance in the function of dynein or its regulation, leads to an accumulation of EEs at the hyphal tip and consequently to the perturbation of hyphal morphology and its elongation rate. Dissection of dynein motors have shown that there is an interdependence between dynein and dynactin subunits for their proper localization at these cargo loading zones (Han *et al.* 2001; Lenz *et al.*, 2006; Zhang *et al.*, 2002; 2003).Additionally, a number of studies in

filamentous Ascomycetes and Basidiomycetes have demonstrated that the targeting of dynein motors to microtubule plus-ends at hyphal tips is dependent on conventional kinesin (Zhang *et al.* 2003; Lenz *et al.* 2006). In fact, while kinesin-3 is known to be involved in the transport of EEs, the main function known to kinesin-1 in filamentous fungi is the transport of dynein motors to the hyphal tip. In fact, if the transport of dynein motor complex by kinesin-1 is blocked, similar abnormalities are observed. Interdependence between kinesin-1 and dynein has been indicated by earliest studies conducted on these motors. In fact, studies in which kinesin-1 was specifically targeted to inhibited anterograde transport, revealed that it leads to the inhibition of retrograde transport carried by dynein as well (Brady, 1999). In Drosophila, it has been shown that the role of dynein in embryo development is tightly linked to DmKHC (kinesin-1 in Drosophila; Duncan *et al.*, 2002; Ligon *et al.*, 2004; Serbus *et al.*, 2005).Direct interaction between DIC and KLC has been shown in Drosophila (Ligon *et al.*, 2004). However, the absence of KLC in fungi pushed many scientists to speculate that the interaction between dynein and kinesin-1 involves a vesicular cargo. In this model, dynein would hitch a ride on the vesicles transported by kinesin-1 to the hyphal tip.

Interestingly, the analysis of three independent partial suppressors of cot-1^{ts}, identified a previously uncharacterized gene which we hypothesized to be involved in the transport of dynein by kinesin-1. In the following sections we present experimental evidence supporting this hypothesis and discuss the role of this novel gene in hyphal growth.

Chapter 2

MATERIALS AND METHODS

2.1. Strains, media, and genetic techniques

N. crassa strains used in this study are listed in Table 1.Genetic procedures and media used for handling *N. crassa* are available through the Fungal Genetic Stock Center (www.fgsc.net; McCluskey *et al.* 2003). The strain FGSC 9717 was used as reference strain for growth rate determinations and protein localization studies. Δ*nkin* was generated by replacement of the *nkin* gene by the hygromycin resistance cassette (hph). Mutants 1.2, a29.4 and 38.5 were identified in a genetic screen for partial suppressors of *cot-1(ts)* mutant (Sivagurunathan *et al.*, 2012). Using sib-selection cloning, the cosmid 'H052H1' from the pLorist library (Kelkar *et al.*, 2001) complemented the ropy (curled hyphae) growth defect of mutant 1.2. Screening available knock-out strains for each of the genes present on this cosmid identified a ropy phenotype in the strain deleted for NCU01277. Heterokarya between ΔNCU01277 and the three mutants did not restore WT characteristics, confirming that these strains were defective in gene function of NCU01277.

2.2. Plasmid construction, fungal expression of tagged proteins and microscopy.

Strains expressing mCherry- or EGFP-tagged proteins were generated using the Marker Fusion Tagging (MFT) technique (Lai *et al.*, 2010), and all fusion proteins were expressed under their own promoters and within their genomic loci. Four different cassettes were generated: mCherry-hph, hph-mCherry, GFP-hph, hph-

GFP. The cassette of choice was then PCR-fused to homology fragments of the gene of interest (primers are listed in Table 2) and electroporated into Δ*mus-51*. Correct clones identified by WT growth, hygromycin resistance, and microscopy/Western blot to confirm expression of the desired fusion protein were backcrossed to Δ*mus-51* for further genetic purification. The proper genomic integrations are verified by PCR amplification and sequencing of 5' and 3' integration junctions. Truncated variants of RO-16 and NKIN were generated using the same method by replacing the sequences to be deleted by hph or hph-mCherry/hph-GFP cassettes for fluorescently tagged truncations. Tagged NKIN(S95L) was generated by integration of tagging cassette in the *col-4* strain. Strains with multiple tags or in combination with other mutation and/or genetic variations were generated by crosses and selection of appropriate progeny. Images were acquired from live hyphae grown on microscope slides coated with media as described (Sivagurunathan *et al.*, 2012). An Olympus BX50 inverted microscope equipped with Plan FI dry 20X (0.50 NA), 40X and oil immersion 100X (1.3 NA) objectives and a SPOT RT-SE 18 camera and SPOT™ software (Diagnostic Instruments) were used for image acquisition. Images were processed using Adobe Photoshop CC and ImageJ (NIH).

2.3. Protein methods.

Liquid *N. crassa* cultures were grown at room temperature, harvested gently by filtration using a Büchner funnel and ground in liquid nitrogen. Crude cell extracts were generated as described (Dettmann *et al.*, 2012). Briefly, the pulverized mycelium was mixed 1:1 with IP-Buffer (50 mM Tris/HCL pH 7.5, 100 mM KCl, 10

mM MgCl2, 0.1% NP-40, 5 mM NaF, 1 mM PEFA bloc SC, 2mM DTT, 1 mM Na3VO4, 25 mM β-glycerophosphate, 2 mM benzamidine, 2 ng/µl pepstatin A, 10 ng/µl aprotinin, 10 ng/µl leupeptin) and centrifuged twice at 4°C (15 min at 4500 g and 30 min at 14000 g) to obtain crude cell extracts. Co-immunoprecipitation experiments were performed as described (Heilig *et al.*, 2014). Cell extracts and protein-decorated beads were washed twice with IP buffer, and immunoprecipitated proteins were recovered by boiling the beads for 10 min at 98°C in 3x Laemmli buffer and separated by 7.5% SDS-PAGE. Monoclonal mouse α-myc (9E10, Santa Cruz), α-GFP (B2, Santa Cruz), GFP trap beads (Chromotek) and polyclonal affinity-purified anti-RO-1 and anti-RO-3 antisera (Minke *et al.*,1999) were used in this study. Precipitates of mock-IPs from WT extracts and of cells expressing only one tagged construct were used as controls.

2.4. Two-hybrid plasmids and methods.

The Matchmaker Two-Hybrid system 3 (Clontech, USA) was used according to the manufacturer's instructions. cDNAs for two- hybrid tests were amplified with primers listed in Table 2, spanning the coding regions of the *ro-16* and *nkin* genes and of the Drosophila kinesin heavy chain (kind gift of Günther Wöhlke, TU Munich, Germany). cDNAs were cloned either into the pGADT7 vector containing the GAL4 activation domain or into the pGBKT7 vector containing the DNA-binding domain. Fusion proteins were co-expressed in *S. cerevisiae* strain AH109 and potential interactions determined by growth tests on SD medium lacking leucine, and tryptophan for plasmid selection and on SD medium lacking adenine, histidine, leucine, and

tryptophan to test for interaction of the fusion constructs as described (März *et al.*, 2012; Dettmann *et al.*, 2013).

Table 1. Strains used in this study		
Strain	Genotype	source
∆mus-51	mat A (or mat a) his-3 ∆mus-51::bar+	FGSC 9717
Strains used for wild-type protein localization		
RO16-mCherry	mat A (or mat a) his-3 ∆mus-51::bar+ ro-16-mCherry-Hygr (C*)	This study
DHC-mCherry	ro-1-Hygr-mCherry(N*)	This study
DIC-mCherry	mat A ro6+-mCherry+	Sivagurunathan *et al*., 2012
DIC-mCherry, Tub-GFP	mat a ro6+-mCherry+his-3+::Pccg-1-Bml+-sgfp+	Sivagurunathan *et al*., 2012
DLIC-mCherry	ro-13-mCherry+	This study
P150-mCherry	ro3-mCherry+	Sivagurunathan *et al*., 2012
Nkin-GFP	Hygr-GFP-Nkin-(N*)	This study
RAB6-GFP	Rab6-gfp-hyg	This study
Tub-GFP	rid(RIP1) mat A his-3+::Pccg-1-Bml+-sgfp+	FGSC #9520
ro-16 mutants		
ro-16 mutant 1.2	his-3 ∆mus-51::bar+ ro-16(224*) cot-1(ts)	This study
ro-16 mutant a29.4	his-3 ∆mus-51::bar+ ro-16(FS127) cot-1(ts)	This study
ro-16 mutant 38.5	his-3 ∆mus-51::bar+ ro-16(FS290) cot-1(ts)	This study
ro-16 knock-out	his-3 ∆mus-51::bar+ ∆ro-16::HygR	FGSC 13840
Ro-16(∆1-51)	his-3 ∆mus-51::bar+ hygr-ro-16(∆1-51)	This study
Ro-16(∆1-110)	his-3 ∆mus-51::bar+ hygr-ro-16(∆1-110)	This study
Ro-16(∆1-110)-mCherry	his-3 ∆mus-51::bar+ hygr-mCherry-ro-16(∆1-110)(N)*	This study
Ro-16(∆1-150)	his-3 ∆mus-51::bar+ hygr-ro-16(∆1-150)	This study
Ro-16(∆1-150)-mCherry	his-3 ∆mus-51::bar+ hygr-mCherry-ro-16(∆1-150) (N)	This study
Ro-16(∆1-293)	his-3 ∆mus-51::bar+ hygr-ro-16(∆1-293)	This study
Ro-16(∆1-293)-mCherry	his-3 ∆mus-51::bar+ hygr-mCherry-ro-16(∆1-293)(N)	This study

Ro-16(Δ293-417)	his-3 Δmus-51::bar+ ro-16(Δ293-417)-hygr	This study
Ro-16(Δ293-417)-mCherry	his-3 Δmus-51::bar+ ro-16(Δ293-417)-mCherry-hygr(C)*	This study
nkin mutants		
Δnkin	mat a his-3 Δmus-51::bar+ Δnkin::HygR	This study
Nkin(rigor)	mat ahis-3 Δmus-51::bar+ Hygr-Nkin(G93E)(N*)	This study
Nkin(rigor)-GFP	mat ahis-3 Δmus-51::bar+ Hygr- GFP-nkin(G93E)(N*)	This study
Nkin(rigor)-mCherry	mat ahis-3 Δmus-51::bar+ nkin(G93E)-Hygr-mCherry(N*)	This study
col-4	mat ahis-3 Δmus-51::bar+ nkin(S95L)	FGSC 943
Nkin(col4)-mCherry	mat ahis-3 Δmus-51::bar+ Hygr-mCherry-nkin(S95L) (N*)	This study
Nkin(Δ740-928)	mat ahis-3 Δmus-51::bar+ nkin(Δ740-928)-Hygr	This study
Nkin(Δ740-928)-mCherry	mat ahis-3 Δmus-51::bar+ nkin(Δ740-928)-Hygr-mCherry(C*)	This study
Nkin(Δ885-928)	mat ahis-3 Δmus-51::bar+ nkin(Δ885-928)-Hygr	This study
Nkin(Δ885-928)-mCherry	mat ahis-3 Δmus-51::bar+ nkin(Δ885-928)-Hygr-mCherry(C*)	This study
Dynein mutants		
Δro-1 (DHC null mutant)	mat a his-3 Δmus-51::bar+ ro-1K770* qde-2::Bml^R	Sivagurunathan *et al*., 2012
Dynactin p150	mat aro□6::hygr	FGSC 14726
Cross progeny between different strains		
DIC-mCherry in Δnkin	ro6+::mCherry+Nkin::Hygr	This study
DIC-mCherry, Nkin-GFP,in Δro-16	ro6+::mCherry+Hyg-GFP-Nkinro-16::Hygr	This study
DIC-mCherry, Tub-GFP, in Δro-16	mat a ro6+-mCherry+his-3+::Pccg-1-Bml+-sgfp+Δro-16::HygRΔmus-51::bar+	This study
DHC-mCherry in Δro-16	mat a his-3 Δmus-51::bar+ro□1-Hygr-mCherryΔro-16::HygR	This study
P150-mCherry in Δro-16	Δmus-51::bar+ ro3-mCherry+ Δro-16::HygR	This study
RO16-mCherry in Δnkin	Δmus-51::bar+ro-16-mCherry-Hygrnkin::Hygr	This study

RO16-mCherry in ∆ro-1	∆mus-51::bar+ro-16-mCherry-Hygrro-1K770*	This study
RO16-mCherry in nkin(∆885-928)	∆mus-51::bar+ro-16-mCherry-Hygr nkin(∆885-928)-hygr	This study
RO16-mCherry in nkin(∆740-928)	∆mus-51::bar+ro-16-mCherry-Hygr nkin(∆740-928)-hygr	This study
RO16-mCherry in nkin(∆885-928) with ∆ro-1	∆mus-51::bar+∆mus-51::bar+ro-16-mCherry-Hygr nkin(∆885-928)-hygr ro-1K770*	This study
DIC-mCherry in Nkin(∆885-928)	∆mus-51::bar+ro6+-mCherry+nkin(∆885-928)-hygr	This study
DIC-mCherry in Nkin(∆740-928)	∆mus-51::bar+ro6+-mCherry+nkin(∆740-928)-hygr	This study
DIC-mCherry in nkin(∆885-928) with ∆ro-16	∆mus-51::bar+ro6+-mCherry+nkin(∆885-928)-hygr ∆ro-16::HygR	This study
RO16-mCherry, Tub-GFP in col-4	∆mus-51::bar+ro-16-mCherry-Hygr his-3+::Pccg-1-Bml+-sgfp+nkin(S95L)	This study
DIC-mCherry, Tub-GFP in col-4	∆mus-51::bar+ro6+-mCherry his-3+::Pccg-1-Bml+-sgfp+nkin(S95L)	This study
DIC-mCherry, Rab6-GFP in col-4	∆mus-51::bar+ro6+-mCherryro6+-mCherry Rab6-gfp-hyg	This study
*N: N-terminal; C:C-terminal		

Table 2. Primers used in this study	
Name	**Sequence 5'-3'**
Generation of mCherry-HphR (CH) tag for C-terminal tagging	
Frag. 1 (C) F	AAGCTTCATCATCATCATCATC
Frag. 1 (C) R	GTGAGTTCAGGCTTTTTCATCTTGTACAGCTCGTCCATG
Frag. 2 (H) F	GCATGGACGAGCTGTACAAGATGAAAAAGCCTGAACTCA
Frag. 2 (H) R	TTATTCCTTTGCCCTCGGACGAG
Generation of HphR-mCherry (HC) tag for N-terminal tagging	
Frag. 1 (H) F	ATGGGTAAAAAGCCTGAACTCAC
Frag. 1 (H) R	GATGATGATGATGATGAAGCTTTTCCTTTGCCCTCGGACGAGTGC
Frag. 2 (C) F	GCACTCGTCCGAGGGCAAAGGAAAAGCTTCATCATCATCATCATC
Frag. 2 (C) R	TGCTCCTGCTCCAGCACCCTTGTACAGCTCGTCCATGC
Generation of GFP-HphR (GH) tag for C-terminal tagging	
Frag. 1 (G) F	GGTGCTGGAGCAGGAGCAATGGTGAGCAAGGGCGAGGAGC
Frag. 1 (G) R	TGAGTTCAGGCTTTTTCATTCCGGACTTGTACAGCTC
Frag. 2 (H) F	GAGCTGTACAAGTCCGGAATGAAAAAGCCTGAACTCA
Frag. 2 (H) R	TTATTCCTTTGCCCTCGGACGAG
Generation of HphR-GFP (HG) tag for N-terminal tagging	
Frag. 1 (H) F	ATGGGTAAAAAGCCTGAACTCAC
Frag. 1 (H) R	GCTCCTCGCCCTTGCTCACCATTTCCTTTGCCCTCGGACGAG
Frag. 2 (G) F	CTCGTCCGAGGGCAAAGGAAATGGTGAGCAAGGGCGAGGAGC
Frag. 2 (G) R	TGCTCCTGCTCCAGCACCTCCGGACTTGTACAGCTCGTC
RO16 tagging with mCherry-Hph (CH), verification and sequencing	
5' RO16 F	GAGGGTCTCCGCGATCTA
5' RO16 R	ATGATGATGATGAAGCTTAGCCTCAACACTCCCTGC
RO16-CH F	GCAGGGAGTGTTGAGGCTAAGCTTCATCATCATCAT
RO16-CH R	CAAGTCTGCCTCCCTCTTGGATCCTCACTATTACTTGTAC

3' RO16 F	GTACAAGTAATAGTGAGGATCCAAGAGGGAGGCAGACTTG
3' RO16 R	TCATGTTTAGTCCCTTCGAATGC
Check 5' F	GTCTTCACCGCAAACACCA
Check 5' R	TTGGAGCCGTACATGAAC
Check 3' F	GCATAACAGCGGTCATTGA
Check 3' R	TGACTACGGCCTAAAGGC
NKIN tagging with HphR-GFP (HG)	
5' NKIN F	GCGAGTAGTGTGTCACATTGA
5' NKIN R	GTGAGTTCAGGCTTTTTACCCATTATGGCGATTCCCTTGCAGC
NKIN-HG F	GGCAGGGAGTGTTGAGGCTATGGGTAAAAAGCCTGAACTCAC
NKIN-HG R	TGCTCCTGCTCCAGCACCTCCGGACTTGTACAGCTCGTC
3' NKIN F	GGTGCTGGAGCAGGAGCAATGTCGTCAAGTGCGAATA
3' NKIN R	TCCATATAGCTGACCCGGAC
Check 5' F	GTTCAGACTGCGTCTCAGGCT
Check 5' R	TACATCGAAGCTGAAAGCAC
Check 3' F	GCAGTGCTTCAGCCGCTA
Check 3' R	TCGGAACCAGCCAGATCGAC
Generation of RO-16 N-terminal truncation constructs	
Upstream homology sequence	
5' RO16 F	ATCCACCTCAACACTTCATCAC
5' RO16 R	CGGTGAGTTCAGGCTTTTTCATGGTGGGCTTGGTGTTTTG
ro16 Δ1-150 F	GCATGGACGAGCTGTACAAGGGACCGAACGGAAGGGGCCACC
ro16 Δ1-150 R	GGTGGCCCCTTCCGTTCGGTCCCTTGTACAGCTCGTCCATGC
ro16 Δ1-241 F	GCATGGACGAGCTGTACAAGGGAGCCCTAACAACCCACCTAACC
ro16 Δ1-241 R	GGTTAGGTGGGTTGTTAGGGCTCCCTTGTACAGCTCGTCCATGC
ro16 Δ1-293 F	GCATGGACGAGCTGTACAAGGGAGCCGCCTTCGCCCAGCTCGC

ro16 Δ1-293 R	GCGAGCTGGGCGAAGGCGGCTCCCTTGTACAGCTCGTCCATGC
ro16 Δ293-417 F	ATGCTGGTCAGCCTCTCGTCAAAGCTTCATCATCATCATCATCACAG
ro16 Δ293-417 R	CTGTGATGATGATGATGATGAAGCTTTGACGAGAGGCTGACCAGCAT
ro16 Δ369-417 F	CTGGAGCGAGAGGCTGCGGAAGCTTCATCATCATCATCATCACAG
ro16 Δ369-417 R	CTGTGATGATGATGATGATGAAGCTTCCGCAGCCTCTCGCTCCAG
ro16 Δ1-110 F Hyg only	CTCGTCCGAGGGCAAAGGAAGGAGAAGAAGAGGACCTGCTCCC
ro16 Δ1-110 R Hyg only	GGGAGCAGGTCCTCTTCTTCTCCTTCCTTTGCCCTCGGACGAG
ro16 Δ1-150 F Hyg only	CTCGTCCGAGGGCAAAGGAAGGACCGAACGGAAGGGGCCACC
ro16 Δ1-150 R Hyg only	GGTGGCCCCTTCCGTTCGGTCCTTCCTTTGCCCTCGGACGAG
ro16 Δ1-241 F Hyg only	GAAGGTGCTGGAGCAGGAGCAGCCCTAACAACCCACCTAACC
ro16 Δ1-293 F Hyg only	GAAGGTGCTGGAGCAGGAGCAGCCGCCTTCGCCCAGCTCGC
ro16 Δ293-417 F hyg only	ATGCTGGTCAGCCTCTCGTCAGGAATGAAAAAGCCTGAACTCACCG
ro16 Δ293-417 R hyg only	CGGTGAGTTCAGGCTTTTTCATTCCTGACGAGAGGCTGACCAGCAT
ro16 Δ369-417 F hyg only	CTGGAGCGAGAGGCTGCGGGGCGGAATGAAAAAGCCTGAACTCACCG
YH-NKIN-726-NdeI-f	CAT ATGATG GCC AAC GGC AAG ACC GTC
YH-NKIN-stop-EcoRI-r	GAA TTC TTA CGA CTT CTG GAA GAA CCA AC
YH-RO16-atg-NdeI-f	CAT ATGATGATG CCG CCG TCT TCA CCG
YH-RO16-stop-EcoRI-r	GAA TTC TCA AGC CTC AAC ACT CCC TGC
YH-RO16-288-NdeI-f	CAT ATGATG CCG CGG CCG TCG ACG
YH-RO16-371-EcoRI-r	**GAA TTC** CTA CTC GCG ATT CCG CAG CCT C
YH-NKIN-774-NdeI-f	**CAT ATG** GTG CTC CGC AGC AGC AAC AAC
YH-NKIN-419-NdeI-f	**CAT ATG** CCT GCT ATC TCA GAC CGA GC

YH-NKIN-582-EcoRI-r	**GAA TTC** CTA GGC AAC TGC CCG TTC GTT ATC
YH-DmKHC-820-EcoRI-f	TAT GGC CAT GGA GGC CAG T**GA ATT C**CT GCG AAA ACT TTT CGT TCA GG
YH-DmKHC-stop-BamHI-r	TCT GCA GCT CGA GCT CGA T**GG ATC C**CT ACG AGT TGA CAG GAT TAA CCT G
Restriction enzyme recognition sites are indicated in bold	

CHAPTER 3

ROPY-16, A NOVEL LINK BETEEN DYNEIN AND KINESIN-1

3.1. Identification of ROPY-16 in *Neurospora crassa.*

The *N. crassa* NDR protein kinase COT-1 and associated proteins are essential for spreading hyphal growth (Steele and Trinci, 1977; Yarden *et al.*, 1992; März and Seiler, 2010), and mutations in components of the dynein complex partially suppress this growth defect (Plamann *et al.*, 1994; Seiler *et al.*, 2006). A large-scale suppressor analysis has specifically defined almost all known subunits of the dynein, dynactin, and LIS-1 complexes (Bruno *et al.*, 1996; Sivagurunathan *et al.*, 2012). However, three mutants (1.2, a29.4, and 38.5) defined a novel complementation group, suggesting that the corresponding gene encodes a previously uncharacterized gene required for dynein function. NCU01277 (named *ropy-16* (*ro-16*)) was identified by sib-selection cloning as described in the Methods section.

3.2. Results

3.2.1. Characteristics of ROPY-16

Sequencing of NCU01277 in the mutants revealed a non-sense mutation at codon 224 for the strain 1.2, while mutants a29.4 and 38.5 contained frameshift mutations at codons 127 and 290, respectively (Fig.11 A). The *ro-16* ORF had one predicted intron, encoding a protein of 417 residues with an estimated molecular mass of 44.8 KDa. Homologs of RO-16 were present in the genomes of filamentous ascomycete fungi (the Pezizomycotina), which include many plant and animal pathogens, but were absent from ascomycete yeasts, basidiomycetes and all other sequenced

eukaryotic genomes (Fig.11 B). Comparison with predicted fungal orthologs identified conserved sequence stretches across RO-16, which included a C-terminal region that is predicted to form a coiled-coil (Fig.12).The rate of mycelial expansion in ∆*ro-16* was reduced to *ca* 60% of wild-type (WT), resulting in a growth defect that was not as severe as those of ∆*ro-1*(dynein heavy chain deletion) and ∆*nkin* (kinesin-1 deletion), where colony expansion is reduced to *ca* 35% of WT (Fig. 13A). This was consistent with the hyphal morphology of ∆*ro-16*, which was not as severely altered as that of ∆*ro-1* and ∆*nkin*. Similarly, nuclear distribution in germlings of ∆*ro-16* was only affected to a minor extent (Fig. 13B). Since nuclear distribution is dependent on functional dynein motors, these results suggest that in the absence of RO-16, the motor activity of dynein is not abolished. However, the dynein-specific *ropy* phenotype of ∆*ro-16* and the partial suppression of *cot-1(ts)* suggested RO-16 as a novel protein involved in dynein function.

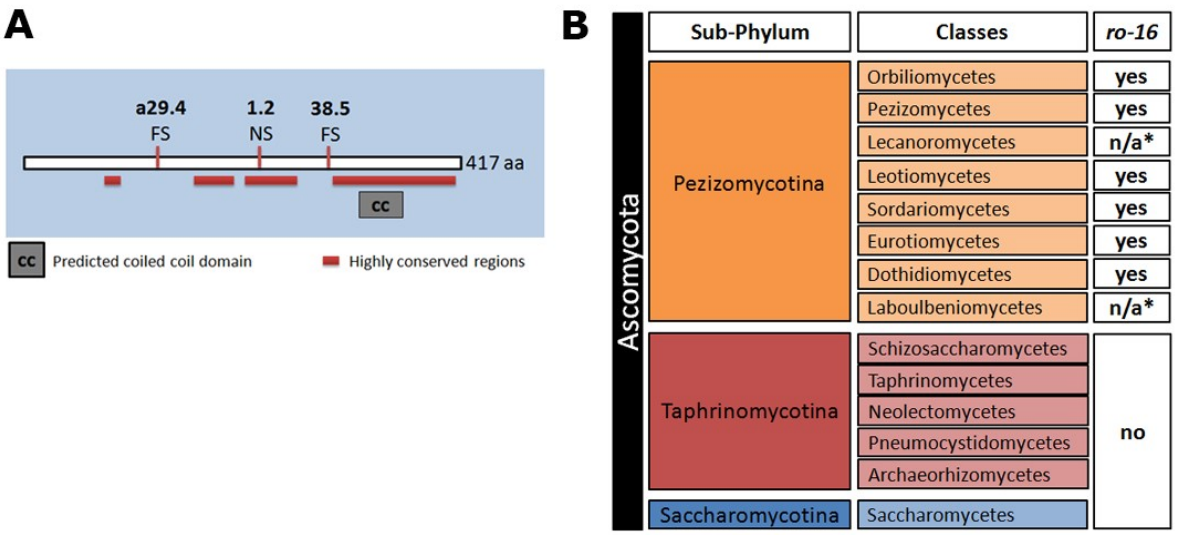


Figure 11. **RO-16 is a conserved fungal protein. (A)** Position and type of mutations in RO-16 isolated as partial *cot-1(ts)* suppressors; a29.4: frame shift (FS), 1.2: non-sense (NS) and 38.5: FS. Conserved sequence regions are indicated by red bars. A carboxy-terminal coiled-coil (CC) domain is found in the C-terminal region. **(B)**Taxonomical distribution of RO-16 within the Ascomycetes. RO-16 is absent from the Taphrinomycotina and Saccharomycotina to which the yeasts, the dimorphic *Candida* species and the filament-forming species *Ashbya gossypii* belong. N/A: RO-16 homologs were not detected in these taxa, because of unavailable or partial genome sequences.


Neurospora
Fusarium
Magnaporthe
Endocarpon
Exophiala
Aspergillus
Dothistroma
Bipolaris
Macrophomina
Botrytis
Pyronema
Arthrobotrys

Figure 12**. RO-16 contains well conserved sequences.** Multiple sequence alignment between RO-16 and putative orthologs from across the sub-phylum Pezizomycotina using Clustal Omega (http://www.ebi.ac.uk/services). *****indicate identical residues identical in all sequences. **:** and **.** indicate conserved and semi-conserved substitutions, respectively. Residues between positions 318 and 370 correspond to predicted Coiled-Coil domain. The accession numbers of RO-16 homologs are: *Neurospora crassa*, XP_960755.2; *Fusarium graminearum*, XP_382083; *Magnaporthe oryzae*, XP_003711429.1; *Endocarpon pusillum*, XP_007799847.1; *Exophiala dermatitidis*, XP_009154365.1; *Aspergillus nidulans*, XP_660348.1 (AN2744.2); *Dothistroma septosporum*, EME49584.1; *Bipolaris oryzae*, XP_007692891.1; *Macrophomina phaseolina*, EKG18317.1; *Botrytis cinerea*, XP_001555764.1; *Pyronema omphalodes*, CCX33862.1 and *Arthrobotrys oligospora*, XP_011123625.1.

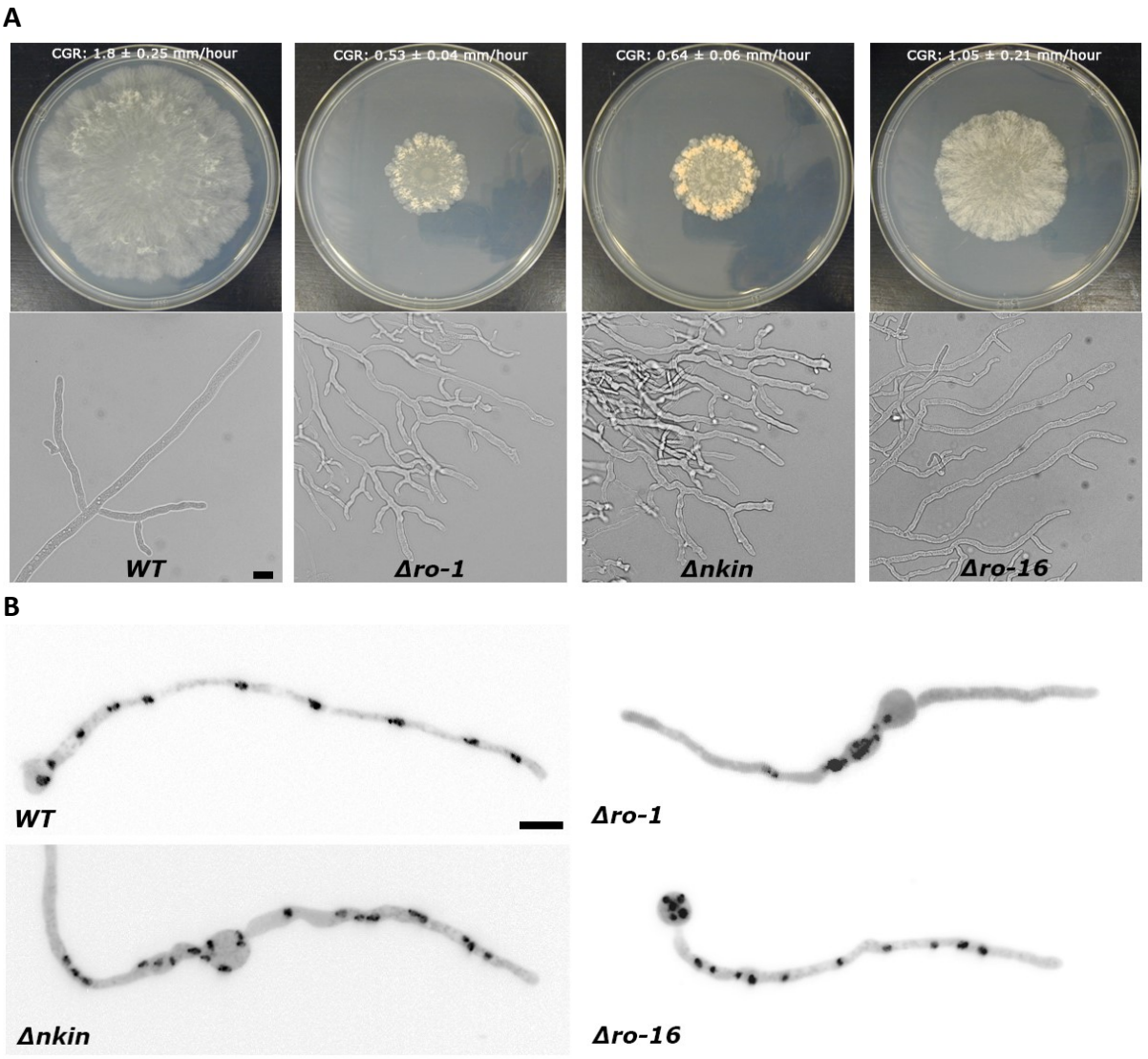


Figure 13**. RO-16 is required for proper hyphal growth but not for nuclear distribution. (A)** Effect of ro-16 deletion on colony (top panel) and hyphal morphology (bottom panel) in comparison to WT, Δro-1 (dynein heavy chain deletion) and Δnkin (kinesin-1 deletion). The colony growth rate (CGR) of Δro-16 is not as severely affected when compared with the two motor mutants.**(B)** Nuclear distribution in germlings of WT, Δ*ro-1*, Δ*nkin*, and Δ*ro-16*. Nuclei were visualized with DAPI. Scale bars are 20 µm.

3.2.2. RO-16 is required for dynein localization at hyphal tips

Dynein motors associate with microtubule(MT) plus-ends and form comet-like structures at the hyphal tip of *N. crassa* and other filamentous fungi (Han *et al.*, 2001; Xiang *et al.*, 2000; Zhang *et al.*, 2002; Lenz *et al.*, 2006; Sivagurunathan *et al.*, 2012; Fig.14 A). Similarly, kinesin-1 in *N. crassa*, and other filamentous fungi, accumulates at hyphal tips (Fig.14 B; Seiler *et al.*, 2000; Schuchardt *et al.* 2005; Sivagurunathan *et al.*, 2012), and its deletion causes dynein to mislocalize to distal regions of growing hyphae (Fig.14C). To test for a possible function of RO-16 on dynein/NKIN distribution, we localized dynein (DIC-mCherry was used as marker)and NKIN-GFP in Δ*ro-16*(Fig.14 D). While NKIN localization was unaffected, the accumulation of DIC at hyphal tips was abolished. DIC accumulated 60 to 80 µm distal of the tip as observed for DIC localization in Δ*nkin* (compare C with D in Fig.14). Also, dynein heavy chain (RO-1) and p150-glued (RO-3) mislocalized subapically in Δ*ro-16* (Fig.14 F-H), confirming mislocalization of the entire dynein/dynactin complex in Δ*ro-16*. Closer examination of mislocalized dynein subunits in Δ*ro-16* and Δ*nkin* revealed linear structures that suggested an association with MTs (Fig.14 F). Thus, we constructed a Δ*ro-16* strain expressing DIC-mCherry and GFP-tagged β-tubulin and confirmed partial colocalization of dynein specles/streaks along MTs (Fig. 15). Since MT interaction is dependent on the proper assembly of dynein complex (Xiang *et al.*, 2000; Zhang *et al.*, 2002; Sivagurunathan *et al.*, 2012), the integrity of dynein motor complex seems uncompromised in the absence of RO-16.

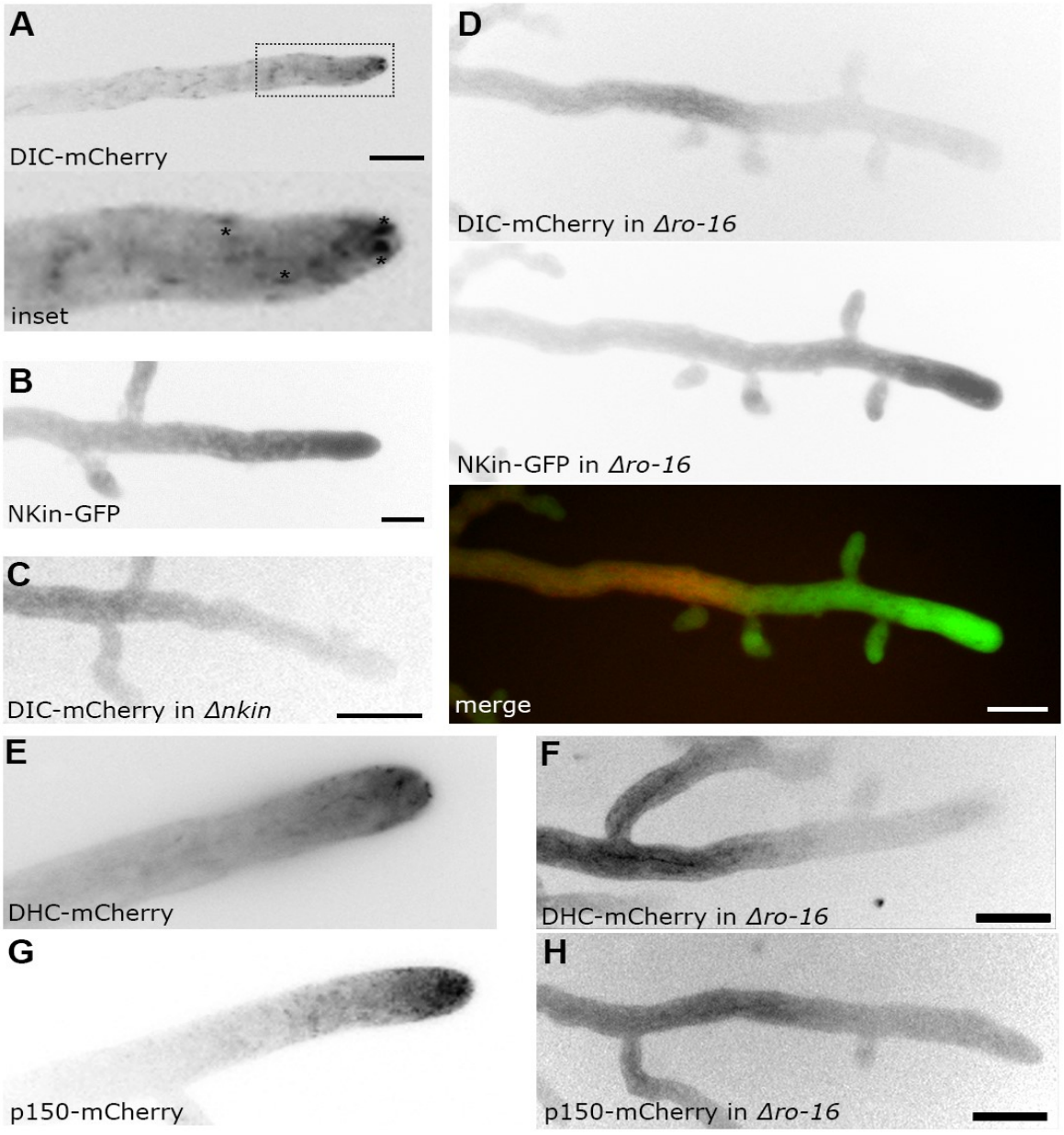

A
DIC-mCherry
inset
B
NKin-GFP
C
DIC-mCherry in Δnkin
D
DIC-mCherry in Δro-16
NKin-GFP in Δro-16
merge
E
DHC-mCherry
F
DHC-mCherry in Δro-16
G
p150-mCherry
H
p150-mCherry in Δro-16

Figure 14. **Accumulation of dynein at the hyphal tip requires NKIN and RO-16. (A)** Dynein accumulates at the hyphal tip in comet-like structures, which correspond to microtubules plus-ends (marked by asterisks in the inset). A DIC-mCherry markeris used to track the motor complex. **(B)** NKIN-GFP localizes in a diffuse manner in the apical region of WT hyphae. **(C)** In the absence of NKIN, DIC is mislocalized to distal regions of the hypha. **(D)** RO-16 is required for DIC localization at the hyphal tip, but is dispensable for accumulation of NKINat the apex. Note that DIC accumulates in a subapical region approximately 70μm distal of the tip in both∆*ro-16* and ∆*nkin*.(E-H) Dynein and dynactin accumulate at hyphal tips of WT but not ∆*ro-16*. Scale bars are 20 μm.

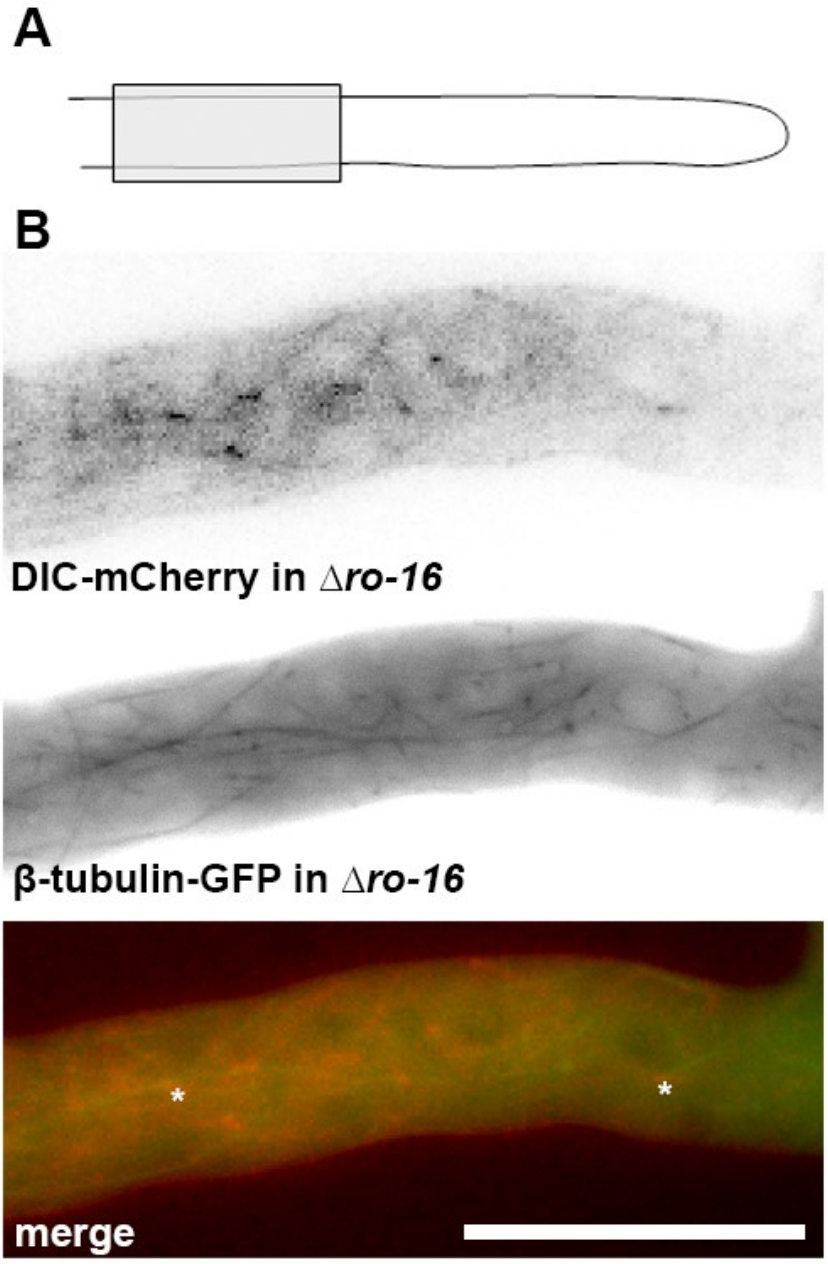


Figure 15. **RO-16 deletion does not affect the ability of dynein to bind microtubules. (A)** Schematic representation of the hyphal zone where dynein mislocalizes in the absence of RO16 (marked by box).**(B)** Deletion of *ro-16*results in subapical mislocalization of DIC that colocalizes with MTs (marked by asterisks). Scale bars are 20 μm.

3.2.3. RO-16 is transported to the hyphal tip by kinesin-1

While RO-16 and NKIN are required for the accumulation of dynein at the hyphal tip, Δ*ro-16* does not affect the localization of NKIN. Thus, we hypothesized that RO-16 regulates the interaction of dynein with NKIN. Therefore, we replaced RO-16 with a RO-16-mCherry construct, which sustained WT growth indicating that the fusion protein is functional. RO-16 accumulated at the hyphal tip in a diffuse manner similar to the localization described for NKIN (Fig.16 A). RO-16 did not show any discrete localization to comet-like structures indicative of an interaction with dynein at microtubule plus-ends. Moreover, RO-16 accumulation at hyphal tips was abolished in Δ*nkin*, (and also in Δ*ro-1*, Δ*ro-3,* or Δ*lis-1*), resulting in a uniform and diffuse distribution along the length of hyphae in these mutant backgrounds (Fig.16 B-E).

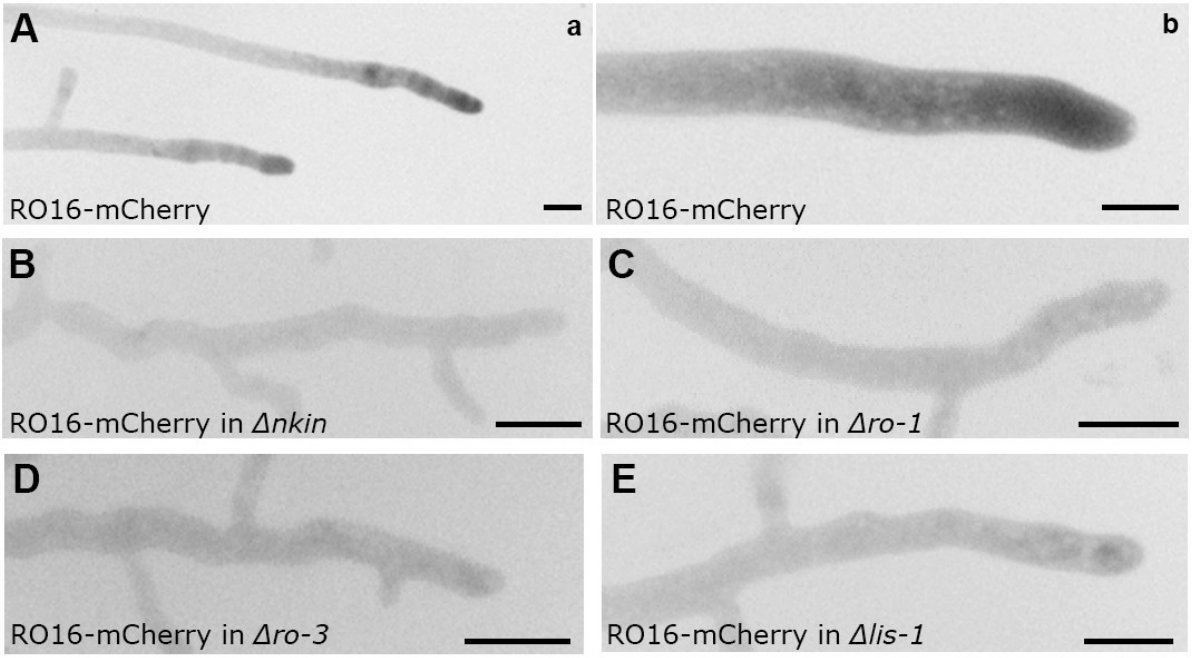


Figure 16. **RO-16 accumulates at the hyphal tip in an NKIN-dependent manner.(A)** RO-16 accumulates in a diffuse manner at hyphal tips (a: overview; b: enlargement of the apical region of the cell). **(B-E)** The accumulation of RO-16 at the apex requires NKIN (B), dynein (C), dynactin (D), and LIS-1 (E). Scale bars are 20 µm.

3.2.4. RO-16 does not interact with MT-bound dynein

The diffuse localization of RO-16 at hyphal tips suggested that it did not interact with dynein motors that are associated with MT plus-ends and/or motors that are engaged in retrograde transport. To further examine this hypothesis, we localized RO-16 in previously isolated *ropy* mutants, in which dynein is blocked along MTs as linear tracks or as MT plus-end-associated comet tails (referred to as class II and class III, respectively; Sivagurunathan *et al.*, 2012). We observed decreased accumulation of RO-16 at the hyphal tip in both dynein mutant backgrounds, but did not detect any discrete localization of RO-16 on either comet-like structures or along MT tracks (Fig.17). Thus, dynein might have a function in maintaining RO-16 at the cell apex yet these data strongly suggest that RO-16 does not interact with dynein that is directly bound to MTs.

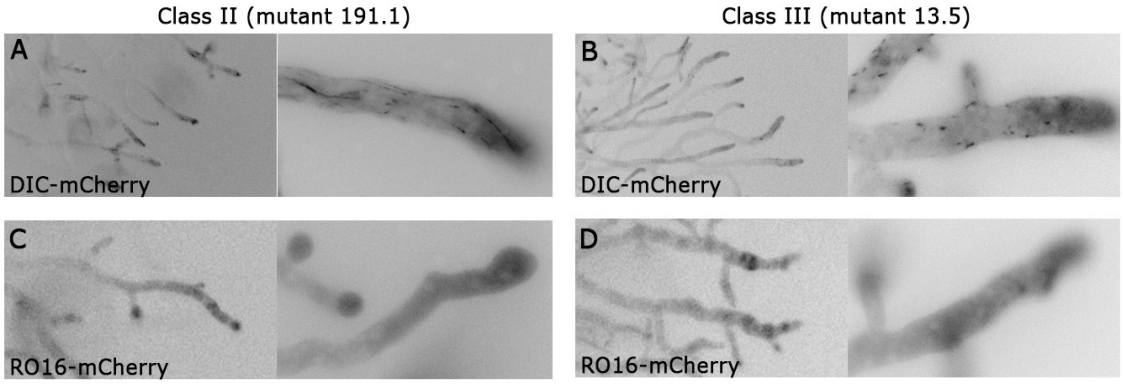


Figure 17. **RO-16 does not interact with MT-associated dynein**. **(A)**Dynein, which is monitored by a DIC-mCherry construct, accumulates in long linear tracks (A) or comet-like structures at the hyphal tips (B) in class II and class III dynein mutants, respectively. **(C-D)** RO-16 localization at hyphal tips is reduced but not abolished in both dynein mutant classes. Note that RO-16 does not associate along linear tracks or comet like structures and does thus not form any stable interaction with MT-bound dynein.

3.2.5. A trimeric RO-16/dynein/kinesin-1 complex appear to form along microtubules during anterograde transport by kinesin-1

The requirement of NKIN for RO-16-dependent tip localization of dynein could indicate that RO-16 functions as part of a tip-directed cargo complex of NKIN. An alternative possibility is that RO-16 is required for establishing the initial dynein-NKIN interaction, but not for the transport process. Therefore, we established an experimental system, in which active NKIN was stably tethered to MTs through introduction of a mutation in the ATP-binding motif that resulted in a "rigor" state and determined the dependence on RO-16 for a stable interaction of NKIN with dynein. Recent sequencing of classical *N. crassa* mutants identified a serine 95 to leucine substitution within the ATP binding motif of NKIN in the colonial mutant *col-4* (personal communication Kevin McCluskey; Fig.18 A). Moreover, early work had detected “lines of vesicles in the cytoplasm” in transmission electron micrographs of *col-4* hyphae (Trinci and Collinge 1974). In order to determine if NKIN(S95L) is a true rigor kinesin, we used targeted homologous recombination to fuse the *col-4*NKIN(S95L) to mCherry. This construct displayed a MT-like localization, confirming its rigor behavior(Fig.18 B). Next, we determined if RO-16 and dynein are tethered to MTs in a *nkin(S95L)* background. Both RO-16 and DIC failed to accumulate at the apex but labeled subapical MTs (Fig.18 C, D). Thus, RO-16 and dynein interact with rigor kinesin-1 that is tethered to MTs, indicating that a stable, trimeric dynein/RO-16/NKIN complex is formed during tip-directed transport by NKIN.

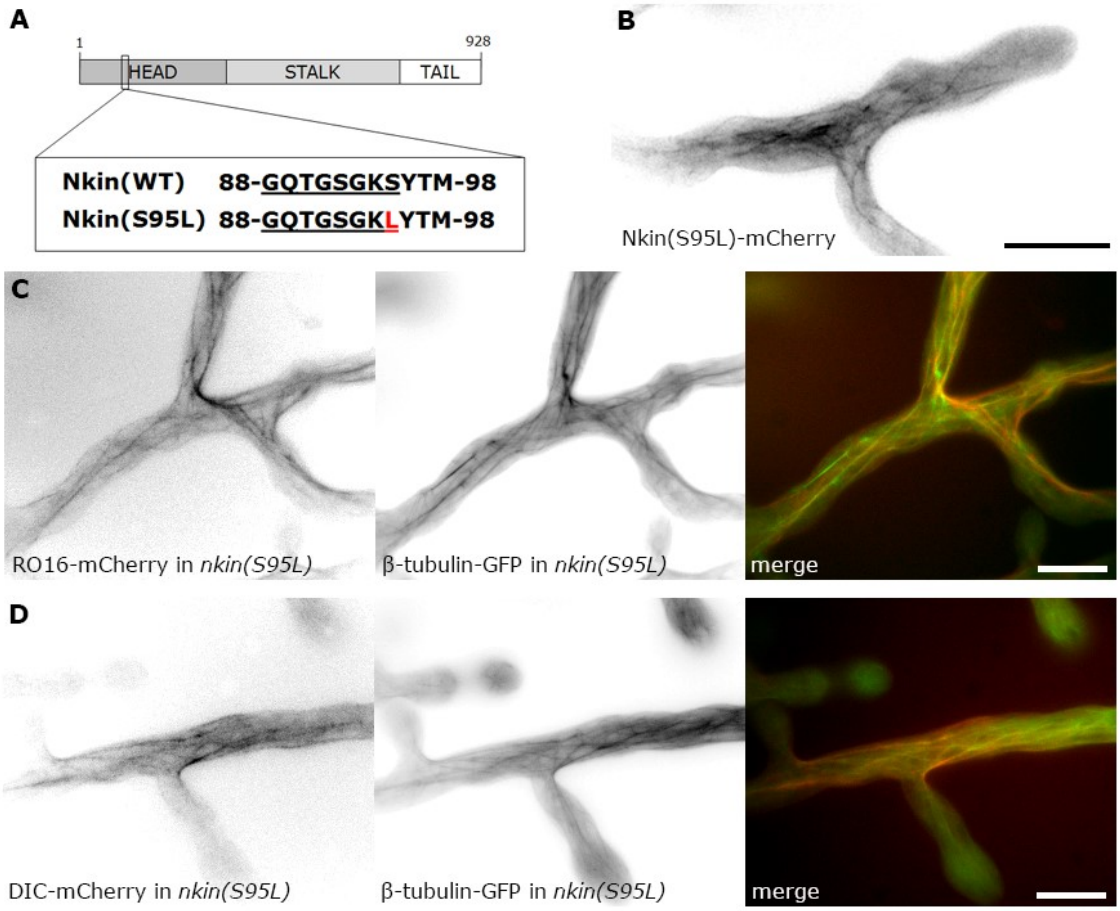


Figure 18. **Rigor kinesin-1 causes RO-16 and dynein to accumulate along microtubules.(A)** Domain organization of NKIN and position of the P-loop motif required for ATP binding (Song *et al.*, 2001). The enlarged box contains the sequence alignement of P-loop motifs (underligned) of WT NKIN and the *col-4*mutant. **(B)**The NKIN(S95L) substitution produces a rigor-state kinesin that accumulates along MTs. **(C, D)** Localization of RO-16-mCherry and DIC-mCherry in *nkin(S95L)*coexpressing GFP-tubulin. Merged channels indicate colocalization of RO-16 and DIC with MTs. Scale bars are 20 μm.

The “lines of vesicles” reported in *col-4* suggested that NKIN(S95L) fixed vesicular cargoes to MTs. In order to determine which vesicular cargo is bound to NKIN(S95L) we localized available GFP-tagged small Rab-GTPases (Bowman *et al.*, 2015) as markers in the *nkin(S95L)* mutant. Among all Rab proteins analyzed, we found that the predicted post-Golgi marker RAB-6 localized along long linear tracks in *nkin(S95L)* (Fig.19 A, B). These MT-fixed RAB-6 vesicles also colocalized with DIC (Fig.19 C), and thus we predicted that RO-16 is required for the interaction of dynein with rigor NKIN. *Ro-16* was therefore deleted in an *nkin(S95L)* strain co-expressing DIC-mCherry and RAB-6-GFP. The association of dynein with MTs was abolished in this strain, while RAB-6-GFP still associated with MT-like tracks (Fig.19 D). Since no other Rab protein displayed this phenotype, the tethering of RAB-6-vesicles to MTs is most likely the consequence of its interaction with kinesin-1and not an indirect consequence to a general blockage in MT-based transport. These data confirm the requirement of RO-16 for the interaction between dynein and NKIN and indicate that RO-16 is not needed for the interaction of RAB-6 vesicles with NKIN.

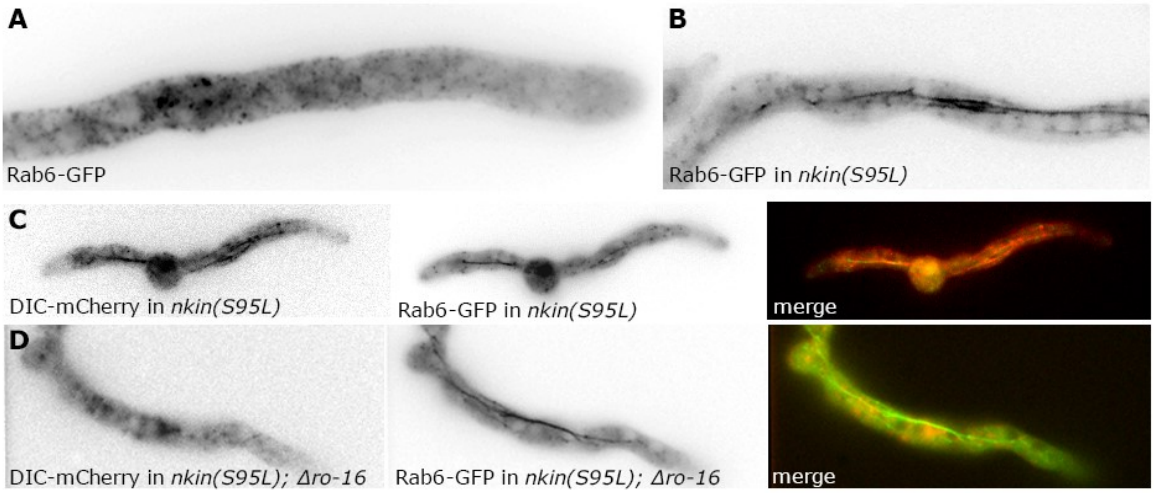


Figure 19. **RO-16 is not required for the interaction of RAB-6-containing vesicles with NKIN**. **(A, B)** Localization of the secretory vesicle marker RAB-6 in WT and *nkin(S95L)*. Rigor NKIN causes Rab-6-containing vesicles to accumulate along MTs. **(C)**RAB-6 and DIC colocalize in a *nkin(S95L)*background.(D) Association of DIC with NKIN, but not of RAB-6-vesicles, is abolished in ∆*ro-16*.

3.2.6. The cargo-binding domain of kinesin-1 is required for tip-directed transport of RO-16 and dynein

The C-terminus of NKIN is required for cargo binding and transport of secretory vesicles to the hyphal tip (Seiler *et al.*, 1997, 1999; Kirchner *et al.*, 1999; Seiler *et al.*, 2000). A cargo-binding domain (CBD) is located roughly between residues 781 and 830. Moreover, a highly conserved, regulatory motif (RD) that controls the ATPase activity of the motor head domain through its interaction with the ATP-binding motif is found between residues 887 and 892 (Fig.20 A; Seiler *et al.*, 2000; Kaan *et al.* 2011). In agreement with previous results (Seiler *et al.*, 2000), we found that mCherry-tagged NKINΔ885-928 (lacking the RD) and NKINΔ740-928 (lacking CBD and RD) accumulate strongly at hyphal tips indicating that their motor activity, although unregulated, is not abolished (Fig.20B).In order to dissect which domain of the tail region of NKIN is required for interaction with RO-16, we engineered strains producing NKIN variants lacking the RD or both the CBD and RD. RO-16 strongly accumulated at hyphal tips in a *nkin(Δ885-928)* background in a manner highly similar to the localization of the truncated NKIN variants, but localized in a diffuse manner throughout the hypha in *nkin(Δ740-928)* (Fig.20 C). Thus, the CBD is required for the RO-16/NKIN interaction and for tip-directed transport of RO-16 by NKIN.

Because apical transport of dynein requires kinesin-1, we also tested which region of the NKIN C-terminus is required for the interaction with dynein by localizing DIC-mCherry in the different *nkin* strains (Fig.20 D). DIC-mCherry localized to comet-like structures within the hyphal apex of WT and *nkin(Δ885-928)* indicating that dynein is

correctly transported to the hyphal tip and is targeted to the MTs plus-ends (Fig.20 D). In contrast, DIC-mCherry localized in the subapical region in *nkin(Δ740-928)*.Similar results were observed when RO-1-mCherry was localized in the two *nkin mutants* (Fig.21). Thus, both the dynein complex and RO-16 require the CBD of NKIN for apical transport. To test if RO-16 mediates the interaction between dynein and NKIN, we constructed a *Δro-16*/*nkin(Δ885-928)* double mutant. DIC-mCherry expressed in this background failed to accumulate at the hyphal tip and was mislocalized to the subapical regions (Fig.20 E). In the reverse experiment we localized RO-16 in an *nkin(Δ885-928)/Δro-1* double mutant background, which showed reduced but not abolished apical localization. This residual accumulation of RO-16 at hyphal tips might be due to an increase in its concentration at the hyphal tip in presence of NKIN(Δ885-928). In summary, these data demonstrate that transport of dynein to hyphal tips requires the CBD of NKIN and that RO-16 mediates this interaction.

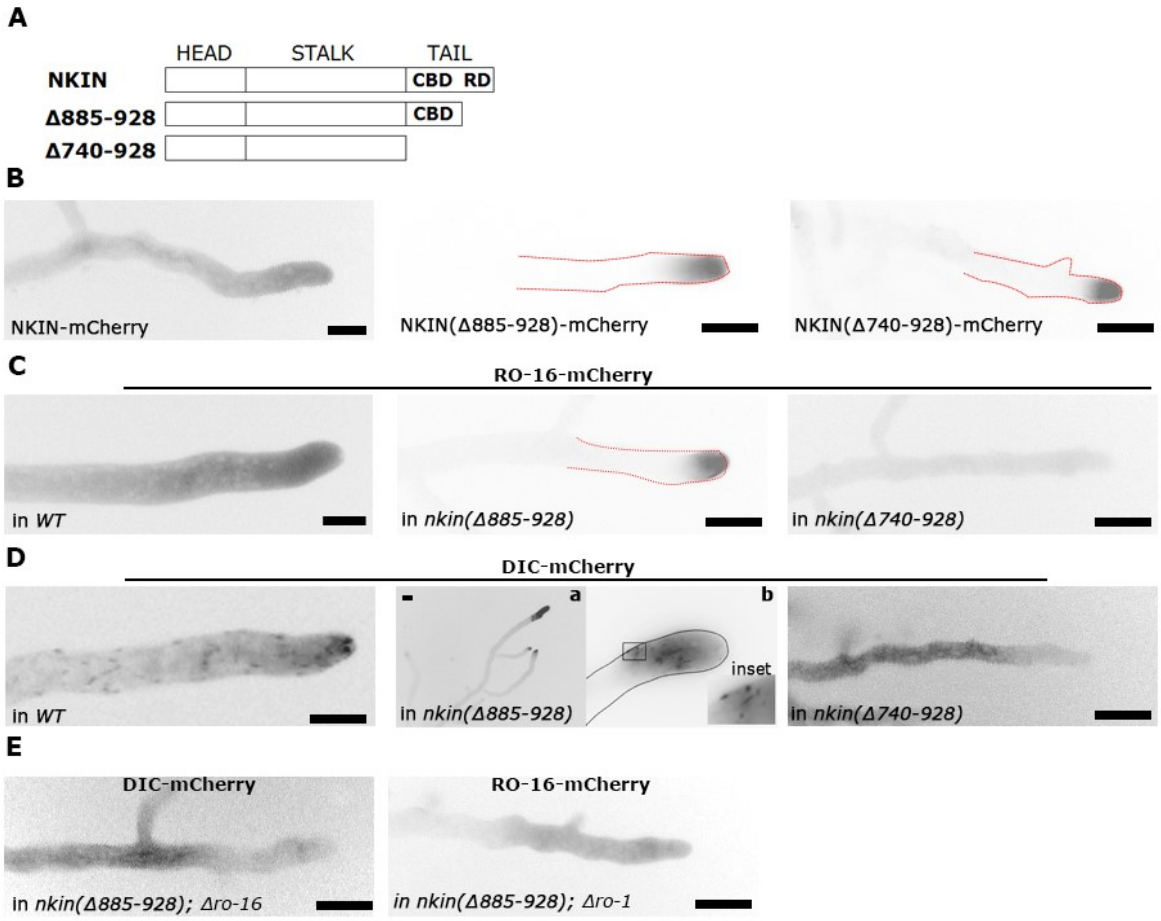


Figure 20. **The cargo-binding domain of kinesin-1 is required for transport of RO-16 and dynein to the hyphal tip. (A)** Schematic representation of thedomain organization of NKIN (not to scale), and of the truncated versions NKIN(∆885-928) and NKIN(∆740-928), in which the regulatory domain (RD) or the RD and the cargo-binding domains (CBD) are removed, respectively (Kirchner *et al.*, 1999; Seiler *et al.*, 2000). **(B)** Localization of mCherry-tagged WT and truncated NKIN variants. As previously reported both mutant kinesin variants strongly accumulate at the hyphal tip. **(C)** Transport of RO-16 to the hyphal tip requires the CBD of NKIN. Note that the strong accumulation of RO-16 at the tip is very similar to that of NKIN(∆885-928).**(D)**DIC localizes to the hyphal tip (a overview) in comet-like structures at MT+ends (b and insert)in WT and *nkin(∆885-928)*, but not in *nkin(∆740-928)*. **(E)**Transport of dynein to the tip by NKIN(∆885-928) requires RO16: DIC mislocalizes in the subapex in *nkin(∆885-928)*when *ro-16* is deleted. Deletion of DHC reduces the accumulation of RO16-mCherry at the tip in the *nkin(∆885-928)*background.Scale bars are 20 µm.

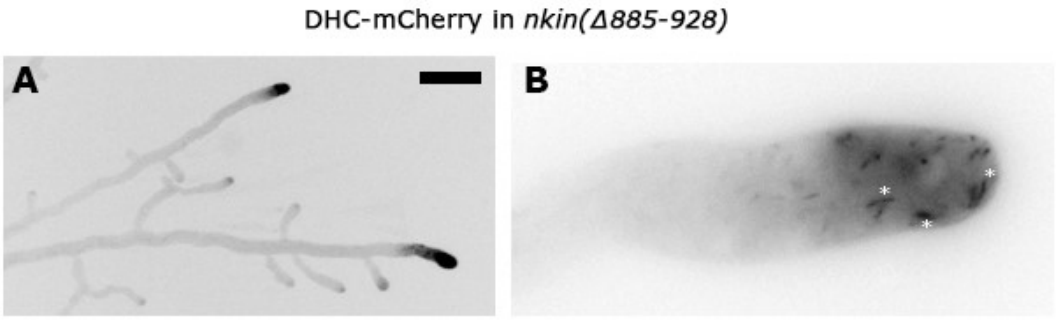


Figure 21. **Constitutively active kinesin-1 packs dynein at the hyphal tips.(A)** overview of the hyphal tip localization of DHC-mCherry (dynein heavy chain) in the *nkin(Δ885-928)* genetic background. (B) a close-up view of a hyphal tip indicate DHC-mCherry comets.

3.2.7. Distinct domains of RO-16 mediate its interactions with kinesin-1 and dynein

In order to map regions of RO-16 that are required for its interaction with dynein and NKIN we analyzed the phenotype of *ro-16* truncations and the localization of DIC-mCherry and truncated RO-16-mCherry variants in these backgrounds (Fig.22). RO-16 constructs lacking the first 110 residues or more were non-functional and resulted in mislocalization of DIC, while RO-16(∆1-51) was fully functional and localized DIC in a WT manner (Fig. 22 A-C). This functional analysis correlated with the presence of a first stretch of conserved amino acids at position 70-90, suggesting that this region is mediating the interaction between RO-16 and dynein. In a similar approach we mapped the region required for interaction of RO-16 with NKIN. In order to obtain clearer localization data, we performed this analysis in a *nkin(∆885-928)* strain, which displayed strong tip accumulation of RO-16. RO-16(∆1-150) accumulated at hyphal tips to a level that was indistinguishable from full length RO-16 (Fig. 22D, E), indicating that the N-terminal region of RO-16 required for interaction with dynein was dispensable for interaction with NKIN. RO-16(∆1-293), which lacked the N-terminal 2/3 of the protein yet still contained a conserved, predicted coiled coil (CC) region was still weakly enriched at the hyphal tip. In contrast, the apical localization of the reverse construct lacking the CC was completely abolished, displaying a cellular distribution similar to the localization of RO-16 in ∆*nkin* (Fig.22 F and G). Thus, RO-16 has a modular structure with discrete domains that are separately mediating the interactions with kinesin-1 and dynein.

Next, we performed reciprocal co-immunoprecipitation assays with cell extracts of *N. crassa* strains co-expressing tagged versions of RO-16 and NKIN, which proved the interaction of NKIN with RO-16 *in vivo* (Fig. 23 A). Using the same approach we also examined if the dynein and dynactin subunits RO-1 and RO-3 co-purified with NKIN and RO-16 by probing the precipitates with anti-DHC (α-RO1) and anti-p150 (α-RO3) antisera. These results provide clear evidence that RO-16, NKIN and dynein/dynactin interact within the same multi-protein complex.

Yeast two-hybrid (Y2H) tests were employed for further mapping of the interacting regions of NKIN and RO-16 in more detail (Fig.23 B). We detect a strong physical interaction between full length RO-16 and NKIN(726-end) (Fig.23 B), which corroborated our genetic and microscopic analysis. As control we used a CC region within the stalk domain of NKIN, which was Y2H negative with RO-16, confirming that the NKIN-RO-16 interaction is specific to the tail region and not an unspecific interaction of two CC motifs (Fig. 23 B). Since the predicted CC forming C-terminus of RO-16 mediated the RO-16/NKIN interaction in the microscopic *in vivo* assays, we tested if this region was sufficient for interaction with the NKIN tail and detected a strong Y2H interaction between the NKIN(726-end)-RO-16(aa288-end) and the NKIN(726-end)-RO-16(288-371) pairs (Fig. 23 B).

A sequence N-terminal of the CBD in the heavy chain of kinesin-1 is involved in binding to the kinesin light chains (KLCs) in metazoans (Diefenbach *et al.*, 1998). Since no KLCs are detectable in fungi we wondered if sequences N-terminal of the NKIN CBD might be involved in binding RO-16 in a manner similar to KLC binding in metazoa. This region also forms a coiled-coil as described for the animal KHC-KLC

interacting region and sequence comparison with other fungal kinesin-1 homologs had identified a highly conserved, yet fungal specific region (Kirchner *et al.*, 1999). When we tested the interaction of RO-16 with an NKIN tail fragment lacking this conserved stretch located N-terminally of the CBD, a positive interaction was still detected between RO-16 and NKIN(774-end), supporting an interaction between the coiled-coil motif of RO-16 and the CBD of NKIN (Fig. 23 B).

The tail regions of fungal and metazoan kinesin-1 are highly homologous. Thus, we predicted that RO-16 was also able to interact with the CBD of the heavy chain of kinesin-1 from *Drosophila melanogaster*. We detected a strong interaction between RO-16 and DmKHC(820-end) and also between RO-16(288-371) and DmKHC(820-end), confirming the evolutionary conservation of the CC-based interactions between the heavy chain of kinesin-1 and its cargo adaptor (Fig. 23 B).

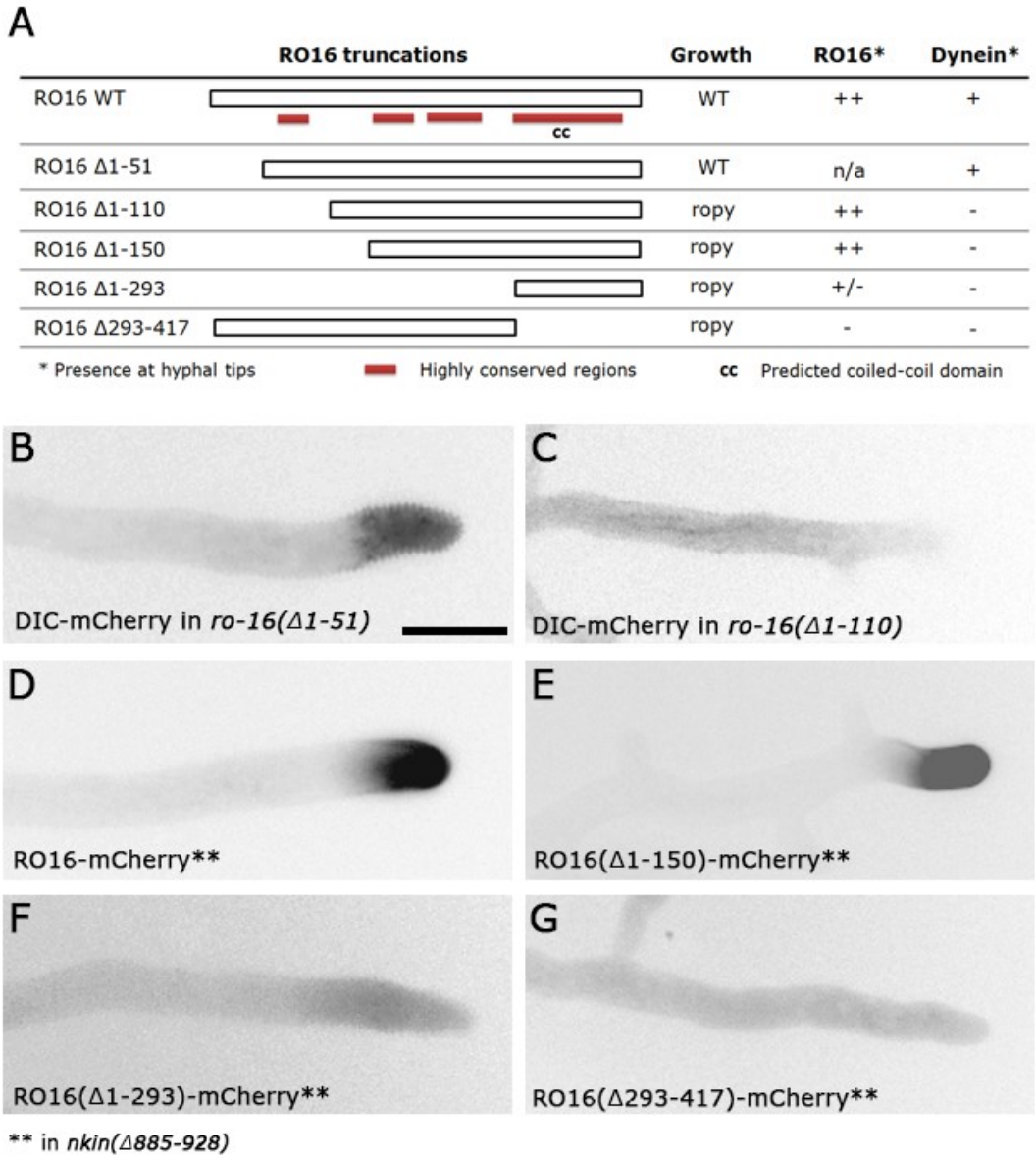
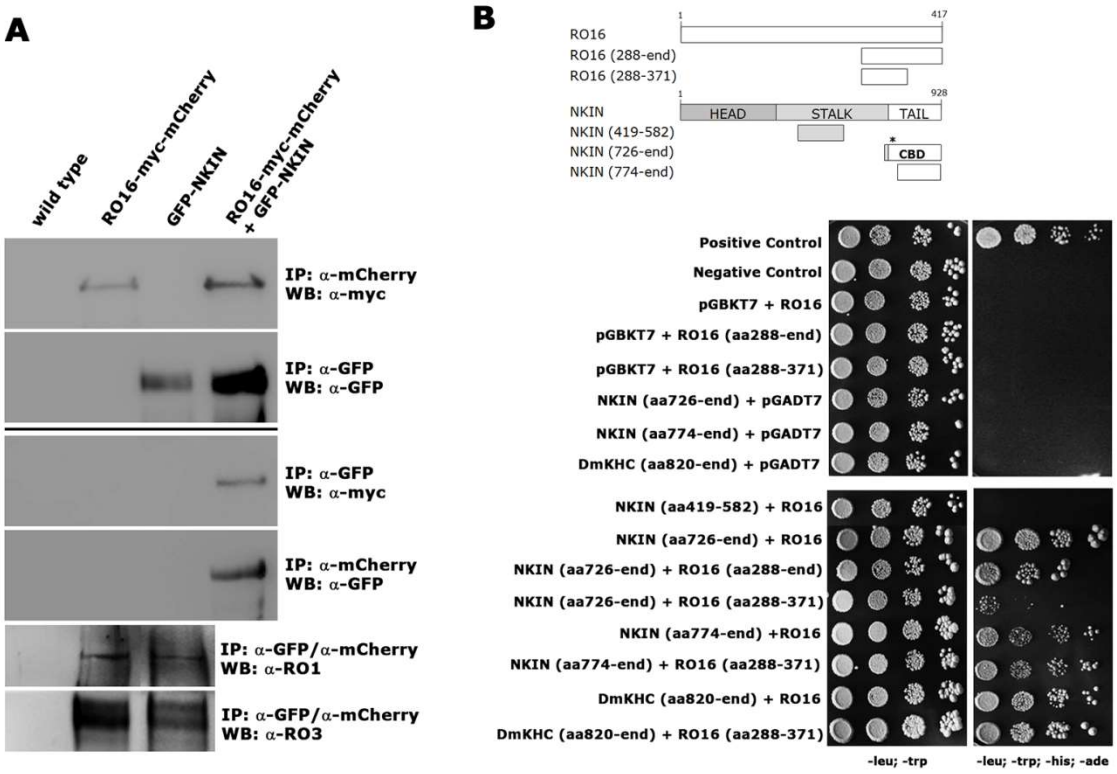


Figure 22. **Distinct domains of RO-16 mediate interactions with kinesin-1 and dynein. (A)** Schematic representation of RO-16 truncations and their effects on growth and apical localization of DIC and truncated RO-16 variants. Red bars and CC indicate conserved regions and the position of the predicted coiled-coil domain, respectively. **(B)** Localization of DIC in *ro-16(Δ1-51)* and *ro-16(Δ1-110)* indicates that the region 50-110 of RO-16 is required for accumulation of dynein at hyphal tips. **(C)**The C-terminus of RO-16 containing the CC motif is essential for its apical localization in a nkin(Δ885-928) background. While the localization of RO16(Δ1-150) is indistinguishable from that of full lenght RO-16, RO-16(Δ1-293) displays reduced apical accumulation and RO-16(Δ293-417) fully fails to accumulate in the tip region. Scale bars are 20 µm.



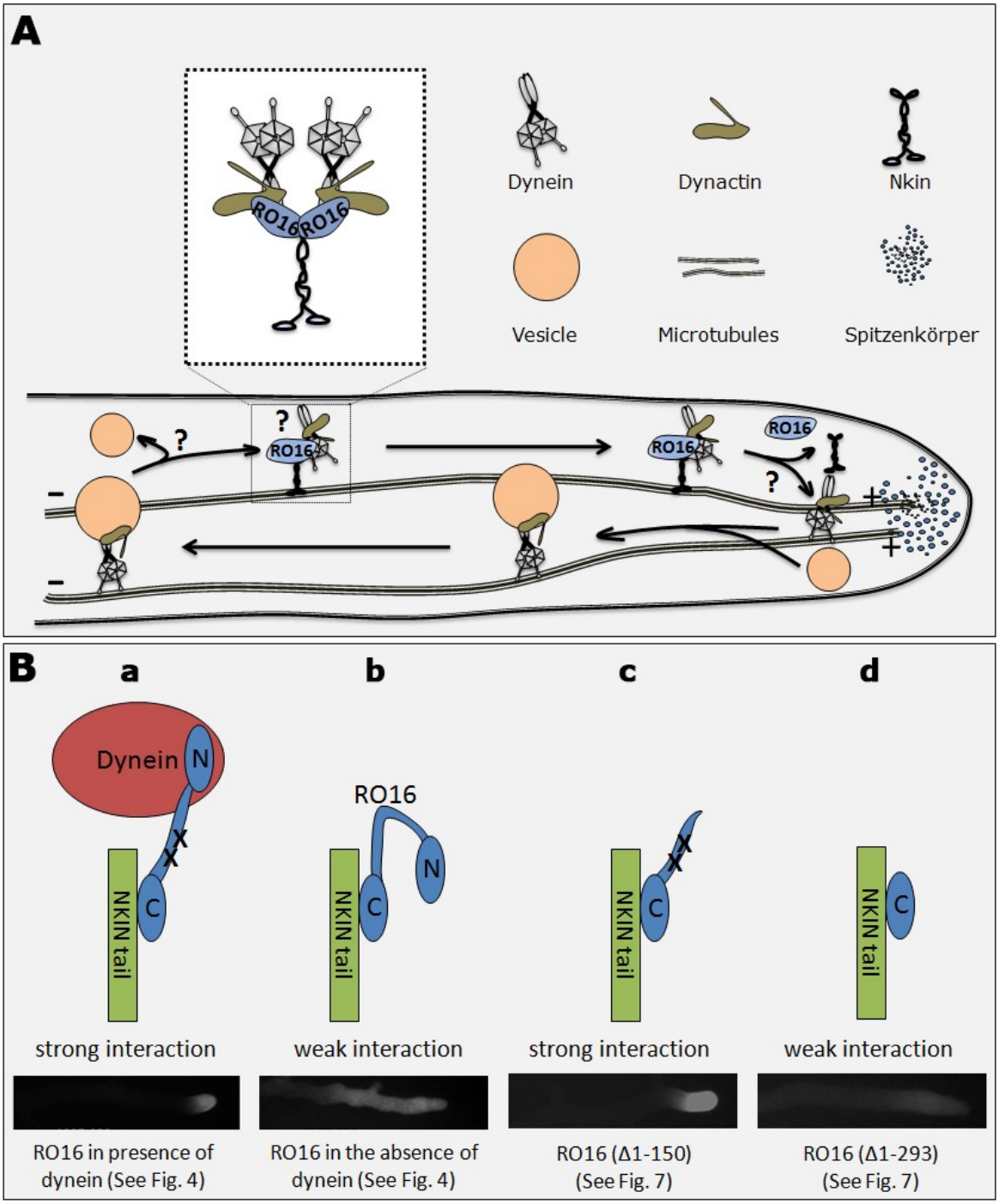
Fiure 23. **Nkin and RO-16 physically interact and copurify with dynein.** (A) Co-Immunoprecipitation experiments between NKIN, RO-16, DHC/RO-1 and the dynactin subunit p150/RO-3. Because the tagged proteins were not detectable in crude cell extracts, the upper two Western blot pannels are controls, indicating the correctness of the strains used and that the lableled proteins can be precipitated from cell extracts using α-mCherry and α-GFP antibodies. The central two panels indicate reciprocal co-precipitation of NKIN and RO-16. The lower two panels indicate that both DHC/RO-1 and p150/RO-3 co-precipitate with RO-16 as well as with NKIN. **(B)**Yeast-two-Hybrid experiments were used to map physically interacting domains ofNKIN and RO-16. A schematic representation of the tested domains, is included. Cargo binding domain (CBD) and KLC homologous binding region (asterisk) are indicated. The upper two panels are controls indicating that none of the constructs used are self-activating. The lower two panels define the tail region of NKIN containing the CBD and RD (aa774-end) and the coiled-coil region of RO-16(aa288-371) as minimal requirements for physical interaction between the two proteins.Note that the interaction of NKIN(aa774-end) with RO16(aa288-371) is stronger than the interaction of NKIN(aa726-end) with RO16(aa288-371), suggesting some inhibitory function in the Nkin region 726-774. The homologous region of the heavy chain of kinesin-1 from *Drosophila melanogaster* (DmKHC; aa820-end) also interacts with RO-16 and with RO-16(aa288-371).In order to exclude that these interactions are due to stickiness between coiled coil regions we used a central NKIN stalk region 419-528 as negative control.

3.3. Discussion

In this chapter we have provided evidence for the role of the novel protein RO-16 in the interaction between dynein and kinesin-1in the model organism *N. crassa*. Our results indicate that RO-16 is required for the transport of dynein to MT plus-ends but not for the assembly or motor activities of kinesin-1 or dynein/dynactin. This is consistent with the fact that dynein-based nuclear distribution and motor activity of NKIN are not abolished in Δ*ro-16*. However, in the absence of RO-16, the accumulation of dynein at hyphal tips is lost and leads to a mild ropy phenotype. Like NKIN and dynein, RO-16 localizes to the hyphal tips and a positive correlation is observed between alterations in the transport of NKIN and the cellular behavior of RO-16 and dynein. In fact, NKIN transports both dynein and RO-16 and deletion of *nkin* leads to a complete loss of the localization of dynein and RO-16 from hyphal tips. Consistent with the role of kinesin-1 in transporting RO-16 and dynein, a rigor kinesin-1 causes dynein to become tethered to MTs in a RO-16-dependent manner which reflects the formation of a stable complex between these proteins. Functional dissection of the tail region of NKIN showed that its cargo binding domain (CBD; aa740-885) is necessary for the interaction with RO16 and dynein/dynactin. On the other hand, systematic truncations in the RO-16 protein, indicate that this protein has a modular structure where a N-terminal sequence is specific to dynein interaction and a C-terminal domain is essential for binding NKIN. This domain contains a highly conserved sequence predicted to form a coiled-coil structure and interacts with residues 774-885 in the CBD of NKIN. In *D. melanogaster*, KLCs bind to a specific domain in the tail region of kinesin-1 (Diefenbach *et al.*, 1998) and can

bind directly to DIC (Ligon *et al.*, 2004; Williams *et al*., 2004). Interestingly, we found that RO-16 can also bind to the tail region of DmKHC, but outside of the KLC-binding domain, suggesting that the binding mechanism of RO-16 to kinesin-1 is different than that of KLCs. The identification of the residues involved in the binding of RO-16 to the tail region of kinesin-1 will be necessary to better understand the molecular mechanistics of RO-16. Our *in vivo* and *in vitro* experiments also show that RO-16 interacts directly with the two motors during anterograde transport and does not involve a vesicular cargo as suggested by previous models. Evidence for a vesicle-independent interaction between dynein and kinesin-1, was provided by co-immunoprecipitation experiments where core subunits of dynein and dynactin were co-precipitated in complex with RO-16 and NKIN. Since the conditions used during protein extractions destroy vesicular bodies, it is evident that the formation of a NKIN-RO16-dynein/dynactin complex is independent of vesicular cargo. Another evidence for the non-requirement of vesicular cargoes, came from our finding that RAB-6-vesicles interact with NKIN independently of RO-16. Using our *in vivo* experimental system, where kinesin-1 is in a rigor state, RAB-6-vesicles became tethered to MTs in a way similar to that of RO-16 and dynein. Since no other known Rab proteins (Bowman *et al.*, 2015) displayed this phenotype, the tethering of RAB-6-vesiclesto MTs is most likely the consequence of its interaction with kinesin-1and not an indirect consequence to a general blockage in MT-based transport. Additionally, since we found that the motor activity of kinesin-1 and its accumulation at hyphal tips are unaffected by the functional disruption of dynein and RO-16 and since, the activation of kinesin-1 requires cargo interaction, it is obvious that kinesin-

1 can be activated by other cellular components. In filamentous fungi, RAB-6 has been shown to be involved in membrane trafficking between the Golgi apparatus, ER, endosomes and the plasma membrane; however, no evidence supports the role of kinesin-1 in this traffic (Pantazopoulou and Penalva, 2011). Our results suggest for the first time that RAB-6-containing vesicles are a possible cargo of kinesin-1. Since in metazoa, RAB-6-vesicles are transported by both kinesin-1 and dynein and a direct interaction between RAB-6 and DLC is detected, this transport is most likely conserved between fungi and animals (Fogerty *et al.* 2016; Grigoriev *et al.*, 2007; Wanschers *et al.*, 2008).RAB-6 is also involved in the regulation of the kinesin KIF1C, and in the initiation of retrograde transport by dynein (Lee *et al.* 2015; Yamada *et al.* 2013). Thus, a regulatory link between kinesin-1 and dynein at MT-plus ends and involving RAB-6 can be envisaged. Confirmation and determination of the possible mechanisms of interaction between RAB-6 and NKIN will provide an important addition to the understanding of cargo traffic and hyphal growth. The direct interaction between RO-16 and NKIN tail, the copurification of dynein-dynactin with NKIN-RO16 and the dispensability of RO-16 in the interaction between NKIN and RAB-6-vesicles, all strongly support a model in which RO-16 is a direct link between dynein/dynactin and NKIN (Fig. 24).


A
RO16
RO16
Dynein
Dynactin
Nkin
Vesicle
Microtubules
Spitzenkörper
?
?
RO16
RO16
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+
B
a
b
c
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Dynein
N
C
NKIN tail
RO16
N
C
NKIN tail
C
NKIN tail
C
NKIN tail
strong interaction
weak interaction
strong interaction
weak interaction
RO16 in presence of dynein (See Fig. 4)
RO16 in the absence of dynein (See Fig. 4)
RO16 (Δ1-150) (See Fig. 7)
RO16 (Δ1-293) (See Fig. 7)

Figure 24. **Model for the role of RO-16 in the transport of dynein by kinesin-1.(A)** NKIN in association with RO-16 transports dynein/dynactin to the hyphal tip. The mechanim(s) by which NKIN and RO-16 initially recognize and interact with dynein, and the stoichiometry of the protein complex, remain to be determined. At hyphal tip, dynein is released from NKIN and is targeted to MT plus-ends where it interacts with vesicular cargoes, i.e. early endosomes, for retrograde transport. **(B)**Efficient accumulation of RO16 at the hyphal tip requires its interaction with dynein(a). When this interaction is abolished (i.e. by loss of dynein) a structural change in RO-16 weakens the RO-16/NKIN interaction (b). When the N-terminal domain of RO-16 (aa 1-150) is removed, this inhibition of the RO-16/NKIN interaction is relieved (c). Potentially this might include exposure of residues destined for post-translational modification (X marks) that would explain the strong accumulation of RO16Δ1-150. When further RO16 sequences are removed (1-293), the accumulation of RO16 at hyphal tip is strongly decreased (d).

CHAPTER 4

CONCLUSION

Since RO-16 is only found in the Pezizomycotina, our model of RO-16-mediated transport of dynein might be a defining feature of this clade. In fact, RO-16 is absent from the Basidiomycota where kinesin-1 is also required for the targeting of dynein motors to MT plus-ends. Whether it is a RO-16-like protein or a vesicular cargo which connects kinesin-1 to dynein in the Basidiomycota remains to be determined. These findings indicate that while the MT-based transport in filamentous fungi is well-conserved, molecular differences exist between fungal groups and might be specific to the biological behavior and habitat of different fungal groups. For instance, the absence of kinesin-1 and kinesin-3 from the unicellular *S. cerevisiae*, might explain the non conservation of RO-16 in yeast. A recent study in *S. cerevisiae*, using *in vitro* reconstitution of dynein targeting to MT plus-ends, identified a role of the yeast homologs of LIS-1 and CLIP-170 (a plus-end tracking protein) in connecting dynein to the kinesin-like protein, Kip2p (Roberts *et al.* 2014). Kip2, which is involved in targeting plus-end tracking proteins to the growing end of MTs, belongs to a separate group of kinesins distinct from kinesin-1. These findings, suggest the existence of species-specific mechanisms linking dynein to its kinesin transporter. This further supports the exclusive role of RO-16 in the long-range MT-based transport. RO-16 might have emerged in the filamentous ascomycetes (or lost from all other eukaryotes) to allow for a fast rate of growth that characterizes members of this group. This is consistant with the reduced mycelial expansions in

the absence of RO-16. In the hyphal tips of *N. crassa*, a concentration of cytoplasmic dynein is maintained throughout growth. In contrast to the basidiomycota, this consitutes a larger pool of dynein motors ready to engage in a larger volume of retrograde transport of vesicles. This in turn would provide three important functions to the hyphae: 1) prevent over-accumulation of vesicles, 2) provide a continuous supply of nutrients, 3) support the formation of the larger vacuoles observed in distal parts of the hyphae. The first function will allow more anterograde transport to occur and thus support a larger supply of vesicles which provide the necessary plasma membrane and cell wall components required for hyphal extension. The second function, should allow the building of new cellular components and the production of sufficient energy to respond to the high energetic demands of the growing hyphal tip. The third function, would provide, by forming larger vacuoles, a pressure force that can generate larger cytoplasm movement accompanying the fast extension of the hyphae. Whether RO-16 was present in other eukaryotes, but lost in the basidiomycetes and metazoa, or if it appeared independently in the Pezizomycetes, is a challenging question worth consideration.

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GLOSSARY

ATP: abbreviation for Adenosine Triphosphate; it is a nucleotide used in living cells to store and transfer energy. Also known as the “molecular unit of currency”, it contain energy-rich phosphate bonds. *See **nucleotide***

Coenocyte (adj. coenocytic): multinucleate mass of protoplasm formed by division of the nucleus, but not the cytoplasm.

Conidium (pl. conidia): asexual propagules of N. crassa and other fungi. They are formed by fission at the apex of specialized **hyphae** (conidiophores).

Eukaryotes: All organisms in which the genetic information, *i.e.* DNA, is contained in a sub-cellular compartment known as the nucleus. This latter is surrounded by a nuclear envelope which contains pores allowing the bidirectional flow of information or macromolecular complexes, between the nucleus and the cytoplasm.

Filamentous fungi: A major group of fungi which is characterized by growth as thread-like structures known as hyphae. See *Hypha*

Hypha (pl. hyphae): in fungi, it’s a coenocytic (see **coenocytes**) tubular filament or thread of a **thallus**, often vacuolated. Increases in length by growth at its tip; but proteins are synthesized throughout the mycelium and transported to hyphal tips by cytoplasmic streaming and molecular motors. Hyphae can be septate or non-septate. New Hyphae arise by lateral branching. (The Penguin Dictionary of Biology).

Phylum (plural phyla): From the Greek word phylon meaning ‘tribe’ or ‘stock’; It is a taxonomic category which is right below a kingdom and above a class. *See*

Taxonomy: the science of hierarchical classification of living organisms based on their shared morphological, physiological or molecular characteristics.The major taxonomic divisions, from highest to lowest, are: Kingdom, Phylum, Class, Order, Family, Genusand Species. The suffix sub- can be added to indicate an intermediate division. Example: sub-kingdom is below kingdom and above phylum.

VITA

Tarik El Mellouki was born on February 11, 1981 in Rabat, the capital of Morocco. He graduated from Abdelkarim Al Khattabi High School in 1999 with a major in experimental sciences. Tarik received a Bachelor of Science (B.S.) degree in Plant Biology from Mohammed V University (Rabat, Morocco) in 2003 and a Master of Science (M.S) degree in Plant Cell Biology and Development from Paris XI University (Orsay, France) in 2005. In 2007, he joined the interdisciplinary Ph.D. program at the School of Biological Sciences, University of Missouri-Kansas City (UMKC). Upon completion of required courses, he received a M.S. in Molecular Biology and Biochemistry in 2009 from UMKC. Tarik has a multidisciplinary education and research background and have been involved in teaching multiple undergraduate and graduate level courses at the UMKC. These included general biology, human anatomy and microbiology. During his graduate education he participated in research projects studying developmental genetics of plants, transcriptional regulation in yeast and the role of microtubule-based transport in filamentous fungi. Tarik prepared this dissertation for his Ph.D. degree under the supervision of Dr. Michael Plamann.

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Bowman B.J., M. Draskovic, R.R. Schnittker, T. El-Mellouki, M.D. Plamann, E. Sánchez-León, M. Riquelme, E.J. Bowman. 2015. "Characterization of a Novel Prevacuolar Compartment in Neurospora crassa." Eukaryot Cell. 14(12):1253-63.

Liu S., P. Kandoth, S. Warren, G. Yeckel, R. Heinz, J. Alden, C. Yang, A. Jamai, T. El-Mellouki, P. Juvale, J. Hill, T. Baum, S. Cianzio, S. Whitham, D. Korkin, M. Mitchum, K. Meksem. 2012. A Soybean Cyst Nematode Resistance Gene Points to a New Mechanism of Plant Resistance to Pathogens. Nature 492(7428):256-60.

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Meksem K., S. Liu, X. Liu, A. Jamai, M. Mitchum, A. Bendahmane, and T. El-Mellouki. 2008. TILLING: A reverse genetics and functional genomics tool in soybean. The Handbook of Plant Functional Genomics: Concepts and Protocols (pp. 251-265). Wiley-VCH, Weinheim (Book Chapter)

He has the following publications in preparation:

El-Mellouki T., R. Schnittker, Y. Heilig, S. Seiler and M. Plamann. "Identification and functional description of a novel link between dynein and kinesin-1".

El-Mellouki T., K. Pirani, D. Belostotsky, and J. Chekanova. "Posttranscriptional perinuclear retention of activated genes in S. cerevisiae: chromatin-independent effects of Sus1 and position dependent effects of H2A.Z".