Tansley review

Tracks for traffic: microtubules in the plant pathogen *Ustilago maydis*

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Tracks for traffic: microtubules in the plant pathogen *Ustilago maydis*

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Contents

Key words: dynein, hyphal growth, kinesin, microtubule array, organelle transport.

Summary

Pathogenic development of the corn smut fungus *Ustilago maydis* depends on the ability of the hypha to grow invasively. Extended hyphal growth and mitosis require microtubules, as revealed by recent studies on the microtubule cytoskeleton. Surprisingly, hyphal tip growth involves only two out of 10 kinesins. Kinesin-3 is responsible for tip-directed (anterograde) endosome motility of early endosomes, which are thought to support hyphal elongation by apical membrane recycling. In addition, kinesin-3, together with kinesin-1 and myosin-5, appear to deliver secretory vesicles to the hyphal tip. Kinesin-1 also affects endosome motility by targeting cytoplasmic dynein to microtubule plus ends. This plus-end localization of dynein is essential for cell body-directed (retrograde) endosome motility, but also allows force generation during spindle elongation in mitosis. Furthermore, kinesin-1 and dynein participate in the organization of the microtubule array, thereby building their own network of tracks for intracellular motility. The recent progress in understanding microtubule-based processes in *U. maydis* has revealed an unexpected complexity of motor functions essential for the virulence of this pathogen. Further studies on structural and regulatory requirements for motor activity should help identify novel targets for fungicide development.

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I. Introduction

1. Fungal tip growth

Fungi are an important group of microorganisms that have enormous impact on our ecosystem. Saprotrophic fungi are crucial for the decomposition of organic material and vegetable matter (Evans & Hedger, 2001), whereas mycorrhizal fungi live in symbiosis with 80–90% of the vascular plants (Smith & Read, 1997). Moreover, many fungi are also harmful plant pathogens and food contaminants that pose a serious threat to the agricultural and food industries (Agrios, 1997; Tournas, 2005). Filamentous tip growth is a hallmark of fungi. Apical expansion of the fungal hypha allows the fungus to invade, explore, and colonize substrate such as soil or tissues (Wessels, 1993), and invasive growth is essential for fungal pathogenesis (Deising *et al.*, 2000) and establishing mycorrhiza symbiosis (Genre *et al.*, 2005). Thus, the molecular mechanisms of polarized fungal growth are of crucial importance for an understanding of the relationship between fungi and plants.

It has long been discussed that the expansion of the tip involves local release or surface exposure of exoenzymes and proteins that participate in wall formation (Bartnicki-Garcia, 2002). Prominent representatives of the latter are chitin synthases. These integral membrane proteins reside on a special class of vesicles, the chitosomes (Bracker *et al.*, 1976; Agrios, 1997; Bartnicki-Garcia, 2006), and are delivered to the growth region at the hyphal tip. Although the molecular details are not known, it is widely assumed that enzyme-containing vesicles are formed at the Golgi apparatus and accumulate in an apical membrane cluster, called the 'Spitzenkörper' (Brunswik, 1924; Girbardt, 1957). From there they are thought to be released for subsequent fusion with the plasma membrane (Bartnicki-Garcia *et al.*, 1989; Bartnicki-Garcia, 2002). Once exposed to the cellular surface, chitin synthases are involved in the formation of chitin microfibrils, which confer rigidity to the fungal cell wall and are therefore essential for shaping the hypha. Defects in the cell wall abolish tip growth, and this fact underlies the crucial importance of some chitin synthases in plant pathogenicity in *Botrytis cinerea* (Soulie *et al.*, 2003, 2006), *Ustilago maydis* (Garcera-Teruel *et al.*, 2004; Weber *et al.*, 2006), or *Fusarium oxysporum* (Madrid *et al.*, 2003). In the yeast *Saccharomyces cerevisiae*, chitin synthases most likely are endocytosed and recycled back to the plasma membranes via early endosomes and the Golgi apparatus (Ziman *et al.*, 1996; Ortiz & Novick, 2006). Although it is likely that they exist, no similar mechanisms have been reported in other fungi. However, in numerous fungi, the endocytic marker dye FM4-64 colocalizes with the Spitzenkörper (Hoffmann & Mendgen, 1998; Fischer-Parton *et al.*, 2000; Crampin *et al.*, 2005; Harris *et al.*, 2005) and in the corn smut fungus *Ustilago maydis* early endosome-based recycling probably supports hyphal growth and the distinct steps of pathogenic development (Wedlich-Söldner *et al.*, 2000; Fuchs *et al.*, 2006). Interestingly, early endosomes reach the hyphal tip in *U. maydis* hyphae by a microtubule (MT)-based transport mechanism. Early ultrastructural studies in *Fusarium acuminatum* demonstrated that filamentous actin (F-actin) and MTs reach into the fungal Spitzenkörper (Howard, 1981) and molecular motors are concentrated in the apex of *U. maydis* hyphae (Weber *et al.*, 2003; Schuchardt *et al.*, 2005), which suggests that the cytoskeleton supports hyphal growth. Indeed, numerous studies imply an essential role of F-actin and MT-based transport in fungal growth and development (Yokoyama *et al.*, 1990; Akashi *et al.*, 1994; Crampin *et al.*, 2005; Fuchs *et al.*, 2005; Horio & Oakley, 2005; Konzack *et al.*, 2005; for an overview see Heath, 1995).

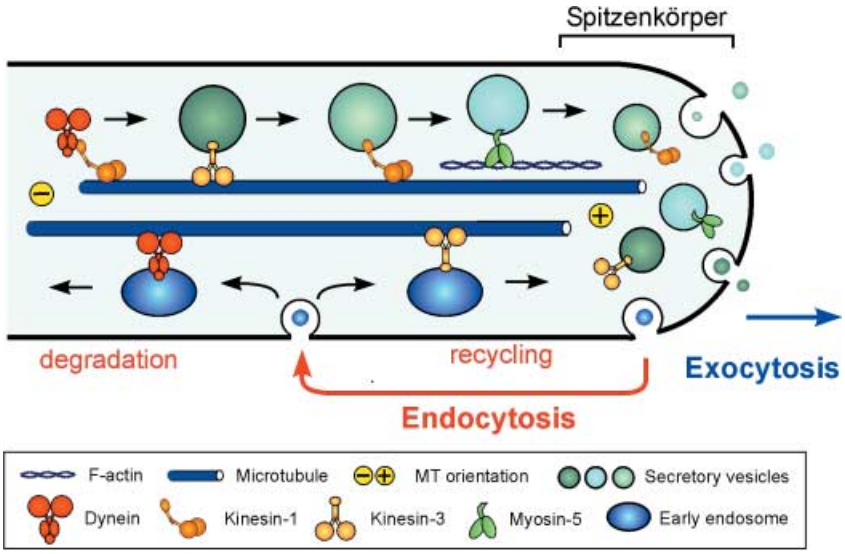

Fig. 1 Motors in membrane traffic during hyphal growth of *Ustilago maydis*. Myosin-5, kinesin-1 and kinesin-3 are thought to cooperate in maintaining the polarity of the hyphal tip cell by delivering growth supplies. In addition, kinesin-1 directly or indirectly takes dynein to microtubule (MT) plus ends. From there dynein takes early endosomes to subapical regions of the cell for sorting of endocytosed material to the vacuole. Early endosomes reach the hyphal tip through the activity of kinesin-3. At the apex, early endosomes most likely participate in polar endocytic recycling processes and sorting of endocytosed material for degradation in the vacuole. Both exocytosis and endocytosis is essential for proper hyphal tip growth.

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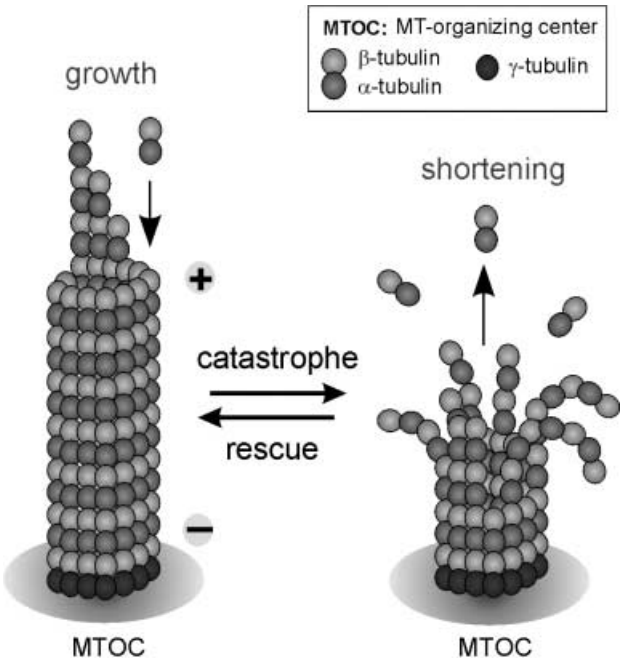

Fig. 2 Dynamics of microtubules (MTs). These consist of α- and β-tubulin dimers that assemble and disassemble at the MT plus end (+). The minus end (–) is usually embedded in a γ-tubulin containing the microtubule-organizing center (MTOC). *In vitro* MTs stochastically switch between phases of growth and shrinkage; the transitions are named catastrophe and rescue. In the cell, MT-associated proteins modify these parameters and thereby stabilize or destabilize MTs arrays. Figure modified from Steinberg (2007).

2. The microtubule cytoskeleton in fungal growth

Maintaining the polarization of fungal hyphae and forming the apical Spitzenkörper require directed transport of membranous organelles and vesicles to the hyphal tip (Gow, 1995a). The individual organelles move at a considerable speed over a long distance, as shown by *in vivo* observation of growing hyphae (Steinberg, 1997; Seiler *et al.*, 1999; Wedlich-Söldner *et al.*, 2000), and this transport is thought to support polar extension of the hyphal apex. In the current view, the protein fibers of the cytoskeleton, F-actin and MTs, serve as 'tracks' for specialized mechanoenzymes – the so-called molecular motors – that carry membranes and proteins towards the hyphal tip or back to the subapical parts of the cell (Fig. 1; see later). These motors are grouped into F-actin-associated myosins and MT-dependent kinesins or dyneins (overview in Schliwa & Woehlke, 2003). Most kinesins move towards the plus ends, whereas dynein 'walks' toward the minus ends, thereby mediating membrane traffic (see overview in Xiang & Plamann, 2003; Gross, 2004; Hirokawa & Takemura, 2005; Soldati & Schliwa, 2006). Thus, in fungal hyphae the orientation of the MTs determines whether kinesins or dyneins are used for delivery of membranes and growth supplies to the expanding apex.

The MTs are hollow cylinders consisting of 13–15 protofilaments, each made of numerous α/β-tubulin dimers (Fig. 2). *In vivo*, MT formation usually begins at microtubule-organizing centers (MTOCs). These centers are often located at the nucleus, such as the spindle pole body (SPB) (Fig. 3a, *S. cerevisiae* is given as an example), but can also be cytoplasmic and nuclear-independent, as found in *U. maydis* yeast-like cells (Straube *et al.*, 2003; Fig. 3b) and in *Aspergillus nidulans* (Konzak *et al.* 2005). The MTOCs contain the distantly related γ-tubulin (Oakley & Oakley, 1989; Oakley, 2000), which is thought to be involved in recruitment of the first tubulin dimers and thereby nucleates MTs, although this view is challenged by more recent results on MT nucleation (summarized in Job *et al.*, 2003). The polymer is elongated by rapid assembly of tubulin subunits at the plus end (Fig. 2), where β-tubulin is exposed; the opposite MT minus end remains in contact with the MTOC (Figs 2 and 3). Assembled MTs stochastically switch from elongation to rapid disassembly (catastrophe) and vice versa (rescue; Fig. 2; Desai & Mitchison, 1997), which was observed in many fungi by labeling MTs with GFP-tubulin (Carminati & Stearns, 1997; Drummond & Cross, 2000; Steinberg *et al.*, 2001; Finley & Berman, 2005). The MT binding proteins that often bind to and modify MT plus ends regulate MT dynamics (overview in Carvalho *et al.*, 2003). In particular, fungal kinesin motors not only move their cargo towards MT plus ends, they also modify MT dynamics, thereby participating in the organization of the cellular MT array (Konzack *et al.*, 2005, overview in Steinberg, 2007; Wu *et al.*, 2006). Thus, MT motors, and in particular kinesins, participate in a broad spectrum of functions in the fungal cell, including membrane transport, spindle elongation in mitosis and regulation of MT dynamics. However, our knowledge of the role of MT-based motors in fungi has been restricted mainly to studies in the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe*, and nothing was known about the role of MTs in plant pathogenic fungi. This situation recently changed as rapid progress was made in understanding the organization and importance of MTs in the plant pathogen *U. maydis*.

3. The model system *Ustilago maydis*

The pathogen *U. maydis* is the causative agent of corn smut disease (Brefeld, 1883; Christen, 1963). Virulence of this basidiomycota fungus is linked to a morphological transition from a nonpathogenic yeast-like cell to an infective hypha during the initial steps of pathogenic development. Our understanding of the molecular regulation and structural requirement has greatly advanced because of numerous technical advantages, including molecular genetic tools, a published genome (Kämper *et al.*, 2006), and excellent live-cell imaging techniques (Becht *et al.*, 2006; Fink *et al.*, 2006; Lenz *et al.*, 2006). Consequently, *U. maydis* has become a valuable model system for plant pathogenicity, which is the subject of several

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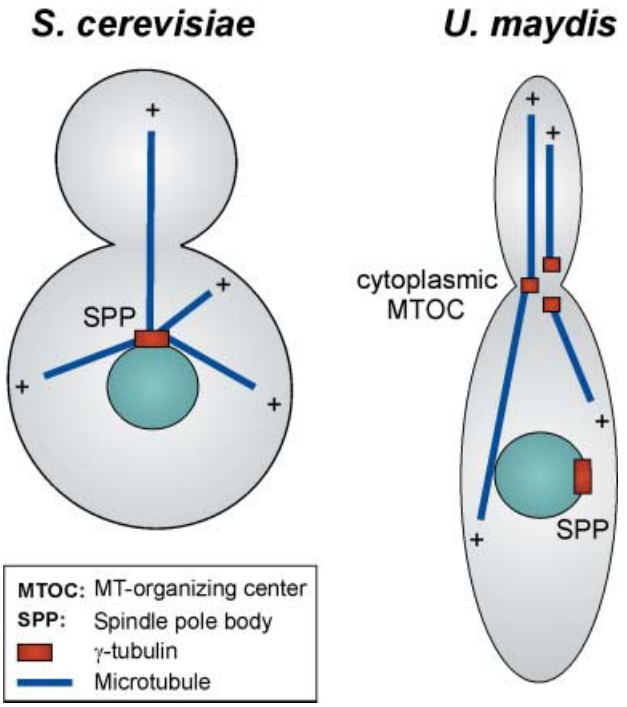

Fig. 3 Microtubule (MT) organization in *Saccharomyces cerevisiae* and *Ustilago maydis*. In bakers yeast a perinuclear microtubule-organizing center (MTOC) organizes the MT array, whereas MTs in *U. maydis* are formed by cytoplasmic nucleation sites at the bud neck. However, in both organisms MTs extend their plus ends towards the growth region.

recent reviews (see Bölker, 2001; Feldbrügge *et al.*, 2004; Perez-Martin *et al.*, 2006).

Outside of its host plant, *U. maydis* forms elongated yeast-like cells that grow by polar budding (sporidia; Fig. 4). Under laboratory conditions, the yeast-like sporidia have a doubling time of about 2 h and can easily be cultivated and experimentally manipulated. Sporidia are saprotrophic and are not harmful to the maize plant. However, the fungus can form galls (usually called 'tumors') on the flowers, leaves or stems. Pathogenic development begins when two compatible sporidia on the leaf epidermis exchange pheromones and recognize each other. The cell cycle arrests in the G2 phase (Garcia-Muse *et al.*, 2003), which leads to continuous tip growth and the formation of long conjugation hyphae (Fig. 4). The conjugation hyphae grow toward each other (Snetselaar *et al.*, 1996), thereby bridging distances of up to 300 μm (Fuchs *et al.*, 2005) before they fuse their cytoplasm at their tip. Subsequently, the dimeric *bE/bW* transcription factor is formed (Gillissen *et al.*, 1992; Kämper *et al.*, 1995), which establishes the formation of a straight hypha (*b*-dependent hypha; Fig. 4; overview in Kahmann *et al.*, 1995; Feldbrügge *et al.*, 2004). This infectious hypha extends over the plant surface, penetrates the epidermis, and colonizes the host plant. Finally, the fungus proliferates and induces the formation of a plant gall, in which diploid spores are formed (Banuett & Herskowitz, 1996). The life cycle is completed when spores are released and germinate to form a promycelium, which undergoes meiosis and buds off haploid sporidia. It is important to note that pathogenic development requires the morphogenic transition from yeast-like to highly polarized hyphal cells. Thus, cytoskeleton-based polarized tip growth is essential for virulence of *U. maydis* (Fig. 4; hyphal growth is indicated by arrows), which also explains the interest in the role of MTs and associated motors in the corn smut fungus.

II. Organization of the interphase microtubule array in *Ustilago maydis*

1. Microtubules in yeast-like sporidia

A deeper understanding of the MT-based transport mechanisms that underlie polar growth and plant infection requires detailed knowledge of the cellular organization of the cytoskeleton. Intensive work along these lines in *U. maydis* has been undertaken. Unexpectedly, these studies reveal that molecular motors play active roles in organizing a polar MT array. In exponentially growing cultures of yeast-like cells, *c.* 50% of the sporidia are in the G2 phase (McCann & Snetselaar, 1997; Garcia-Muse *et al.*, 2004). At this stage, the cells are actively growing at one cell pole and contain three to six MT tracks (Fig. 5a; Steinberg *et al.*, 2001), each consisting of individual or bundled MTs (Straube *et al.*, 2006). *In vivo* observation of green fluorescent protein (GFP)-α-tubulin and growing MT plus ends labeled with a fluorescent protein fused to Peb1, a plus end-binding homologue of EB1 (Straube *et al.*, 2003), indicate that MTs within growing cells have a uniparallel orientation, with more than 85% of the plus ends extending to the cell poles, whereas unbudded cells contain antiparallel MT bundles (Fig. 5a; Straube *et al.*, 2003). The MT plus ends extend into the growing bud of sporidia, which suggests that MT- and kinesin-based transport processes participate in apical growth during the G2 phase. Indeed, in the absence of MTs, cell buds occasionally form at the side of the mother cell (Steinberg *et al.*, 2001), a phenotype also found in kinesin-1 mutants (Straube *et al.*, 2003). Thus, MT-based transport processes appear to participate in bud site selection; this conclusion is further supported by the loss of the bipolar budding pattern in kinesin-3 null mutants (Wedlich-Söldner *et al.*, 2002a). However, bud formation itself does not require MTs. By contrast, cells arrest in a large budded mitotic phase when MTs are disrupted by the inhibitor benomyl (Fuchs *et al.*, 2005) or when MT nucleation is impaired in γ-tubulin mutants (Straube *et al.*, 2003). These findings indicate that MTs are essential for mitosis (see later) and participate in determining cell polarity, but are dispensable in polarized growth in sporidia.

Surprisingly, MTs are not nucleated at the nucleus, but rather appear at the neck region (Straube *et al.*, 2003; Figs 3

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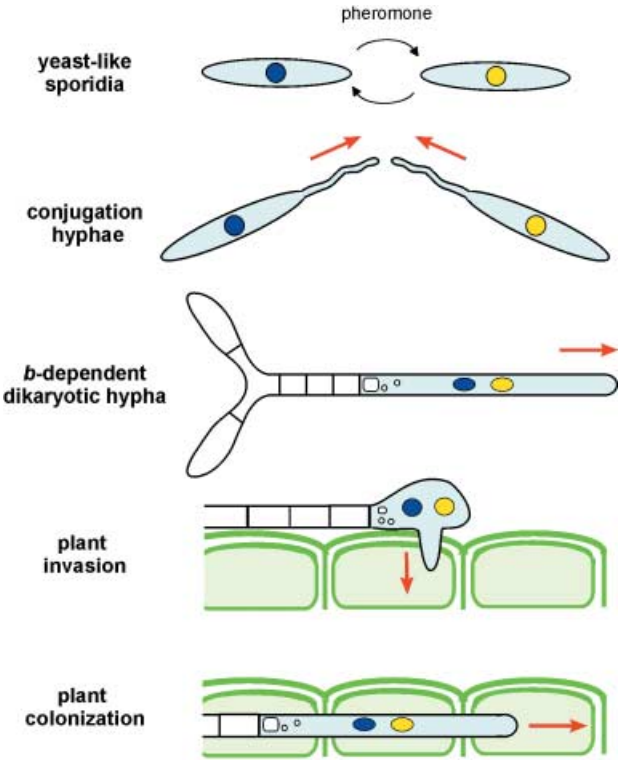

Fig. 4 Early stages of the pathogenic life cycle of *Ustilago maydis*. On the plant surface, compatible haploid sporidia exchange pheromones and undergo a morphological transition from yeast-like cells to tip-growing conjugation hyphae. Hyphae fuse to give rise to a *b*-dependent dikaryotic hypha that consists of a tip cell and subapical vacuolated and empty-appearing section. Upon invasion, the hypha changes its growth direction, enters the epidermis, and subsequently colonizes the plant tissue. Note that polarized tip growth, indicated by arrows, is essential for the pathogenic development of *U. maydis*. Figure modified from Weber *et al.* (2006).

and 5a), which indicates that cytoplasmic nucleation sites organize the MT array. This conclusion is confirmed by antibody studies that indicate that γ-tubulin, an essential compound of MT nucleation sites, is concentrated in the neck region (Straube *et al.*, 2003). Hence, the transition from randomly oriented MTs in unbudded cells to a polarized array in growing cells appears to be based on a recruitment of cytoplasmic nucleation sites to the neck region at the onset of budding.

It was noticed early on that assembled MTs move throughout the cytoplasm of *U. maydis* sporidia at rates typical for motor-driven transport (Steinberg *et al.*, 2001). In neuronal cells, a similar motility of MTs has been described (Tanaka & Kirschner, 1991); this motility is thought to be driven by the cytoplasmic minus-end-directed motor dynein (Ahmad *et al.*, 1998) and underscores the polar organization of the MT array in axons (for an overview see Baas, 2002). *Ustilago maydis* contains a dynein motor (Straube *et al.*, 2001), and extensive analysis of its cellular importance has revealed that dynein is involved in a broad spectrum of processes, including nuclear migration (Straube *et al.*, 2001) and removal of the mitotic nuclear envelope in prophase (Straube *et al.*, 2005), motility of the endoplasmic reticulum (Wedlich-Söldner *et al.*, 2002b), endosome traffic (Wedlich-Söldner *et al.*, 2002a; Lenz *et al.*, 2006) and spindle elongation in anaphase B (Fink *et al.*, 2006; see later). Thus, dynein is a good candidate for powering the motility of assembled MTs in *U. maydis*.

In vivo observation of Peb1-labeled MTs demonstrated that MTs move with their plus end leading (Fink & Steinberg, 2006). Dynein is concentrated at the plus ends of moving MTs and appears to be offloaded to the cell cortex during MT sliding. In the current model, this offloading to the cell cortex results in cortical anchorage and activation of dynein, and the subsequent minus-end-directed activity of dynein pushes MTs through the cytoplasm (Fink & Steinberg, 2006). Such a mechanism was initially described for dynein in nuclear migration in *Saccharomyces cerevisiae* (Lee *et al.* 2003; Sheeman *et al.*, 2003) and has also been suggested to support nuclear migration and spindle elongation in *U. maydis* (Straube *et al.*, 2005; Fink *et al.*, 2006). Interestingly, the polarity of MTs is lost in dynein mutants and after disruption of MTs by the inhibitor benomyl (Fink & Steinberg, 2006), which suggests that cells require intact MTs and dynein-based motility to maintain the nucleation sites within the neck region. Furthermore, MT motility is strongly enhanced in benomyl-treated cells (Fink & Steinberg, 2006). These results suggest that MTs interact with each other to stabilize the MT arrays. Indeed, most MT bundling is found in the neck region of budding cells, which indicates that nucleation sites are kept in place by MT–MT cross-linking proteins. The molecular basis of MT bundling is not known, but it has been recently shown that another motor, kinesin-1, participates in cross-bridging MTs in *U. maydis* (Straube *et al.*, 2006). This result is most surprising, as kinesin-1 motors are well known for their role in membrane traffic.

2. Microtubules in hyphae

Conjugation and *b*-dependent hyphae of *U. maydis* consist of a living tip cell of 100–150 μm long (Steinberg *et al.*, 1998; Fuchs *et al.*, 2005) which expands at the apex, while leaving collapsed sections behind (Fig. 5b). Long and often bundled MTs reach to the growing tip and into the vacuolated region near the basal septum (Straube *et al.*, 2001). Quantitative analysis of the motility and distribution of growing plus ends labeled with fluorescent EB1 homologues (Straube *et al.*, 2003) has demonstrated that most plus ends extend towards the growing tip of conjugation hyphae (Fuchs *et al.*, 2005) and to the tip and the septum of *b*-dependent hyphae (Schuchardt *et al.*, 2005; Lenz *et al.*, 2006; Fig. 5b). Similar to yeast-like cells, the SPBs are not active in hyphae, and a large zone of antiparallel oriented MTs covers the middle part of the hypha (Fig. 5b; Lenz *et al.*, 2006). Hence, sporidia and hyphae are

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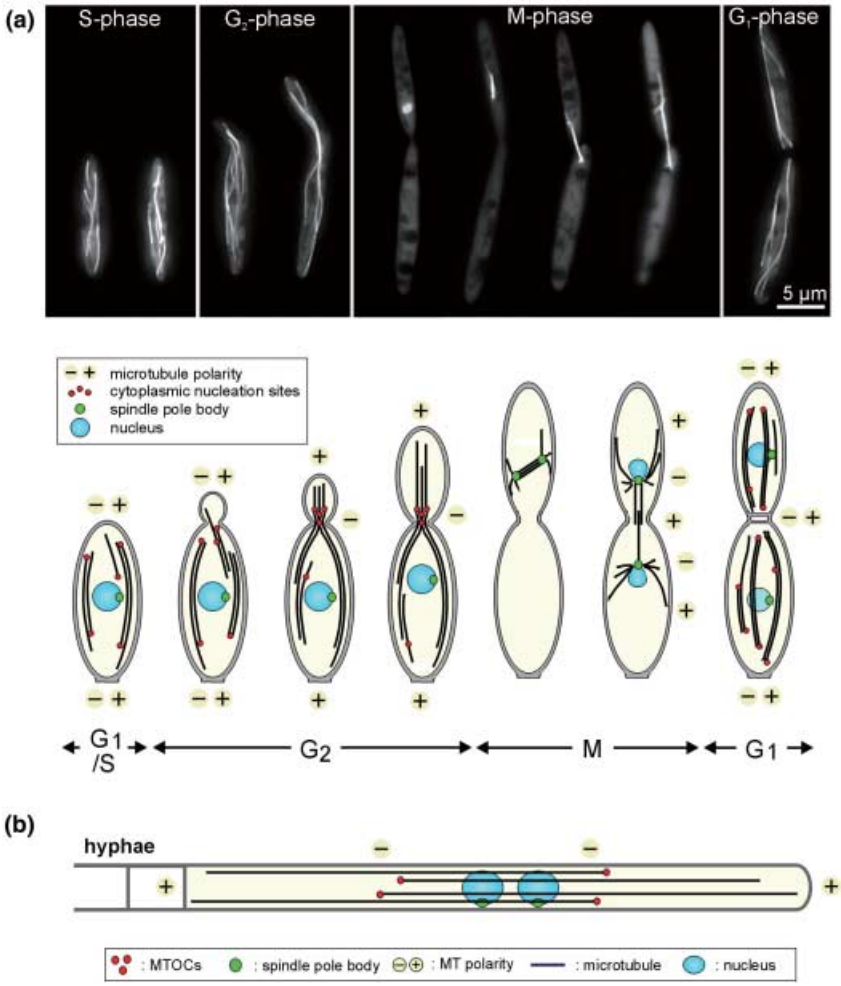

Fig. 5 Microtubule (MT) organization in sporidia and hyphae of *Ustilago maydis*. (a) In the S-phase, cells are unbudded, the nucleus-associated spindle pole body (SPB) is inactive and MTs are nucleated by cytoplasmic microtubule-organizing centers (MTOCs), resulting in an antiparallel microtubule array (orientation of microtubules is indicated by + and –). At the onset of budding (G2 phase), nucleation sites are focused at the neck region. As a consequence, the plus ends are directed to the cell poles. In prophase, the interphase MT array disappears, and the SPBs become active and forms long astral MTs that reach into the bud (not shown). Dynein pulls the SPBs into the bud, and a metaphase spindle is formed. This spindle elongates in anaphase, thereby segregating the chromosomes to the mother and the daughter cell. In the G1 phase, two septa are formed, and antiparallel microtubule arrays are re-established. (b) In hyphae MTs are nucleated near the central nucleus (in case of a dikaryon the central pair of nuclei). About 80–90% of all plus ends are directed towards the cell poles and the subapical septum. Note that molecular motors use the orientation of the microtubules and, consequently, kinesins are involved in polarized growth of *U. maydis* hyphae.

similar in that plus ends are directed to the cell poles and MTs are organized by cytoplasmic nucleation sites. Almost nothing is known about the mechanisms by which the hyphal MT array is organized, but similar to yeast-like cells, hyphal MTs undergo rapid motility (Steinberg *et al.*, 2001), which suggests that a motor-dependent mechanism polarizes the hyphal MT array.

It is generally assumed that MTs are involved in long-distance transport, which might be of particular importance in elongated hyphae. Disruption of hyphal MTs by benomyl consistently drastically decreased the rate of hyphal elongation (Fuchs *et al.*, 2005). Nevertheless, MT-deficient conjugation and the *b*-dependent hyphae continued tip growth, indicating that MTs are not essential for filamentous growth *per se*. However, hyphae stop growing at a length of 40–60 µm, indicating that MTs are essential for extended filamentous growth. The obvious question is: Which MT-based process becomes essential when hyphae reach a length of *c.* 50 µm? The nuclei start migration into the elongating hypha at *c.* 50 µm, and in the absence of MTs, nuclei remain in the mother cell. Nuclear migration in filamentous fungi depends on dynein (Plamann *et al.*, 1994; Xiang *et al.*, 1994; Alberti-Segui *et al.*, 2001) and dynein mutants also arrest at a hyphal length of *c.* 50 µm (Fuchs *et al.*, 2005), which indicates that MT-based nuclear migration is a prerequisite for extended hyphal tip growth. However, extended hyphal tip growth is also impaired in kinesin-3 mutants (Schuchardt *et al.*, 2005), but no role of kinesin-3 in nuclear migration is known. Thus, the MTs may have additional essential roles in extended hyphal growth.

An interesting new aspect of MT function in the hyphae of *U. maydis* was recently provided the work of M. Feldbrügge and co-workers. In a genome wide approach they identified 27 open reading frames that show significant sequence similarity with known RNA-binding proteins (Becht *et al.*, 2005). Surprisingly, only three out of 18 deletion mutants showed a phenotype that ranged from slower growth on agar plates at low temperature (Δ*khd4*) to aberrant cell morphology in yeast-like sporidia (Δ*rrm1*) and impaired mating and reduced virulence (Δ*rrm1* and Δ*rrm4*; Becht *et al.* 2005). Subsequent localization studies demonstrated that a fusion protein of Rrm4 and the green fluorescent protein formed particles that rapidly moved along MTs in a bidirectional fashion (Becht

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Table 1 Cellular role of microtubule (MT)-based motors in *Ustilago maydis*

Type	Name	Localization	Cellular function	Reference
Kinesin-1	Kin1[a]	Hyphal apex; cytoplasm	Secretion; dynein targeting; vacuole formation; organizing MT bundles	1, 2, 3, 4, 5
Kinesin-3	Kin3	Hyphal apex; early endosomes	Secretion; motility of early endosomes	1, 6
Kinesin-4	Kin4	Unknown	Unknown	1
Kinesin-5	Kin5	Mitotic spindle	Spindle elongation in anaphase A	1, 7
Kinesin-6	Kin6	Unknown	Unknown	1
Kinesin-7a	Kin7a[b]	Unknown	Unknown	1
Kinesin-7b	Kin7b	Unknown	Unknown	1
Kinesin-8	Kin8	Unknown	Unknown	1
Kinesin-9	Kin9	Unknown	Unknown	1
Kinesin-14	Kin14	Mitotic spindle	Mitotic (?)	1, 13
Dynein	Dyn1/Dyn2	At plus ends and along MTs, at the mitotic MT; mitotic spindle	Spindle elongation in anaphase B; spindle positioning; removal of nuclear envelope in prophase; regulating MT dynamics in interphase and mitosis; transport of interphase MTs and nucleation sites; motility of early endosomes and the endoplasmic reticulum	7, 8, 9, 10, 11, 12

References: 1, Schuchardt *et al*. (2005); 2, Lehmler *et al*. (1997); 3, Steinberg *et al*. (1998); 4, Lenz *et al*. (2006); 5, Straube *et al*. (2006); 6, Wedlich-Söldner *et al*. (2002a); 7, Fink *et al*. (2006); 8, Straube *et al*. (2001); 9, Straube *et al*. (2006); 10, Adamikova *et al*. (2004); 11, Fink and Steinberg (2006); 12, Wedlich-Söldner *et al*. (2002b); 13, C. Schuberth & G. Steinberg (unpublished).
[a]Previously named Kin2 (Lehmler *et al*., 1997).
[b]Previously named Kin1 (Lehmler *et al*., 1997).

et al., 2006). This was most prominent in hyphae, where Rrm4 showed increased RNA binding, suggesting that this putative RNA-binding protein has an essential role in the filamentous growth of *U. maydis*. Indeed, deletion of *rrm4* led to a bipolar growth of hyphae. The cargo of Rrm4 is presently unknown, but an attractive possibility is that Rrm4 shuttles between the tip and nucleus in order to deliver mRNA to the translation machinery in the hyphal tip. This notion is supported by ultrastructural data that indicate that ribosomes are enriched in the hyphal apex (Girbardt, 1969; Howard, 1981). Taken together it becomes obvious that MT-based long-distance transport of vesicles, organelles and most likely RNA is essential for filamentous growth and therefore pathogenic development of *U. maydis*.

III. Molecular motors in hyphal tip growth of *Ustilago maydis*

During plant infection, *U. maydis* forms appressoria (Snetselaar & Mims, 1992, 1993; Brachmann *et al*., 2003), and it is thought that polar secretion of lytic enzymes supports the penetration of the host epidermis. The importance of protein secretion in pathogenic development is further illustrated by the recent analysis of the published genome of *U. maydis*, which revealed that secretory proteins appear to participate in the establishment of the biotrophic phase during early colonization of the host tissue (Kämper *et al*., 2006). It is thought that the enzymes are secreted at the hyphal tip (Gow, 1995a), and numerous lines of evidence strongly indicate that the cytoskeleton and associated motors play a crucial role in the secretory pathway. Filamentous fungi contain *c*. 15 different motors, which includes four myosins (classes I, II, V and a myosin–chitin synthase), 10–12 kinesins (classes 1, 3, 4, 5, 6, 7, 8, 14 and some orphans) and a single dynein. Conditional or deletion mutants of most motors have been generated in *U. maydis,* and their participation in hyphal growth has been analysed (Lehmler *et al*., 1997; Steinberg *et al*., 1998; Straube *et al*., 2001; Wedlich-Söldner *et al*., 2002a; Wedlich-Söldner *et al*., 2002b; Weber *et al*., 2003; Schuchardt *et al*., 2005; Lenz *et al*., 2006; Weber *et al*., 2006; Table 1). Surprisingly, only a small subset of motors is involved in tip growth, which includes the F-actin-based myosin-5 and members of the kinesin-1 and kinesin-3 family (Fig. 1). Interestingly, the last two are not present in the small yeast *S. cerevisiae* but have important roles in membrane transport in elongated neuronal cells (Bloom, 2001; Hirokawa & Takemura, 2005). Thus, it is tempting to speculate that the need for long-distance transport in hyphae led to conservation of the underlying machinery in *U. maydis* hyphae and elongated animal cells.

1. Microtubule-based kinesins in secretion

Consistent with the predicted role in long-distance transport, null mutants of kinesin-1 and kinesin-3 form only short and slow-growing hyphae (Schuchardt *et al*., 2005). Such a phenotype resembles that found in benomyl-treated cells that lack MTs (Fuchs *et al*., 2005), which indicates that both kinesins have essential roles in MT-based transport. The cargo of kinesin-1 has not yet been identified, but several

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lines of evidence indicate that this motor participates in delivery of secretory vesicles to the Spitzenkörper in the growing hyphal tip. The apex of infectious hyphae of *U. maydis* contain a cluster of vesicles that are thought to support local exocytosis during tip growth (Lehmler *et al.*, 1997). In kinesin-1 null mutants, the apical membrane accumulation is lost (Lehmler *et al.*, 1997) and mutant hyphae are impaired in secretion of the marker enzymes invertase (I. Schuchardt and G. Steinberg, unpublished). Moreover, GFP-fusion proteins of kinesin-1 localize to a distinct apical spot (Schuchardt *et al.*, 2005), which suggests that the motor is bound to the Spitzenkörper vesicles. Taken together, it is therefore most likely that kinesin-1 participates in the delivery of secretory vesicles towards the MT plus ends in the hyphal tip. A similar role in transport towards the tip has been suggested for myosin-5, which also locates in the hyphal apex (Weber *et al.*, 2003; Schuchardt *et al.*, 2005). Single mutants in kinesin-1 and myosin-5 maintain cell polarity and form hyphae, albeit at very low growth rates. However, this ability to grow polarized is lost in kinesin-1/myosin-5 double-mutant hyphae (Schuchardt *et al.*, 2005), which indicates that both MT- and F-actin-based transport cooperate in secretion during hyphal tip growth. A similar phenotype is found in kinesin-3/myosin-5 double mutants (Schuchardt *et al.*, 2005), and kinesin-3 but not kinesin-1 single-mutant hyphae show defects in acid phosphatase secretion (Schuchardt *et al.*, 2005). It is therefore likely that kinesin-1, kinesin-3 and myosin-5 deliver distinct classes of secretory vesicles to the growth region in *U. maydis* (Fig. 1). The results of these studies together demonstrate that MT-and F-actin-based transport mechanisms cooperate to establish and maintain polarized growth of infectious hyphae. The molecular details of this interaction remain to be elucidated, and the identification of their cargo is a challenge for the nearer future.

2. Bidirectional motility of early endosomes

Morphogenesis and pathogenicity of *U. maydis* depends on the ordered formation of the cell wall, and this is best illustrated by the severe growth defects and impaired virulence in the absence of some chitin synthases that localize to the tip of growing sporidia and hyphae (Garcera-Teruel *et al.*, 2004; Weber *et al.*, 2006). Chitin synthases are delivered to the tip in secretory vesicles – so-called chitosomes (Bracker *et al.*, 1976; Ruiz-Herrera *et al.*, 1977), which emphasizes the importance of secretion in tip growth. However, chitin synthases in *S. cerevisiae* undergo endocytic recycling (Ziman *et al.*, 1996; Ortiz & Novick, 2006) and preliminary evidence exists that chitin synthases are also recycled in *U. maydis* (U. Fuchs and G. Steinberg, unpublished). This suggests that exo- and endocytosis support hyphal growth of *U. maydis*. Screening for morphological mutants in *U. maydis* provided the first indication that endocytic recycling participates in polar growth (Wedlich-Söldner *et al.*, 2000). This genetic approach has led to the identification of *yup1*, whose gene product is predicted to be a t-SNARE that resides on a target membrane, where it is thought to mediate membrane fusion with arriving transport vesicles (Wedlich-Söldner *et al.*, 2000). Interestingly, Yup1-GFP localizes on organelles that can be labeled with the endocytic marker dye FM4-64 (Wedlich-Söldner *et al.*, 2000) and that carry Rab5-like GTPases (Fuchs *et al.*, 2006), which suggests that these organelles are early endosomes. Indeed, *yup1*^{ts} mutants are impaired in endocytosis of FM4-64 (Wedlich-Söldner *et al.*, 2000) and fail to recycle the pheromone receptor Pra1 back to the surface (Fuchs *et al.*, 2006). As a consequence, the level of exposed Pra1 is reduced, which results in impaired sensitivity to compatible pheromone and abolishes the initiation of the pathogenic program (Fuchs *et al.*, 2006). These results strongly suggest that receptors and synthetic enzymes cycle between the surface and the early endosomes for several rounds of usage. However, except for the pheromone receptor, no other cargo for endocytic recycling is known.

Early endosomes in sporidia and hyphae rapidly move bidirectionally along MTs (Wedlich-Söldner *et al.*, 2000; Lenz *et al.*, 2006). This motility is thought to support recycling processes at the hyphal tip (Lenz *et al.*, 2006), but also underlies the cell-cycle-dependent rearrangement of early endosomes in yeast-like sporidia (Wedlich-Söldner *et al.*, 2002a). In both cell types, bidirectional motility is mediated by plus end-directed kinesin-3 and minus-end-directed cytoplasmic dynein (Wedlich-Söldner *et al.*, 2002a; Lenz *et al.*, 2006); therefore, kinesin-3 and dynein probably counteract by equally distributing rapidly moving early endosomes within the hyphal cell (Lenz *et al.*, 2006). This balance between the plus end-directed kinesin-3 and the minus end-directed dynein is nicely illustrated by overexpression of kinesin-3, which increases the run-length of individual organelles (Wedlich-Söldner *et al.*, 2002a) and results in a clustering of early endosomes at MT tips in the hyphal apex (Lenz *et al.*, 2006). Consequently, deletion of kinesin-3 leads to increased dynein activity and shifts endosomes towards the minus ends within the cell center (Lenz *et al.*, 2006). These and other results demonstrate that kinesin-3 and dynein are indeed counteracting motor systems that are held in a dynamic balance of force in order to move early endosomes. Interestingly, retrograde dynein-dependent motility usually starts at apical MT plus ends, where dynein and its putative activators Lis1 and dynactin are highly enriched and are loaded onto arriving endosomes that reach the MT plus end by the activity of kinesin-3 (Lenz *et al.*, 2006). These observations led to the concept of an apical loading zone for cytoplasmic dynein, which is maintained in an inactive state until an endosome reaches the hyphal tip to bind dynein and trigger its activation. Such a hypothesis is supported by the significant increase of dynein in the hyphal tip and the inability of the endosomes to leave the hyphal apex and form immobile apical clusters upon depletion of the dynein activator Lis1 (Lenz *et al.*, 2006). However, Lis1 also accumulates at the MT plus ends. This indicates that additional, as yet unidentified

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factors travel on endosomes from where they interact with Lis1, which in turn activates dynein.

In addition to kinesin-3, another kinesin participates in endosome motility, but in an unexpected way. Fungal members of the kinesin-1 family are plus end-directed motors (Steinberg & Schliwa, 1996; Steinberg, 1997) and a role of kinesin-1 in apical secretion is likely (Lehmler *et al.*, 1997; Schuchardt *et al.*, 2005; see earlier). In addition, kinesin-1 is thought to participate in vacuole formation at the basal septum of *b*-dependent hyphae (Steinberg *et al.*, 1998). Surprisingly, in the absence of kinesin-1, early endosomes are enriched at plus ends within the hyphal tip (Lenz *et al.*, 2006), which indicates that kinesin-1 supports minus end-directed dynein-dependent endosome motility. Further studies have demonstrated that occurs because of the role of kinesin-1 in targeting dynein and dynactin to the apical plus ends of MTs (Fig. 1). This transport establishes the apical dynein loading zone, which in turn is essential for retrograde endosome transport (Lenz *et al.*, 2006). As kinesin-3 travels back to MT minus ends on dynein-driven endosomes for another round of usage (Lenz *et al.*, 2006), reduced kinesin-1 activity indirectly affects this kinesin-3 recycling and thereby reduces the activity of kinesin-3. These results demonstrate that molecular motors not only cooperate to maintain polarized growth, they also depend on each other. As our knowledge of motors in fungi increases, more such networks are likely to be discovered. Whereas these findings illustrate an exciting new level of complexity, they also raise doubts about simple conclusions from phenotypic analysis of motor mutants in fungi.

IV. Mechanisms of mitosis

1. Removal of the nuclear envelope during open mitosis

The mitotic spindle of eukaryotic cells is composed of MTs. It is therefore not surprising that *U. maydis* cells fail to go through mitosis when MTs are disrupted (Steinberg *et al.*, 2001; Fuchs *et al.*, 2005). It was noted early on that at the onset of mitosis, DNA migrates into the bud, where the mitotic spindle is formed (Fig. 5a; O'Donell & McLaughlin, 1984; O'Donell, 1992). This premitotic chromosome migration is initiated by the activation of the SPBs at the nucleus (Straube *et al.*, 2003). While the interphase array disappears, the SPBs extend long astral MTs into the bud (Straube *et al.*, 2005). Dynein locates at the plus ends of these MTs, and reaches the cell periphery of the daughter cell by elongation of the astral MTs. In the daughter cell, dynein appears to establish stationary contact with the cell cortex, becomes activated, and pulls the SPBs into the daughter cell by moving towards the MT minus end (Straube *et al.*, 2005). Surprisingly, this does not result in nuclear migration, but stretches the nucleus by up to 10 μm in length. At this stage, chromosomes condense (prophase) and migrate within the envelope towards the SPBs within the daughter cell. Subsequently, the nuclear envelope breaks at the tip and chromosomes leave the envelope, which collapses back into the mother cell. Thus, *U. maydis* undergoes an 'open mitosis'. Ultrastructural data suggest that the envelope is generally removed in basidiomycete fungi, but not in ascomycete fungi. Consequently, the model fungi *S. cerevisiae*, *S. pombe*, *A. nidulans,* and *N. crassa* form an intranuclear spindle during 'closed' mitosis (Heath, 1980). However, it is important to note that the nuclear pore complexes in *A. nidulans* partly disassemble in mitosis, which challenges the term 'closed' mitosis (Osmani *et al.*, 2006).

In the mechanism described, dynein exerts the force that takes the SPBs into the daughter cell. Spindle pole body migration into the daughter cell does not occur in dynein mutants (Straube *et al.*, 2005), but mitotic spindles form in the mother cell (Straube *et al.*, 2005). As the cell cycle continues, this results in multinucleated mother and anucleated daughter cells (Straube *et al.*, 2001). Surprisingly, these spindles are formed inside a nuclear envelope. This and other observations indicate that the envelope is only removed when the SPBs pass the neck constriction. It has been speculated that the SPBs sense their position in the cell and trigger envelope removal when they reach the bud (Straube *et al.*, 2005). A similar sensing mechanism underlies the exit from mitosis in *S. cerevisiae*, where Tem1p on the SPB is activated when entering the bud and activates the mitotic-exit network (MEN; for overview see Smeets & Segal, 2002). Indeed, mutants in *U. maydis* Tem1p and in downstream MEN/SIN signaling compounds are defective in removal of the nuclear envelope (Straube *et al.*, 2005; Sandrock *et al.*, 2006), which indicates that the MEN/SIN pathway participates in this process early in mitosis.

2. Force generation in anaphase

In metaphase and anaphase A, the short spindles formed slowly elongate and thereby separate the chromosomes (Fink *et al.*, 2006). When spindles reach a length of *c.* 2 μm, anaphase B starts with the formation of long astral MTs and an approximate fivefold increase in elongation rate. This leads to spindles of up to 25 μm in length that segregate the chromosomes between mother and daughter cell. Whereas anaphase A occurs independently of dynein, the rapid elongation in anaphase B is dependent on dynein, which appears at the astral MT tip in anaphase B (Fig. 6). The mechanism by which dynein exerts forces on the spindle is similar to that described for prophase (see above). *In vivo* observation of dynein and laser-cut experiments have revealed that dynein becomes offloaded from the tip of the astral MTs to the cortex and pulls on the SPB by moving to the minus end of the MT. By exerting force on both poles of the spindle, the cell is able to position the spindle, but also to support rapid elongation in anaphase B. By contrast, the slow and early stages of anaphase are independent of astral MTs and dynein, but require internal spindle forces at least partly exerted by the mitotic kinesin-5 (Fink *et al.*, 2006). Such a

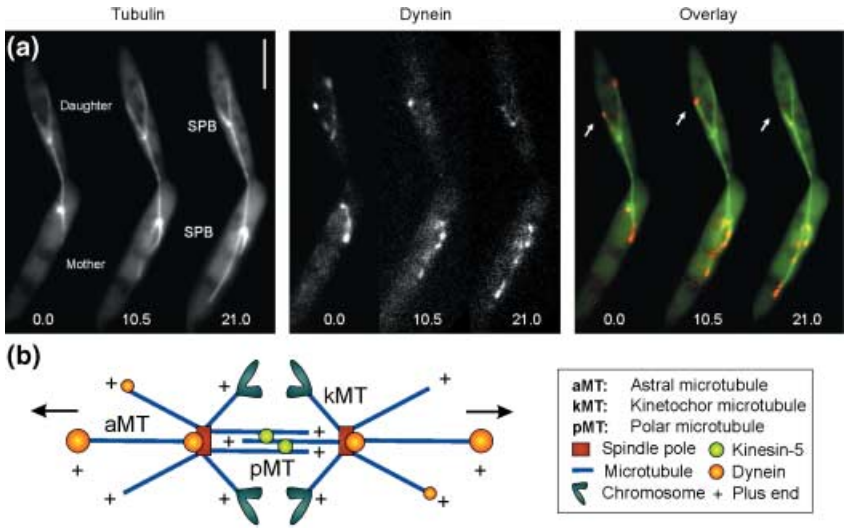

Fig. 6 Dynein at plus ends of astral microtubules in anaphase B. During anaphase A, dynein does not localize to astral microtubules and early spindle elongation is independent of dynein. However, in anaphase B, dynein accumulates at long astral microtubules that exert pulling forces on the spindle pole bodies (SPBs), thereby supporting spindle positioning and rapid spindle elongation. Note that the signal at the plus ends is dynamic (arrow in overlaid images). Elapsed time is given in seconds. Bar, 5 μm.

kinesin-5-based mechanism is also found in *S. cerevisiae* and *S. pombe* (Hagan & Yanagida, 1990; Hoyt *et al.*, 1992) but these yeasts lack a dynein-based rapid anaphase. However, dynein is involved in anaphase B in animal cells, which again reveals unexpected similarities of *U. maydis* and higher eukaryotes.

V. Questions and future perspectives

During the last decade, the results obtained with GFP and its derivatives have changed our view of the fungal cell. Careful quantitative ultrastructural studies predict that hyphal growth is supported by enormous membrane traffic and fusion at the tip, and we are now able to observe intracellular dynamics in the living fungal cell. Studies of the cytoskeleton of *U. maydis* reveal an unexpected complexity of this intracellular transport. It has long been assumed that molecular motors deliver secretory vesicles to the growing hyphal tip, but a role of organelle transporters, such as dynein and kinesin-1, in organizing their own MT tracks was highly unexpected. It was surprising to find that *U. maydis* contains 10 kinesin motors, yet only kinesin-1 and kinesin-3 show defects in filamentous growth. Whereas the role of most other kinesins is elusive (Table 1), other MT-based motors have been shown to participate in numerous essential processes. This is most obvious for dynein, which arranges MTs (Adamikova *et al.*, 2004; Fink & Steinberg, 2006), moves tubules of the endoplasmic reticulum (Wedlich-Söldner *et al.*, 2002b), removes nuclear membranes (Straube *et al.*, 2005) and drives early endosome motility (Wedlich-Söldner *et al.*, 2002a; Lenz *et al.*, 2006); it also exerts force on the anaphase spindle (Fink *et al.*, 2006). All these dynein functions require the localization of the motor at MT tips, and plus end targeting of dynein to MT plus ends requires kinesin-1. Without kinesin-1-based dynein targeting, endosomes are trapped in the hyphal tip and kinesin-3 cannot be recycled (Lenz *et al.*, 2006; U. Fuchs and G. Steinberg, unpublished). Thus, studies on *U. maydis* have revealed an unexpected functional network of motors that depend on each other for recycling. The identification of such functional networks has important consequences for conclusions drawn from analysis of mutant strains. Kinesin-3 mutants have a severe defect in filamentous growth of hyphae, which is phenotypically almost identical to that of kinesin-1 mutants (Schuchardt *et al.*, 2005). As kinesin-3 recycling depends on kinesin-1-based dynein targeting, the observed phenotype of kinesin-1 mutants might reflect an essential function of kinesin-3. A further level of complexity is added by the cooperation of F-actin and MT-based transport systems in maintaining polarized growth. Although it appears that the number of motors involved in such functional networks is limited to a few candidates, our understanding of their cooperation is currently incomplete. Finally, the question arises why fungi encode 10 kinesins, of which only two have an obvious role in morphogenesis and two others might participate in mitosis (Table 1). More detailed insights into the cellular role of these remaining kinesins might uncover additional surprising insights into functional redundancies during tip growth. Thus, the field is wide open and much needs to be learned about the mechanism by which the cytoskeleton supports polarized growth and pathogenicity of fungi.

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