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On the move: Endosomes in growth and pathogenicity *of Ustilago maydis*

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Abstract

Fungi invade substrates, such as host tissues, through hyphal tip growth. This article focuses on the corn smut fungus *Ustilago maydis*, in which tip growth and pathogenicity involves apical endocytic recycling via early endosomes. These organelles rapidly move bi-directionally along microtubules, which is mediated by opposing molecular motors. This motility appears to be essential for extended hyphal growth, possibly because it focuses the endocytic machinery at the hyphal tip and mediates communication between the tip and the sub-apical nucleus.

Introduction

Hyphae of filamentous and dimorphic fungi explore the environment and invade substrates by local extension of the hyphal apex, a process termed hyphal tip growth. The molecular and structural basis of hyphal growth has been studied for many years. In the currently accepted model, molecular motors— large mechanoenzymes that hydrolyze ATP to fuel "walking" along the fibrous tracks of the fungal cytoskeleton — deliver Golgi-derived secretory vesicles to the Spitzenkörper (Box 1). The Spitzenkörper directs tip growth by controlled release of the secretory vesicles[1,2]. These fuse with the apical plasma membrane, thereby supplying membranes, secreting exoenzymes, or delivering enzymes involved in cell wall synthesis, such as chitin synthases, to the hyphal tip[3-5]. However, the Spitzenkörper also stains with the endocytic dye FM4-64 (Box 1; Refs. [6-8]), indicating that the Spitzenkörper has also roles that are related to endocytosis.

Much is known about the molecular basis and role of endocytosis in the baker's yeast *Saccharomyces cerevisiae* (for further reading see Refs.[9-11]). Studies in this model system have demonstrated that chitin synthases are endocytosed and recycled back to the plasma membrane[12]. Such endocytic recycling is well known in higher eukaryotes, where it serves regulatory functions and also allows cells to reuse receptors after ligand binding (summarized in Ref. [13]). In addition, it was shown that endocytic recycling participates in polarization of plasma membrane proteins[14]. Whether endocytosis and endocytic recycling occur in filamentous fungi has long been a matter of debate[15]. Recent analyses of fungal genomes, however, reveals that fungi contain components of the endocytic machinery found in animal cells[15,16], and experiments using endocytic marker dyes, such as FM4-64[6,8,15,17], strongly suggest that endocytosis is common in filamentous and dimorphic

fungi. In the biotrophic plant pathogen *Ustilago maydis* mutants defective in endocytic reccling[18] are heavily impaired in polarized growth, which suggests that endocytic recycling processes are occurring at the growing hyphal apex. Our understanding of the recycled factors required for hyphal growth is in its infancy, but recent progress in fungal cell biology has begun to shed light on the importance of early endosomes in the polarized growth and pathogenicity of fungi. Most of what is known about early endosomes and recycling in hyphae comes from studies in the corn smut fungus *U. maydis*. Consequently, I will focus on this model system and cover recent advances in our understanding of the molecular mechanism of by early endosomes reach the hyphal tip, where they are thought to support endocytic recycling. In addition, I will speculate on possible roles of endosome motility in cell polarization and retrograde signalling to the nucleus.

The dimorphic model fungus *U. maydis*

The basidiomycete *U. maydis* is the causal agent of corn smut disease in maize (see Ref.[19]). The most obvious symptom of a *U. maydis* infection is the appearance of so-called tumours, which are actually fungal-induced galls that contain large amounts of fungal hyphae. These hyphae fragment and transform into massive amounts of diploid teliospores (Fig. 1)[20]. The spores are released and form a promycelium that produces haploid sporidia. These yeast-like cells can be easily cultivated and are amenable to molecular genetics, which makes this fungus an excellent model organism for molecular phytopathology[19,21,22] and also for studying many aspects of modern cell biology, including the mechanisms of open mitosis (where the nuclear envelope breaks down then reforms[23,24]) and the organization of microtubules by molecular motors[25,26].

Under stress conditions, such as nutrient starvation, haploid *U. maydis* cells switch from budding to filamentous growth (Fig. 1). This illustrates that the individual yeast-like sporidia contain all of the factors required to form a hypha and grow filamentously. Under natural conditions, however, filamentous growth of *U. maydis* is linked to the initiation of pathogenic development on the plant surface, which starts with the recognition of a compatible pheromone secreted by a partner cell. Pheromone perception involves specific receptors and triggers a morphogenic yeast-hypha transition[27]. Long conjugation hyphae arrested in the G2-phase[28] are then formed that search for each other [29], while continuously exposing the pheromone receptor at

the tip[30]. Compatible conjugation hyphae fuse and form a dikaryotic filament consisting of a long tip cell and vacuolated sections that are devoid of cytoplasm and contains a Spitzenkörper-like vesicle accumulation in its apex[31]. It invades the plant epidermis to colonize the host tissue. The cell-cycle arrest is released, and the fungal hyphae massively proliferate, which finally results in the production of teliospores.

Taking the life cycle of *U. maydis* into consideration, it is apparent that pathogenicity is closely associated with cell-cell communication and polarized hyphal growth. Pathogenicity is accompanied by a yeast-hyphal transition, similar to other fungal pathogens such as *Candida albicans* and *Histoplasma capsulatum* (overview in Ref. [32]). The rationale behind this morphogenic switch in *U. maydis* is that hyphal growth enables the walled and immobile sporidial cell to overcome the distance to a mating partner and also allows rapid escape from host defence mechanisms. The latter strategy in *U. maydis* is thought to be supported by the release of exoenzymes that suppress the plant defence response and thereby establish the biotrophic phase during early infection[33].

Exocytosis in the pathogenicity of *U. maydis*

Some pathogenic fungi adhere to and penetrate host tissues with the aid of secreted lytic proteins, such as aspartic proteases, aminopeptidases, carboxypeptidases, and phospholipases, and also hydrophobins; these proteins are therefore virulence factors (summarized in Ref.[34-36]). Biotrophic fungi, such as *U. maydis*, in contrast, secrete only limited amounts of lytic proteins[37], and appear to establish a biotrophic interaction by secreting proteins[33]. It is generally assumed that the secretion of exoenzymes and polarized hyphal growth requires the motor-based tip-ward movement of exocytic vesicles along the fibres of the fungal cytoskeleton[38]. For *U. maydis,* this concept is strongly supported by two observations: both microtubules (MTs) and F-actin are required for the polarized growth of hyphae[39] and a class V myosin is essential for the initial steps of plant invasion[40]. Why myosin-V is essential is not yet known, but it is tempting to speculate that it participates in the secretion of factors that suppress plant defences. A component of the fungal cell wall might also be involved. A major compound of the fungal cell wall is chitin, which is formed by chitin synthases[41]. An important class of these enzymes contains an N-terminal myosin motor

domain that is fused to a chitin synthase domain. Mutants defective in a class V myosin-chitin synthase fusion protein of *Fusarium oxysporum* are more sensitive to the tomato plant defence system than wild type[42]. Consistently, deletion mutants in Mcs1, a *U. maydis* myosin-chitin synthase[4] grow normally outside the plant, but *in planta* they swell and die shortly after penetrating the epidermis. Taken together, these results argue that myosin-based exocytosis of as-yet unknown factors is essential during initial invasion, and this in turn emphasizes the importance of the cytoskeleton in fungal pathogenicity.

Endocytosis in the pathogenicity of *U. maydis*

The existence of endocytosis in filamentous fungi has been controversial[15]. However, there is now a strong consensus from a number of studies (e.g. Refs. [6,8,17,18,30]) that it does occur in these organisms. Evidence has also recently been obtained that indicates that recycling of endocytosed material contributes to fungal virulence. The analysis of endocytic recycling in *U. maydis* became possible once mutations in the *yup1* gene were identified. *Yup1* encodes a tSNARE **(**Box 2**)** that contains a PX-domain, which is predicted to bind to lipids that are typical early endosomes and vacuoles[43,44]. Yup1-GFP indeed localizes to vacuoles and rapidly moving organelles[18]. That the latter are indeed early endosomes is supported by the observation that the endocytic marker FM4-64 passes these organelles on its way to the vacuole[18]. Moreover, it was recently shown that Yup1 colocalizes with Rab5-related small Ras-related GTPases (Box 2) that are characteristic for early endosomes in higher eukaryotes[45,46]. A temperature-sensitive mutation in *yup1* (*yup1*^{ts}) has no effect in vacuoles[18], but leads to a dissociation of the mutant tSNARE from the endosomal membrane. Consequently, the fusion of endocytic transport vesicles with early endosomes is impaired. This defect blocks sorting to the vacuole, as well as the endocytic recycling pathway (Fig. 2), and causes morphological defects in sporidia and hyphae[18,30]. As these mutant strains do not induce the symptoms of infection at restrictive temperatures, endocytic recycling is thought to be essential for *U. maydis* virulence[30]. It was shown that the *yup1*^{ts} mutants are unable to perceive a compatible mating pheromone. Consequently, *yup1*^{ts} cells do not form conjugation hyphae in the presence of the mating partner or exogenous synthetic pheromone[30]. In other words, *U. maydis* cells that are defective in endocytic recycling are "blind" to the compatible mating pheromone.

A central component of the pheromone recognition system in *U. maydis* are the pheromone receptors Pra1 and Pra2[47], which transmit the pheromone signal to a mitogen-activated protein kinase cascade that triggers downstream effects and initiates pathogenic development[19,48] (Fig. 2). The homologous pheromone receptor in *S. cerevisiae* undergoes endocytic recycling[49]. It was therefore speculated that *U. maydis* Pra1 cycles between the plasma membrane and early endosomes. Indeed, it was found that a functional fusion protein between Pra1 and the green fluorescent protein (GFP) localizes to the surface of yeast-like sporidia and is constitutively endocytosed for degradation in the vacuole or recycling back to the plasma membrane[30] (Fig. 2A). In *yup1*^{ts} mutants, the fusion of Pra1-carrying endocytic transport vesicles with early endosomes is blocked, whereas the early steps of endocytosis still operate (Fig. 2B). Consequently, the receptor becomes trapped in primary endocytic transport vesicles and cannot recycle back to the surface. This finally leads to depletion of the receptor from the surface, which explains the insensitivity to compatible mating pheromone and the inability to form conjugation hyphae.

This model gained further support from the rescue of the pheromone perception defect in *yup1*^{ts} mutants and the initiation of the formation of conjugation hyphae when excess Pra1 is synthesized by expressing the gene from a strong constitutive promoter[30]. However, the hyphae formed are unable to undergo cell-cell fusion, which indicates that endocytic recycling has additional and essential roles in subsequent steps of the pathogenic development of *U. maydis.* Interestingly, in the absence of endocytic recycling, colonization of plant tissues is only slightly affected, whereas spore formation and spore germination are almost abolished[30].

The molecules that cycle between the plasma membrane and the early endosomes during cell-cell fusion or spore development have not yet been identified. However, these studies demonstrate that the initiation of pathogenic development requires endocytic recycling of the pheromone receptor via early endosomes, which, together with additional recycling processes that are still to be identified, is crucial for *U. maydis* virulence. The upcoming challenge is to identify the proteins that are recycled in order to support essential aspects of *U. maydis* biology and pathogenicity.

The molecular basis for endosome motility

The results of studies with early endosomes labelled with a Yup1-GFP or a GFP-Rab5a fusion protein in *U. maydis* made it evident that these organelles rapidly move along MTs in sporidia and hyphae[18,30]. In both cell stages, motility is abolished in the absence of MTs but is unaffected in cells treated with F-actin inhibitors. Motility of early endosomes is therefore exclusively MT-based.

MTs are polar structures with dynamic plus ends and a minus end that is usually anchored at an MT-organizing centre. In sporidia, these MT-organizing centres are initially located at the new growth site and later remain in the boundary between the mother and daughter cells[26,50]. As a consequence, MT plus ends in yeast-like cells are oriented toward the cell poles. In hyphae, MTs are similarly organized; the MT plus ends extend toward the hyphal tip and the septum, and MTs in the middle region of the hyphal tip cell have a bi-polar orientation[51]. Owing to the uni-polar orientation of MTs in both yeast-like and hyphal cells, bi-directional transport of early endosomes is expected to be mediated by opposing motor systems, such as minus-end-directed dynein and plus-end-directed kinesins. Indeed, early endosome anterograde motility towards the hyphal tip or the cell poles of sporidia is driven by kinesin-3, whereas retrograde motility towards the cell centre is mediated by dynein[51,52]. Despite anterograde and retrograde motility being supported by fundamentally different motors, the velocity and the frequency of minus-end-directed and plus-end-directed transport are almost identical[52]. This is because the activity of kinesin-3 and dynein is balanced, which results in an equal distribution of early endosomes in yeast-like sporidia and hyphae[51,52].

How the two motors regulate each other to balance their counteracting activities is not known. Different concepts for the activity of opposing motors in bi-directional organelle traffic in animal cells have been discussed[53,54]: (1) the motors co-localize on the organelle and counteract simultaneously (Fig. 3A, tug-of-war model); (2) the motors regulate each other's activity (Fig. 3A, coordination model); or (3) only one motor acts on an organelle at a given time (Fig. 3A, exclusionary presence model).

In hyphae of *U. maydis*, early endosome traffic combines elements of both the coordination and the exclusionary presence models (Fig. 3B). This is mainly because of the activity of kinesin-1. In *A. nidulans*, kinesin-1 is required for dynein targeting to the MT plus end[55]. In *U. maydis*, kinesin-1 also targets dynein and associated dynactin to MT plus ends within the hyphal apex (Fig. 4)[51]. Dynein remains inactive there until the early endosomes reach the tip through the activity of kinesin-3. It is thought that early endosomes rapidly exchange membranous material with the tip and trigger dynein activation, which is accompanied by the transfer of the endosome to the retrograde transport machinery (Fig. 4). Subsequently, dynein takes both the endosome and the associated kinesin-3 back to sub-apical regions. Full activity of dynein requires the presence of kinesin-3[52], which suggests that both motors could interact on retrogradely moving endosome membranes. Taken together, anterograde transport fits the exclusionary presence model, whereas retrograde traffic shows characteristics of the coordination model (Fig. 3B). The regulatory details of this process remain to be elucidated. Research in this area not only promises to extend our understanding of the role of the cytoskeleton in hyphal tip growth, it will also give insights into the basic mechanisms of motor coordination in the bi-directional endosome motility of higher eukaryotes.

Why do endosomes move bi-directionally?

In *U. maydis* and *Aspergillus oryzae*, early endosomes move bi-directionally along MTs[18,56]. In *U. maydis* this motility is mediated by the molecular motors dynein and kinesin-3 (see above). In animal systems, both motors operate in small groups of at least 2–3 motor complexes[57,58], each of which hydrolyzes ATP for each step along the MT. Thus, continuous bi-directional endosome motility is energetically costly for the cell, and, therefore, is expected to serve a biological function. Unfortunately, little is known about the role of early endosome traffic in *U. madyis.*

In the yeast-like sporidia, early endosomes transiently cluster at the small bud, where MT minus ends are focused[50]. It was speculated that the endosomes support local recycling during early bud growth[18]. However, in cells with medium-sized buds, the balance between dynein and kinesin-3 activity is slightly modified, and endosomes cluster at the MT plus ends at the rear cell pole. Interestingly, besides being localized at the growth region, chitin synthases are also found at

the rear cell end[4]. This indicates that cell wall modifications occur at the distal cell pole, while the bud continuously enlarges at the opposite pole. During budding, *U. maydis* switches between the cell poles[59] and this alternating budding pattern appears to require rearrangement of the early endosomes, as kinesin-3 null mutants form new buds at the same cell pole[52]. In addition, early endosome clustering at septa during cytokinesis requires kinesin-3 activity, and this is essential for cell separation[52]. A function of early endosomes in cytokinesis is further supported by the presence of a conserved FYVE domain in Don1, a guanine-nucleotide-exchange factor required for septation[60]. This domain most likely mediates binding to lipid that is characteristic of early endosomes[61] (Box 2). Unfortunately, more detailed information on the role of early endosomes in bud site selection and cell separation is lacking. An attractive hypothesis is that bi-directional transport of endosomes lies behind the rearrangement of the recycling machinery during the cell cycle and serves basic cellular processes, such as cytokinesis.

In hyphae, the reason for bi-directional endosome motility is by far less clear. In *U. maydis* conjugation hyphae, a functional Pra1–GFP fusion protein concentrates at the growing hyphal tip[30], which suggests that pheromone perception occurs at the apex. Furthermore, in *yup1*^{ts} mutants, transport vesicles accumulate in the hyphal apex at restrictive temperatures (see above; Fig. 2B)[30]. Therefore, endocytic recycling probably also occurs at the hyphal tip. Thus, in theory, it would be most efficient to position early endosomes close to the expanding hyphal tip, where they could participate in membrane recycling during extended hyphal tip growth. However, in reality, endosomes stay at the apex only transiently, and after reaching the MT plus ends in the hyphal tip, they usually quickly switch to retrograde traffic (see above; Fig. 4). The biological reason for this is not yet understood, but here I speculate on three possible roles of endosome motility that might all occur in the cell and that are currently under investigation.

(1) The most obvious role is to deliver endocytosed material, such as receptors, via long-distance transport along MTs to vacuoles for degradation. Indeed, vacuoles containing Pra1-GFP are scattered along the length of the hypha[30]. In *yup1*^{ts} mutant hyphae, Pra1-GFP does not reach the vacuoles[30], which suggests that vacuolar sorting requires passage through the early endosomes that undergo retrograde transport along MTs in order to take material to the sub-apical vacuoles (Fig. 5).

(2) Another possible role is to concentrate receptors, such as Pra1, or other endocytosed membrane components at the hyphal tip by continuous endocytosis along the length of the cell and subsequently to deliver these components to the hyphal tip by anterogradely moving endosomes (Fig. 5). An endocytosis-based polarization mechanism has been suggested for the *S. cerevisiae* pheromone receptor Ste3p[62] and the exocytic SNARE Snc1[14], which might allow polarization and directed growth toward pheromone sources. However, polarization of membrane components could also be achieved by local secretion or anchorage of membrane components to specialized membrane regions, such as lipid rafts[63].

(3) Finally, another possible role might be to mediate communication between the hyphal tip and the nucleus. The need for such long-distance communication is nicely illustrated by the perception of the mating pheromone at the hyphal tip by the pheromone receptor. In contrast, gene expression in response to this is triggered in the hyphal nucleus, which is located about 50 µm away in the sub-apical region of the hyphal tip cell[39] (Fig. 5). The distance between the site of signal perception and the reacting nucleus makes it very unlikely that the signal is transmitted by passive diffusion[64]. A challenging alternative explanation is that long-distance transport of early endosomes along MTs could mediate tip-to-nucleus communication. In hyphae early endosomes rapidly move at ~1.9 µm/s (U. Fuchs and G. Steinberg, unpublished results), which would allow to overcome this distance from the tip to the nucleus in ~26 seconds. One possibility could be that MAP kinases, which are essential in pheromone signal transduction (see Fig. 2), are bound to early endosomes and by retrograde endosome traffic transmit the signal from the receptor to nucleus. Currently, there is no evidence that such a mechanism exists in fungi. In contrast, in animal neurons, which face a similar problem, retrograde, dynein-dependent, long-distance transport of endosomes transmits signals from the apical synapsis to the nucleus in the cell body[65,66]. However, mutants defective in endosome motility, such as kinesin-1 and kinesin-3 null mutants, stop tip growth at a hyphal length of about 50 µm. This can be taken as a promising indication that communication between the tip and the nucleus is disturbed over long distances. However, many compounds involved in endosome motility, such as dynein and its regulators, also support nuclear migration. Thus, it remains to be seen whether retrograde traffic of endosomes mediates long-distance communication in fungal hyphae.

Conclusions

The current concepts of polarized hyphal growth must be reconsidered and must take into account the participation of endocytic recycling in hyphal tip growth. The identity of most of the recycled proteins, however, remains elusive. Moreover, the route and regulation of endocytic recycling in filamentous fungi is not known. Studies in *U. maydis* have demonstrated that the pheromone receptor Pra1 undergoes endocytic recycling, and that this process is crucial for initiation of the pathogenic program of this plant pathogen. These studies have also emphasized that endocytic recycling has a crucial role in subsequent cell-cell fusion, spore formation, and germination, but again the recycled cargo is unknown. Thus, a major challenge ahead is to define the recycled proteome of fungal cells. In *A. oryzae* and *U. maydis* the microtubule cytoskeleton supports the rapid bi-directional transport of early endosomes[18,56], which probably also holds true for the rapid motility of early endosomes observed in *A. nidulans*[17]. The reason for this bi-directional motility is obscure, but perhaps anterograde endosome transport supports cell polarization by focussing recycled compounds at the growing tip of hyphae. Retrograde endosome traffic most likely supports sorting of proteins for degradation in the vacuole. A very attractive idea is that dynein-driven early endosome motility also ensures communication between the tip and the nucleus, a process that is intensively investigated in animal neurons[65]. Experimental evidence for such a role of early endosomes in fungi does not exit, yet. However, research in fungal cell biology is ready to take on this fascinating challenge, which promises to change our view of fungal hyphae.

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Figure legends

Figure 1. Life cycle of *Ustilago maydis*.

Diploid teliospores are released from the plant and germinate to form a promycelium that, after meiosis produces haploid yeast-like sporidia. These cells are saprotrophic and grow by polar budding. Under stress conditions, the cells switch to filamentous growth. On the surface of the plant, compatible cells (indicated by the colour of the nuclei) recognize each other by pheromone exchange and switch to tip growth. This leads to the formation of conjugation hyphae that fuse and give rise to a dikaryotic hypha that invades the plant tissue. Inside the host, the cell-cycle arrest is released and long hyphae colonize the plant and induce the formation of plant "tumours". After proliferation, nuclear fusion occurs and hyphae fall apart, forming teliospores.

Figure 2. Recycling of the pheromone receptor Pra1 in wild-type and Yup1^{ts} mutants.

A. The receptor cycles via primary endocytic transport vesicles between the early endosomes and the plasma membrane and is partially sorted to the vacuole for degradation. Compatible mating

pheromone 2 is perceived and induces a signal cascade that involves an MAP-kinase module. As a consequence, the stimulated cell secretes mating pheromone, forms conjugation hyphae, and initiates the pathogenic program. **B**. At restrictive temperature, the mutated Yup1^{ts} tSNARE leaves the endosomal membrane, and endocytic transport vesicles are unable to fuse with the organelle. This blocks both the degradation and the recycling pathway. Initial steps of endocytosis are not affected, which results in an accumulation of primary endocytic transport vesicles and a depletion of the pheromone receptor Pra1 from the surface. Consequently, mating pheromone 1 cannot be detected, and pathogenic development is initiated. Note that Yup1 also localizes to vacuoles, which raises the yet unlikely possibility that the observed phenotype in Yup1^{ts} mutants is due to unknown vacuolar functions.

Figure 3. Concepts of microtubule-based bi-directional organelle transport.
A. Bi-directional organelle traffic is common in eukaryotic cells and is mediated by opposing motor proteins. Dynein moves its cargo toward minus ends that usually are embedded at the nucleation site and bind γ-tubulin, whereas kinesin motors move toward the plus end, where polymerization and depolymerization occur. There are currently three concepts on how these motors mediate motility. (1) Tug-of-war model: both motors are located on an individual organelle and are simultaneously active. This results in a "tug-of-war" and leads to stochastic switching between plus-end-directed and minus-end-directed motility. (2) Coordination model: kinesin and dynein are located on the same organelle, but only one motor is active at the time. This coordinated activity results in bi-directional motility. (3) Exclusionary presence model: finally, either kinesin or dynein bind to the organelle, and directionality is controlled by the affinity of the motors to the organelle.
B. Rapid bi-directional motility of early endosomes in *U. maydis* combines features of the coordination model and the exclusionary presence model, as both dynein and kinesin-3 bind to organelles that move toward the minus ends, whereas only kinesin binds to anterogradely moving organelles.

Figure 4. The role of kinesin-1, kinesin-3, and dynein in early endosome motility and apical recycling in *U. maydis*.

In the current model, the bi-directional motility of early endosomes (EEs) in *U. maydis* requires the establishment of an apical accumulation of dynein at MT plus ends within the hyphal tip. This is mediated by kinesin-1,which either directly or indirectly binds the dynein/dynactin complex and targets it to the MT tip (1). Kinesin-3 moves early endosomes to the hyphal tip (2), where rapid membrane exchange with the plasma membrane occurs (3) before the endosomes are loaded onto dynein (4), which becomes activated and moves the early endosomes back to the sub-apical regions, thereby recycling attached kinesin-3 for another round of usage (5). Note that the recycling pathway of kinesin-1 is not known.

Figure 5. Potential roles for long-distance transport of early endosomes in pheromone signalling. The pheromone receptor Pra1 is concentrated at the apex of conjugation hyphae, where it undergoes recycling between early endosomes (EE) and the plasma membrane. In pheromone signalling the long-distance transport of early endosomes might be important in three ways: (1) it is likely that retrograde early endosome traffic supports vacuolar sorting of endocytosed receptors and this could regulate the level of surface-exposed Pra1; (2) receptor that diffuses away from the tip (red arrow) might be focused at the hyphal tip because of the plus-end-directed motility of early endosomes, which take up endocytosed receptors at sub-apical regions and deliver it to the tip; and (3) the nucleus, located ~50 µm sub-apically, must be informed that the mating pheromone has been detected in order to trigger gene expression. Thus, retrograde early endosome traffic might have several essential functions in pheromone signalling.

Box 1: The fungal "Spitzenkörper"

In early light microscopical experiments Brunswick identified a dark body in the hyphal tip of *Coprinus cinereus* that he named the "Spitzenkörper"[67]. Subsequently it was shown that the Spitzenkörper is a dynamic accumulation of micro- and macrovesicles, ribosomes and elements of the cytoskeleton that is found in growing hyphae of the basidiomycetes, ascomycetes and deuteromycetes (e.g.[68-70]). Live cell imaging strongly indicated that the relative position of the Spitzenkörper within the hyphal apex determines the growth direction of the hypha[68,71], which suggests that the Spitzenkörper has essential roles in hyphal tip expansion of fungi. A milestone in

understanding the cellular role of the Spitzenkörper was provided by the work of Bartnicki-Garcia and co-workers, who first suggested that the Spitzenkörper is a "vesicle supply center" (VSC)[72]. Their concept predicts that the VSC releases exocytic vesicles in all directions while it moves forward, thereby generating an exocytosis gradient that shapes the hypha. Indeed, some experimental evidence and sophisticated mathematical simulations support this view (summarized [73]). Thus, it is now widely accepted that the Spitzenkörper is a membrane reservoir for controlled exocytosis at the hyphal apex. In addition, the Spitzenkörper contains actin-binding formins[7,74] that are known to support actin assembly (overview in [75]). This suggests that compounds of the Spitzenkörper might help to polarize the cytoskeleton towards the hyphal apex, which in turn organizes the fibrous tracks of the cytoskeleton towards the growing tip for motor-based tip-ward delivery of exocytic vesicles. Such a model is supported by the fact that actin-associated myosin-5 motors and a regulatory myosin light chain, as well as microtubule-dependent kinesin-1 are enriched in the Spitzenkörper[7,40,76]. Both mosin-5 and kinesin-1 are required for proper hyphal growth in *U. maydis*[40,76], suggesting that they participate in the delivery of vesicles to the apical Spitzenkörper and apical plasma membrane from the Spitzenkörper. One, yet to be confirmed view, is that microtubules are primarily responsible for the long-distance transport of secretory vesicles to the Spitzenkörper, while actin microfilaments primarily control vesicle organization within the Spitzenkörper and transport to the plasma membrane[2]. It is important to note that the content of the Spitzenkörper vesicles is not yet known. It is assumed that the Spitzenkörper vesicles are Golgi-derived and contain secretory proteins and wall-precursors[68,69,73]. In addition, evidence accumulates that endocytic marker dyes concentrate in the Spitzenkörper[2,6,8]. This raises the possibility that endocytic membranes take part in the apical vesicle accumulation.

Box 2: Rab-GTPases, SNAREs and organelle identity

The organization and survival of the eukaryotic cell requires ATP-dependent traffic of transport vesicles or tubules between functionally distinct organelles. Membrane traffic is a four-step process that involves vesicle formation, motor-dependent transport, tethering and fusion of the vesicle with target membrane[77]. An essential requirement for this process is that each organelle has a distinctive identity. Identity is provided by the activity of small Ras-like GTPases of the Rab family[44]. Rab GTPases are molecular switches that cycle between an cytosolic inactive GDP-

bound form and an membrane-bound active GTP-bound state[78]. This cycle is regulated by guanine nucleotide exchange factors (GEFs) and GTPas-activating proteins (GAPs). After membrane binding GTP-Rab proteins recruits effectors, such as thethering factors[77] or motor proteins[79], which execute specific functions. Among these effectors are lipid modifying enzymes that change the lipid composition in the membrane. The presence of certain lipids and Rab-effector complexes confer the identity of the organelle. This is best understood for the mammalian early endosome. After binding and activation of the early-endosome specific Rab5-GTPase[45,46], Rab5-effectors are recruited that generate a specific microdomain[78]. One effector is a phosphatidylinositol (PI) 3-OH kinase[80], which catalyzes the formation of PI 3-phosphate (PI3P). This early endosome-specific membrane lipid is now a target for proteins that contain PI3P-binding PX-domains[43] or FYVE-domains[81]. Among these is the kinesin Kif16B, which is involved in motility of early endosomes[82]. Specialized membrane domains might also recruit the basic machinery for membrane fusion. The central compound of this machinery are soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), which are classified into Qa-, Qb-, Qc- and R-SNAREs[83]. These proteins contain conserved 60-70 amino acid stretches that are arranged in heptad repeats. It is thought SNAREs from partner membranes form of a hetero-oligomeric four-helix bundle thereby promoting membrane fusion. Rab-GTPases participate in recruiting SNAREs[84], which in case of the *S. cerevisiae* Vam7 and *U. maydis* Yup1 most likely occurs by binding to lipids via a PI3P-binding PX-domain[18,85]. Indeed, the PX-domain of Yup1 is essential for targeting the SNARE to rapidly moving organelles[18] that also carry Rab5-GTPases, indicating that they are early endosomes[30]. Furthermore, *U. maydis* contains a FYVE-finger domain containing a homologue of Rabenosyn-5[16], which is an effector of Rab5[86], which suggests that the mechanism of conferring Rab5-mediated early endosome identity is conserved from animals to fungi.

Figure 1_Steinberg

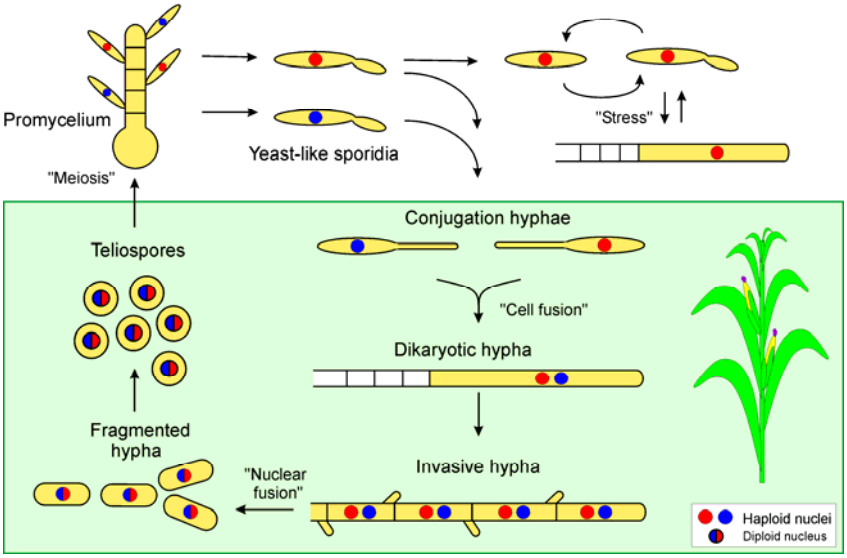

Figure 2 _Steinberg

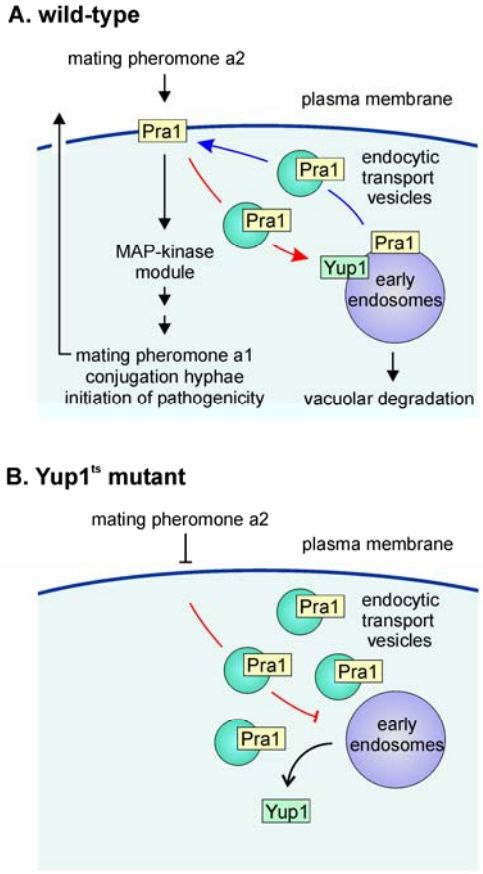

Figure 3_Steinberg

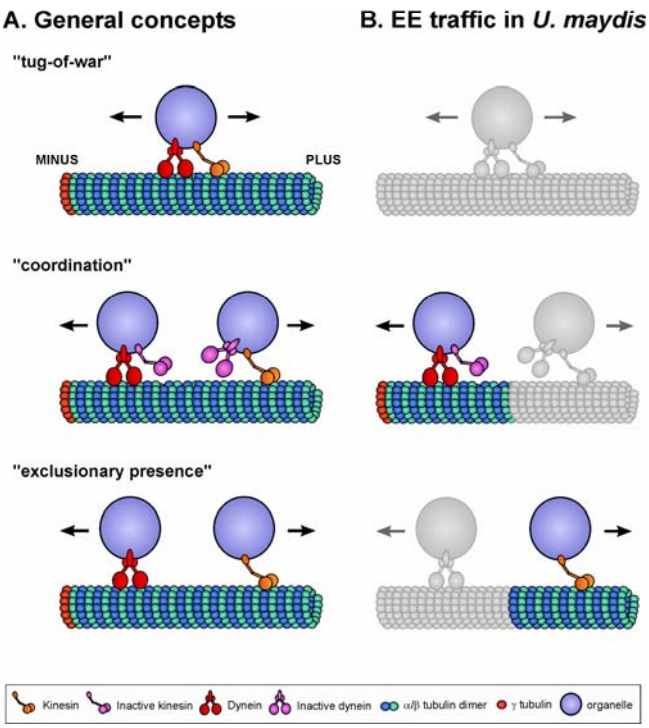

Figure 4_Steinberg

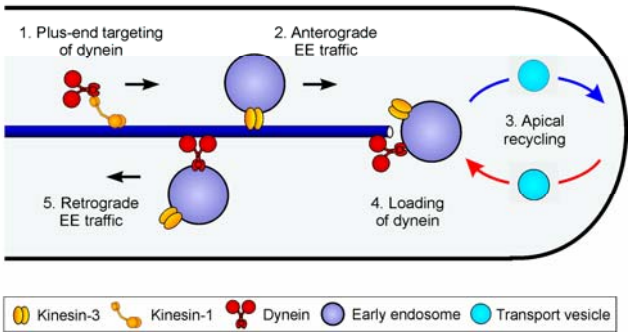

Figure 5_Steinberg