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Microtubule-dependent membrane dynamics in *Ustilago maydis*

Trafficking and function of Rab5a-positive endosomes

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Abbreviations: ESCRT, endosomal sorting complex required for transport; ER, endoplasmic reticulum; FYVE, PtdIns(3)P-binding domain (Fab1, YOTB, Vac1 and EEA1); GEF, guanine nucleotide exchange factor; Gfp, green fluorescent protein; mRNP, messenger ribonucleoprotein; PH, pleckstrin homology; RRM, RNA recognition motif; SNARE, soluble N-ethylmaleimide-sensitive-factor attachment receptor

Long-distance trafficking of membranous structures along the cytoskeleton is crucial for secretion and endocytosis in eukaryotes. Molecular motors are transporting both secretory and endocytic vesicles along polarized microtubules. Here, we review the transport mechanism and biological function of a distinct subset of large vesicles marked by the G-protein Rab5a in the model microorganism *Ustilago maydis*. These Rab5a-positive endosomes shuttle bi-directionally along microtubules mediated by the Unc104/KIF1A-related motor Kin3 and dynein Dyn1/2. Rab5a-positive endosomes exhibit diverse functions during the life cycle of *U. maydis*. In haploid budding cells they are involved in cytokinesis and pheromone signaling. During filamentous growth endosomes are used for long-distance transport of mRNA, a prerequisite to maintain polarity most likely via local translation of specific proteins at both the apical and distal ends of filaments. Endosomal co-transport of mRNA constitutes a novel function of these membrane compartments supporting the view that endosomes function as multipurpose platforms.

Introduction

Eukaryotic cells depend on vesicle-mediated endocytosis and secretion to interact and communicate with their environment. Such membrane-based cellular processes are dynamic as well as closely interconnected and thus require complex cellular logistics.[1] Small vesicles, for example, cycle between the endoplasmic reticulum (ER), Golgi and larger spherical compartments termed endosomes.[2,3] These endosomes are actively transported along polarized microtubules by the action of kinesin and dynein motor proteins to cover long distances. Kinesins mainly transport their cargo to the plus ends of microtubules whereas dynein mediates transport in the opposite direction.[1]

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In simple eukaryotes such as filamentous fungi secretion is essential for growth and nutrition.[4-6] Membrane trafficking is necessary to support fast expansion of growth cones at the hyphal tip. At the apex, a particular active zone of secretion is visible as Spitzenkörper,[7] which is proposed to act as a vesicle supply center.[8,9] Adjacent to this structure the polarisome assembles actin cables and, most likely, anchors microtubules to the membrane thereby directing the cytoskeleton-dependent membrane traffic toward the tip.[4,5,8,9] Secretory vesicles provide membrane material to the fast-growing tip. They also deliver biosynthetic enzymes such as chitin and glucan synthases to sustain the formation of cell walls as well as hydrolytic enzymes for nutrient acquisition and cell wall remodeling.[8,9] In addition, pathogenic fungi secrete a battery of effector proteins to interact with their animal or plant host.[10,11]

Trafficking in the reverse direction by endocytosis is required for the uptake of membranes and membrane-associated proteins such as trans-membrane receptors. Protein cargo can be either recycled to the plasma membrane or directed to the vacuole for degradation.[6,12] In fungi, membrane trafficking is supported by active microtubule-dependent transport of endosomes. However, the current knowledge on functionally important cargo is scarce.[6,8,12]

The basidiomycete *Ustilago maydis* has been established as a fungal model system that is particularly well-suited to study the mechanism and biological function of microtubule-dependent membrane dynamics.[13-15] This phytopathogenic fungus causes smut disease in corn.[11,16,17] Important for infection is a developmental switch from saprophytic yeast cells, which proliferate by budding (**Fig. 1A**), to filamentously growing cells, the infectious form (**Fig. 1B**). The key transcriptional regulator that triggers filamentous growth is the heterodimeric transcription factor bW/bE.[18] To constitute an active heterodimer subunits must be derived from different alleles provided by compatible mating partners. Thus, in wild type cells plasmogamy of the mates is a prerequisite for generating the active transcription factor.[16] However, monokaryotic lab strains have been established that express an active bW/bE heterodimer under control of regulated promoters (induced by either arabinose or nitrate). In these haploid strains hyphal growth,

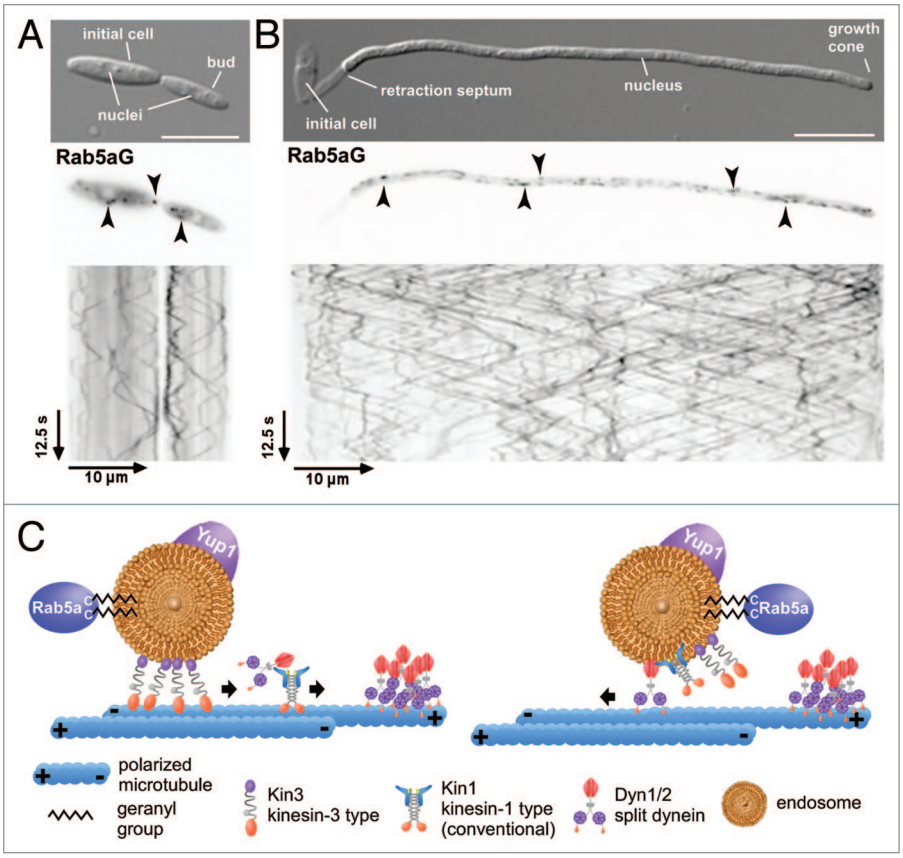

Figure 1. Microtubule-dependent shuttling of Rab5a-positive endosomes. (A) Yeast and (B) filamentous form of strain AB33Rab5aG expressing an active bW2/bE1 variant under the control of a nitrogen-source regulated promoter and the green fluorescent protein (Gfp) fused to the N-terminus of Rab5a (filamentous growth was induced by changing the nitrogen source of the medium; size bars, 10 µm). Rab5aG-positive endosomes (arrowheads in the inverted image detecting Gfp fluorescence) shuttle bi-directionally along microtubules (kymograph in the lower panel). In the kymograph time is plotted vs. distance. Thus, motion of Rab5aG is visible as defined tracks (note the highly processive movement and the reversal of shuttling at the poles). (C) Model depicting the motor-dependent mechanism (three motor system) of endosome transport (endosomes carry the small G protein Rab5a and the SNARE Yup1; symbols are explained in the inlay).

which closely resembles that of wild type filaments, can be elicited synchronously and reproducibly by switching the carbon or nitrogen source of the medium (**Fig. 1A and B**).[19]

In *U. maydis*, microtubule-dependent transport of endosomes is important for various steps of the life cycle: triggering cell separation during budding, receptor recycling during pheromone signaling and maintenance of polarity during filamentous growth.[20-22] Here, we review recent advances on the detailed transport mechanism as well as on the biological function of endosomes in signaling, endocytosis and mRNA transport. For further information on microtubule transport and *U. maydis* biology we refer to other, more comprehensive reviews.[5,11,15,23-26]

Motor-Dependent Transport of Rab5a-Positive Endosomes Along Microtubules

As mentioned above, the processes involving membrane trafficking are tightly interwoven and highly complex. A common concept in investigating endosomal compartments is the identification of characteristic marker proteins that differentiate endosomes.[1] At present, two different classes of endosomes are known in *U. maydis*: Mcs1- and Rab5a-positive endosomes. Mcs1, the fungal class 17 myosin, consists of two domains: an N-terminal myosin motor domain and a C-terminal chitin synthase domain.[27] Transport of Mcs1-positive endosomes does not depend on the myosin motor domain of Mcs1 and is mediated by other actin- and microtubule-dependent motors. The integral motor domain of Mcs1 is thought to tether the membrane units to the hyphal tip. Membrane-based Mcs1 trafficking is needed for its secretion by exocytosis, a process apparently required for cell wall remodeling and virulence.[27,28]

Rab5a is a small G protein belonging to the family of Rab proteins, which serve as specific markers of distinct classes of endosomes.[2] In *U. maydis*, Rab5a-positive endosomes were suggested to be early endosomes because they can also be stained with the styryl dye FM4–64, which has been used to study the endocytic pathway.[29] However, FM4–64 is not specific for endocytosis because it also stains secretory vesicles of the fungal Spitzenkörper[8] as well as secretory vesicles downstream of the trans-Golgi network in plant cells.[30] Moreover, it has not so far been reported that Rab5a-positive endosomes maturate into late endosomes in *U. maydis*, and there is a second Rab5 protein, Rab5b, encoded in the genome.[21] Therefore, we use the more generic term Rab5a-positive endosomes throughout this review.

An important component of Rab5a-positive endosomes is the t-SNARE-like protein Yup1 (related to Vam7p from *Saccharomyces cerevisiae*) that has been proposed to function in fusing endocytic vesicles with endosomes.[21,31] Its precise role is yet unclear but loss of Yup1 results in altered cell morphology[31] and reduced endocytic recycling of the pheromone receptor Pra1 (see below).[21] Moreover, Yup1 is important for endosome movement, because their motility is drastically impaired in temperature-sensitive *yup1*^{ts} mutants under restrictive conditions.[22]

In the last decade, endosomal transport in *U. maydis* has been studied in great detail, both in the yeast form and in filaments.[23] Rab5a-positive endosomes shuttle along microtubules throughout the cell moving in a highly processive fashion. At the poles they change direction without extensive pause (**Fig. 1A and B**). Each cell contains two to four microtubule bundles that consist of single microtubules oriented in an antiparallel fashion.[32] Near the poles of filaments (i.e., about 10 µm from both ends)

microtubules are not arranged in antiparallel bundles but form single unidirectional extensions with plus ends facing to the poles (**Fig. 1C**).[32]

Plus-end directed movement of endosomes along antiparallel bundled as well as single microtubules is mediated by Kin3 (**Fig. 1C**),[32,33] a Kinesin-3 type motor that is related to Unc104 and KIF1A from nematodes and mammals, respectively. The motor contains a C-terminal pleckstrin homology (PH) domain for interaction with phosphatidylinositol lipid containing membranes. The PH domain is essential for Unc104-dependent transport in neurons of *C. elegans*[34,35] and Kin3-driven movements of endosomes in filaments of *U. maydis*.[22] Each endosome contains about three to six Kin3 and these are responsible for the highly processive bidirectional movement along the antipolar bundles in filaments (**Fig. 1C**).[36]

Minus-end directed transport of endosomes is mediated by the dynein motor Dyn1/2 (**Fig. 1C**). This becomes important at the poles of filaments because in the unidirectional region of the microtubule cytoskeleton, minus end-directed transport is the only option for endosomes to return to the antiparallel array.[32,37] This can nicely be seen in temperature-sensitive mutants of *dyn2* where Rab5a-positive endosomes accumulate at the poles under restrictive conditions.[22,32]

Dyn1/2 can also move in the minus-end direction independently of Rab5a-positive endosomes. If such a minus-end directed Dyn1/2 motor meets an oncoming endosome, Dyn1/2 is able to reverse the direction of the moving endosome toward the minus end.[38] This process has been proposed to protect endosomes from "falling off the track."[23]

How are motors recycled once they reach the ends of microtubules? Kin3 associates with Rab5a-positive endosomes in both directions of transport. This holds true, even at the poles, where unidirectional microtubules and no longer antiparallel bundles are present. In this area Dyn1/2 mediates minus-end directed transport of endosomes. Here, Kin3 is a cargo and no specific recycling system for Kin3 is needed.[22] In contrast, dynein is actively transported back to the plus-ends of microtubules by conventional kinesin.[39,40] Therefore endosomes accumulate at the poles in the absence of conventional kinesin because dynein required for minus-end directed transport is missing at the poles.[22,40,41] In summary, a tripartite motor system consisting of Kin3, dynein and conventional kinesin manages the complex logistics of long-distance transport of endosomes along a highly organized array of microtubules (**Fig. 1C**).

Biological Functions of Rab5a-Positive Endosomes During Endocytosis, Signaling and Posttranscriptional Regulation

Interestingly, Rab5a-positive endosomes exhibit rather diverse functions throughout the life cycle of *U. maydis*. In yeast cells they play an important role in septum formation and cell separation. *kin3*Δ strains form donut-like colonies (**Fig. 2A**) reminiscent of the mutants *don1* and *don3* that were originally identified owing to their striking colony morphology.[42] The explanation for this mutant phenotype is the failure to form a secondary septum,

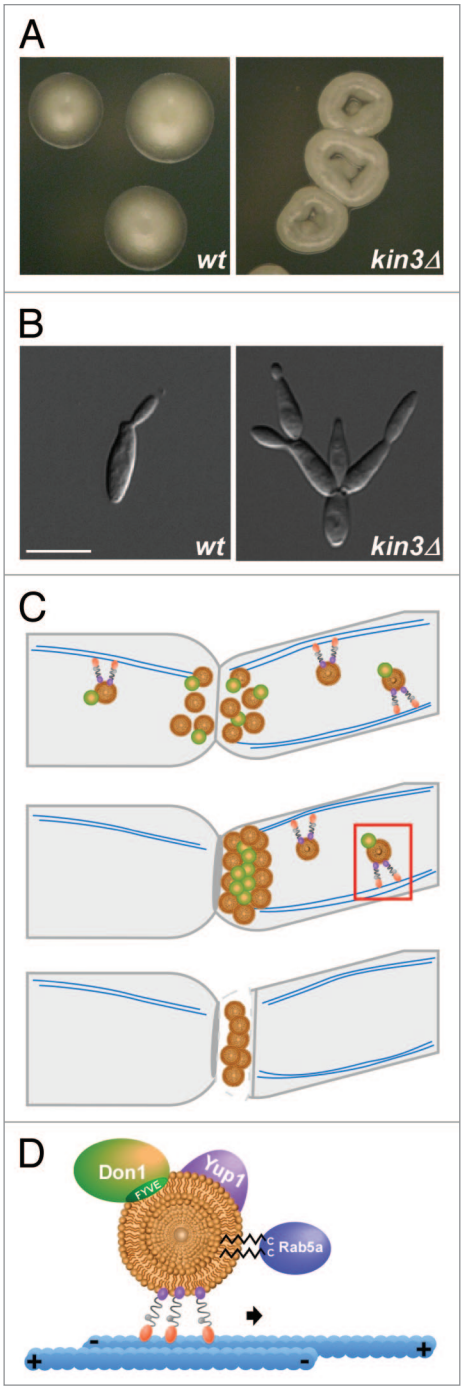

Figure 2. Rab5a-positive endosomes are essential for cytokinesis. (A) Colony and (B) yeast cell morphology of strain AB33kin3Δ in comparison to the progenitor strain (size bar, 10 μm). (C, D) Model depicting the function of Rab5a-positive endosomes in the delivery of Don1 to the site of septation, a process essential for formation of the secondary septum. The red rectangle shown in the middle panel of C is enlarged in D.

which is essential for cell separation after cytokinesis (**Fig. 2B and C**). *don1* encodes a guanine nucleotide exchange factor (GEF) specific for the small GTP binding protein Cdc42 and Don3 is a Ste20-like protein kinase acting parallel to this signaling module.[42,43] Don1 carries a C-terminal FYVE domain[44] that

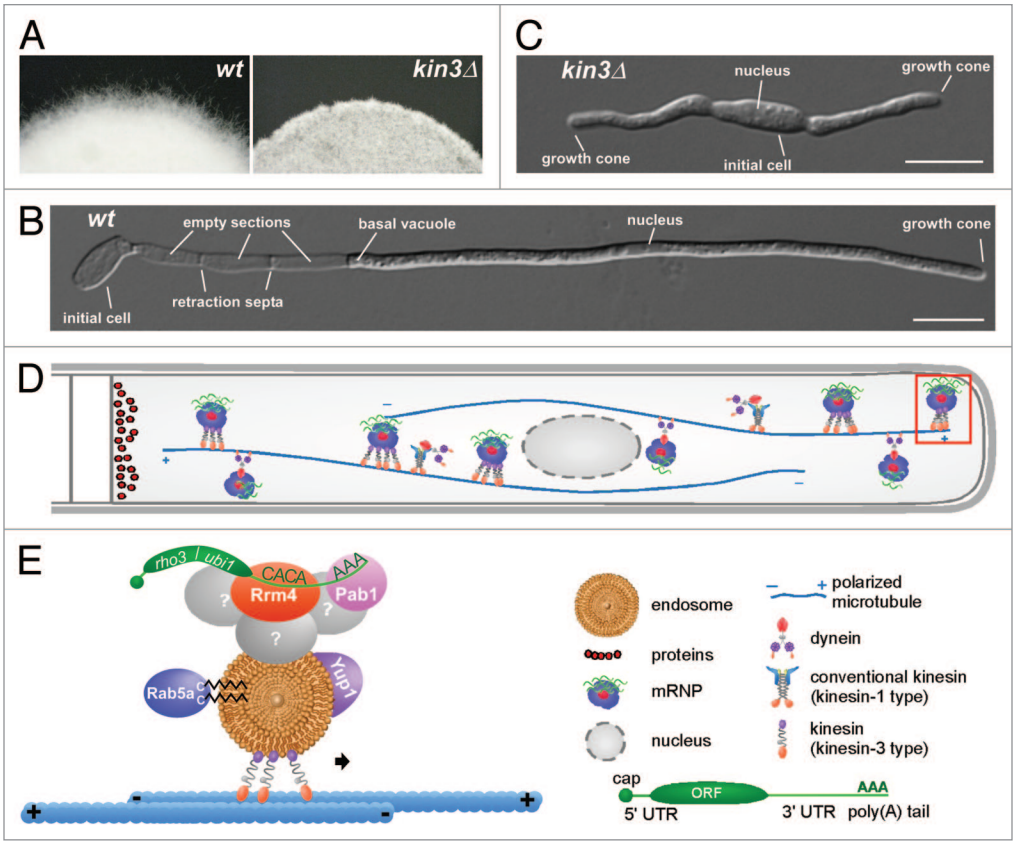

Figure 3. Rab5a-positive endosomes are important for filamentous growth. (A) Colony and (B, C) cell morphology of *kin3*Δ filaments. The edges of colonies growing under filament-inducing conditions are shown in (A). Monokaryotic filament carrying wild type allele of *kin3* (B) is compared with the bipolarly growing filament of a *kin3*Δ (C) strain grown under filament-inducing conditions (size bars, 10 μm). (D, E) Model depicting the function of Rab5a-positive endosomes during transport of mRNPs. Red rectangle shown at the growth cone is enlarged in E (symbols are explained in the inlay).

is specific for the recognition of phosphatidylinositol-3-phosphate lipids and thus targets Don1 to Rab5a-positive endosomes. Kin3-mediated transport of these Don1-loaded endosomes is critical for cell separation in order to synchronize Cdc42-triggered secondary septum formation with the accumulation of vesicles involved in lysis of the fragmentation zone (**Fig. 2C and D**).[20]

During mating Rab5a-positive endosomes are involved in endocytic recycling of the pheromone receptor Pra1.[21] Pra1 accumulates in endocytic vesicles inside the cell in temperature-sensitive *yup1*^{ts} mutants under restrictive conditions and thus is missing at the plasma membrane of conjugation tubes. This correlates with a reduced recognition of compatible mating partners and results in a fusion defect of conjugation tubes.[21] Thus, endocytic recycling of Pra1 mediated by Rab5a-positive endosomes appears to be important for polarized accumulation of the receptor at the growth cones of mating hyphae that orient their growth toward pheromone sources.[12,45]

During filamentous growth microtubule-dependent motor function is important for maintaining unidirectional growth of the tip cell and the concomitant formation of retraction septa at the distal end of the hyphae. *kin3*Δ strains form shorter (**Fig. 3A**) and predominantly bipolar filaments lacking retraction septa, indicating that unipolar growth is disturbed (**Fig. 3B and C**). Similar observations were made in the absence of functional dynein and conventional kinesin or in filaments treated with the microtubule inhibitor benomyl.[46]

It is noteworthy that loss of RNA-binding protein Rrm4 causes a similar growth defect: mutant filaments grow mainly bipolar and fail to insert retraction septa.[41] This RRM-type (RNA recognition motif) RNA-binding protein constitutes an integral part of a transport machinery mediating microtubule-dependent long-distance transport of mRNAs.[47,48] Rrm4 binds to distinct sets of target mRNAs encoding cytotopically related proteins including polarity factors such as Rho3 and ribosomal proteins such as Ubi1, a natural fusion protein of ubiquitin and Rpl40 of the large ribosomal subunit.[47] Rrm4-mediated mRNA transport is believed to promote subcellular accumulation of Rho3 at the retraction septum by local translation (**Fig. 3D and E**).[48]

Interestingly, Rrm4 is almost exclusively co-transported with Rab5a-positive endosomes.[22] Co-transport is observed throughout the whole filament, and co-localization with these endosomes does not depend on the presence of Kin3. Thus, the Rrm4/endosome interaction appears to be independent of molecular motors.[22] In the absence of Rrm4, endosomal movement is not drastically impaired suggesting that Rab5a-positive endosomes shuttle independently of Rrm4 and its mRNA cargo. Nevertheless, loss of Rrm4 causes defects in filamentous growth resembling mutants with defects in microtubule function. Thus, mRNA transport

appears to be an important function of trafficking Rab5a-positive endosomes, particularly during filamentous growth (**Fig. 3D and E**).[15,22]

This novel function of endosomes has broad implications for mRNA transport in general.[15] In *U. maydis* we could demonstrate unambiguously that microtubule-dependent mRNA transport is mainly endosome dependent (**Fig. 3D and E**).[17,22] But it has been observed before that mRNA transport can be tightly linked to membrane trafficking.[49] In *S. cerevisiae* actin-dependent transport of mRNAs can be achieved by hitchhiking on the ER.[50] During development of *Drosophila melanogaster*, the endosomal marker Rab11[51] as well as components of ESCRT-II (endosomal sorting complex required for transport)[52] are important for correct subcellular localization of mRNAs encoding morphogens. Therefore, it is speculated that endosomes might be directly involved in short range mRNA transport.[53]

Perspectives

Research in recent years has revealed that endosome action is a lot more complex than previously anticipated.[1,54] This is well supported by recent findings on the variety of endosomal functions in the fungal model system *U. maydis*: along with their function in endocytosis, Rab5a-positive endosomes are important for cell separation, pheromone signaling and mRNA transport. The connection to mRNA transport, in particular, adds a novel aspect to the growing list of endosomal functions, thus substantiating current views that these endomembrane compartments function as multipurpose platforms.[54]

This increased level of complexity may also influence the design of future experiments. If, for example, mRNAs encoding proteins involved in endocytosis are themselves transported by endosomes,[47] alterations of endosome function might disturb endocytosis not only directly but also indirectly via mRNA transport. In the future, joint efforts of scientists focusing on mRNA and membrane trafficking are needed to explain both the mechanistic and functional aspects of this complex and interlaced molecular sorting machinery.

Disclosure of Potential Conflicts of Interest

There is neither a conflict-of-interest nor a financial interest in publishing this work.

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