Rab5 regulates motility of early endosomes on microtubules

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focus on MEMBRANE TRAFFIC

The small GTPase Rab5 regulates membrane docking and fusion in the early endocytic pathway. Here we reveal a new role for Rab5 in the regulation of endosome interactions with the microtubule network. Using Rab5 fused to green fluorescent protein we show that Rab5-positive endosomes move on microtubules *in vivo*. *In vitro*, Rab5 stimulates both association of early endosomes with microtubules and early-endosome motility towards the minus ends of microtubules. Moreover, similarly to endosome membrane docking and fusion, Rab5-dependent endosome movement depends on the phosphatidylinositol-3-OH kinase hVPS34. Thus, Rab5 functionally links regulation of membrane transport, motility and intracellular distribution of early endosomes.

In mammalian cells, endocytosis is responsible for the uptake of essential nutrients from the external environment and the maintenance of cellular homeostasis through the retrieval of proteins and lipid delivered to the plasma membrane by secretion[1]. After internalization, the efficient sorting of endocytosed proteins and lipids into pools destined for degradation or recycling to the plasma membrane occurs through interactions between a series of biochemically and morphologically distinct compartments. Dynamic associations with actin and microtubule cytoskeletal networks mediate both the generation and the movement of vesicular carriers, as well as maintenance of the characteristic spatial distribution and morphology of endocytic organelles within the cell. This implies a mechanistic link between the membrane targeting, docking and fusion machinery and the machinery that governs organelle movement and position.

Although little is known about the molecular basis for such coordination, these processes have been individually extensively studied. In fibroblasts, the radial arrangement of microtubules determines the distribution of the endoplasmic reticulum and the Golgi and facilitates transport along the secretory pathway[2]. In the endocytic pathway, association with microtubules and action of microtubule-associated motor proteins are necessary for the proper positioning of late endosomes, lysosomes and the pericentriolar-recycling compartment[3–5]. Efficient transport of cargo between early and late endosomes and transcytosis in polarized epithelial cells also depend on microtubule-associated motors[6,7]. Additionally, actin is implicated in early events in endocytosis in both yeast and mammalian cells[8,9]. In mammals, expression of an active mutant of the small GTPase RhoD which causes rearrangements of the actin cytoskeleton inhibits movement of endosomes *in vivo*[10].

Rab GTPases regulate vesicle docking and fusion events during both endocytic and biosynthetic transport in yeast and mammalian cells[11]. These proteins act through recruitment of specific effector proteins involved in membrane tethering and docking[12–14], and have been proposed to modulate the activity of SNAREs, integral membrane proteins mediating membrane fusion[15]. One member of this GTPase family, Rab5, regulates membrane traffic into and between early endosomes. The recent demonstration that at least 20 cytosolic proteins specifically interact with active Rab5 highlights the complexity of the downstream regulation of this GTPase and raises the possibility that Rab5 might regulate other aspects of endosome function in addition to docking and fusion[13].

The present study addresses observations made during the phenotypic analysis of cells overexpressing wild-type Rab5 or the activated mutant Rab5Q79L[10,16,17]. Stimulation of Rab5 activity results in formation of enlarged early endosomes, which is consistent with a role for Rab5 in fusion of endosomal structures. Interestingly, Rab5 overexpression also results in redistribution of endosomes from punctate structures dispersed throughout the cytoplasm to a juxtanuclear localization[10,16,17]. This, combined with the observation that enlarged endosomes are disrupted and redistributed into the cell periphery upon microtubule depolymerization[18], led us to investigate whether Rab5 might regulate interactions of early endosomes with microtubules in mammalian cells.

Results

EGFP–Rab5 labels early endosomes that associate with microtubules *in vivo*. In order to study the dynamics of Rab5-positive early endosomes *in vivo*, wild-type Rab5 fused to the carboxy terminus of enhanced green fluorescent protein (EGFP) was expressed in stably transformed A431 cells. EGFP–Rab5 was observed primarily on punctate cytoplasmic structures distributed throughout the cells as well as accumulated in the juxtanuclear region. These structures co-localized with fluorescently labelled transferrin (Fig. 1a) and with the early-endosome-associated protein EEA1 (Fig. 1b). However, no significant co-localization with EGFP–Rab5 was observed if cells were probed with antibodies against the Golgi marker Giantin (Fig. 1c) or the late-endosome marker Rab7 (Fig. 1d). Therefore, most EGFP–Rab5 is correctly targeted to early endosomes in these cells.

We then determined whether EGFP–Rab5 endosomes co-localized with cytoskeletal elements. Whereas little co-localization between EGFP–Rab5 endosomes and F-actin was detected (Fig. 1e), we often observed Rab5 structures distributed in linear tracks in the cytoplasm (Fig. 1e, arrows) that co-localized with microtubules (Fig. 1f, arrows). Upon actin depolymerization with cytochalasin D, the juxtanuclear localization of endosomes was not affected. However, peripheral EGFP–Rab5 structures now overlapped significantly with residual cortical F-actin patches (Fig. 1g). In contrast, depolymerization of microtubules with nocodazole resulted in accumulation of enlarged or clustered EGFP–Rab5 endosomes in the extreme periphery of the cell (Fig. 1h). These results suggest that both actin and microtubule networks govern the intracellular distribution of the Rab5-positive endosomal compartment.

Long-range movement of early endosomes *in vivo* is microtubule dependent. To visualize the dynamics of the EGFP–Rab5 compartment and to determine the role of microtubules in this process, we

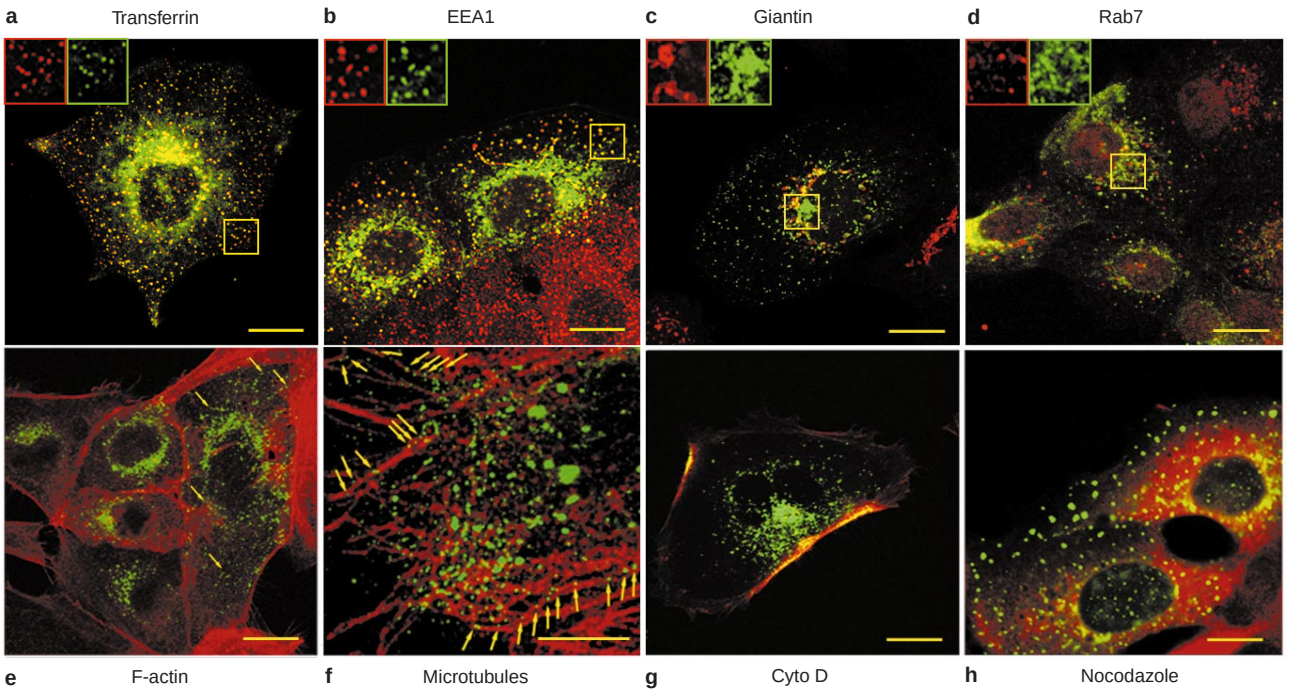

Figure 1 **EGFP–Rab5 co-localizes with early endosomes in A431 cells.** Transfected cells expressing EGFP–Rab5 were processed for immunofluorescence and analysed by laser scanning confocal microscopy to detect the extent of co-localization of EGFP fluorescence (green) with several other cellular proteins (red). Inset boxes show specific examples of the extent of co-localization of EGFP fluorescence (green box) with rhodamine fluorescence (red box). Cells were allowed to internalize rhodamine-labelled transferrin for 5 min (**a**), probed with antibodies to early-endosome-associated protein EEA1 (**b**), antibodies to the Golgi protein Giantin (**c**), antibodies to the late-endosome-associated GTPase Rab7 (**d**), or anti-α-tubulin antibodies to visualize microtubules (**f, h**), or were probed with rhodamine-labelled phalloidin to visualize F-actin (**e, g**). The arrows in **e** indicate linear tracks of Rab5 structures in the cytoplasm. Similar structures co-localize with microtubules as indicated by the arrows in **f**. To assess the effect of depolymerization of cytoskeletal elements on cellular localization of EGFP–Rab5, actin (**g**) or microtubules (**h**) were disrupted with cytochalasin D (Cyto D) or nocodazole respectively. Scale bars represent 10 μm.

used video-enhanced fluorescence microscopy (Fig. 2). We observed two distinct types of movement of EGFP–Rab5 endosomes in the cell. A short-range nonlinear motion was most evident in the cell periphery or in lamellipodia. The second type of motion was observed primarily in the cell body, and consisted of long-range linear movements; these are presented in Fig. 2a as a merged stack of successive frames of a movie. In this representation, moving endosomes appear as a linear series of dots (Fig. 2a, arrows; see also Supplementary Information). These long-range motions were often bidirectional and were abolished when microtubules were depolymerized with nocodazole (Fig. 2b and Supplementary Information).

When F-actin was disrupted with cytochalasin D, long-range linear motion was not impaired, but rather was often stimulated (Fig. 2c and Supplementary Information). This effect was accompanied by an accumulation of EGFP–Rab5 in a juxtanuclear region. A similar effect was observed in cells expressing high levels of EGFP–Rab5. We observed by video microscopy that this redistribution correlated with increased endosome movement towards the centre of the cell (Fig. 2d and Supplementary Information). This observation is consistent with previous studies showing that overexpression of wild-type or constitutively active Rab5 results in accumulation of enlarged endosomes in the perinuclear region[16,17]. The redistributed EGFP–Rab5 compartment contained the endosome-tethering protein EEA1 (ref. 13), and was labelled by internalized transferrin with similar kinetics to those of the peripherally localized endosomal structures[16] (Fig. 1a and data not shown). Altogether, these results suggest that Rab5 participates in the intracellular distribution of early endosomes by regulating their microtubule-dependent movement towards the centre of the cell.

Rab5 stimulates association of early endosomes with microtubules *in vitro*. The effect of Rab5 on endosome motility implies that this GTPase may regulate the association of endosomes with, and/or their motility along, microtubules. Given that earlier studies demonstrated binding of endosomes to microtubules[19], we first tested whether Rab5 altered this interaction. Briefly, purified early endosomes were mixed with cytosol and taxol-stabilized microtubules. Microtubules were then sedimented through a sucrose cushion to separate endosomes associated with microtubules from unattached endosomes and cytosolic components, and the pellet fraction was subjected to immunoblot analysis. Cytoskeletal proteins present in the cytosol did not contribute significantly to the sedimentation of endosomes as almost no transferrin receptor was detected in the pellet without addition of microtubules (Fig. 3, lane 5). Recruitment of endosomes to microtubules was stimulated by cytosol (Fig. 3, compare lanes 6 and 7)[19]. Although recruitment was further enhanced in the presence of millimolar ATP concentrations (Fig. 3, lane 8), the nucleotide was not essential for association, as depletion of ATP by hexokinase and glucose did not reduce endosome recruitment to levels lower than in cytosol alone (Fig. 3, compare lanes 7 and 9). As motor proteins release from microtubules in the presence of ATP[20], the association of endosomes with microtubules must also be regulated by other factors, such as microtubule-associated proteins or protein and lipid kinases.

We next tested whether the absence or presence of Rab5 affected the recruitment of endosomes onto microtubules. Addition of Rab-GDP-dissociation inhibitor (RabGDI) alone at a concentration (1 μM) that removes Rab5 from endosomal membranes[21] resulted in a significant reduction in the number of

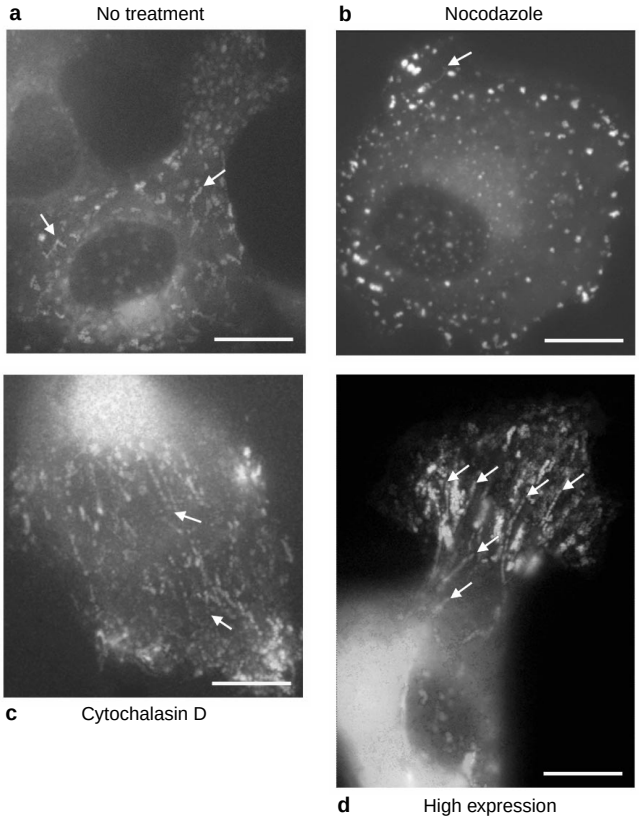

Figure 2 **Endosomes positive for EGFP–Rab5 move in a microtubule-dependent fashion *in vivo*.** A431 cells stably expressing EGFP–Rab5 were imaged using time-lapse fluorescence video microscopy to visualize movement of GFP-labelled endosomes. **a–d**, Images generated by merging a stack of overlaid photos collected at 2-s intervals (see also Supplementary Information). When represented in this manner, a moving object will shift position, creating a linear series of overlapping or closely associated spots. Arrows indicate examples of long-range endosome movements. Images were collected after no treatment (**a, d**), after incubation with nocodazole (20 μM) for 30 min (**b**) or after incubation with cytochalasin D (0.5 μg ml–1) for 30 min (**c**). **d**, Image collected from a cell expressing high levels of EGFP–Rab5. Scale bars represent 10 μm.

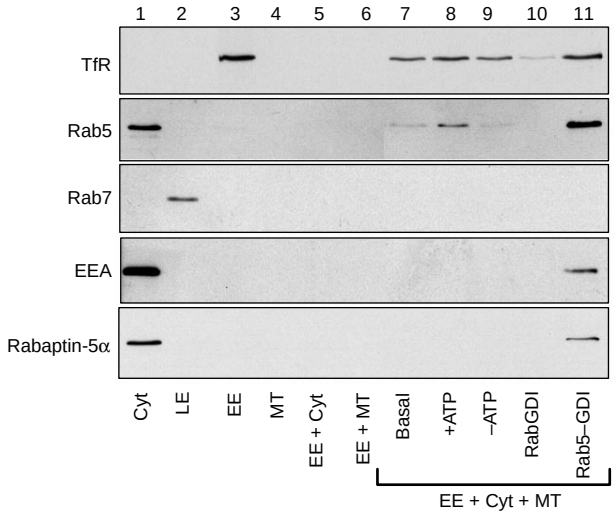

Figure 3 **Rab5 regulates endosome association with microtubules.** Reactions containing cytosol (2 mg ml^{-1}; Cyt, lane 1) and purified early endosomes (15 μg; EE, lane 3) were incubated at room temperature for 20 min then mixed and incubated for 10 min more with taxol-stabilized microtubules (MT, lane 4), and spun through a sucrose cushion. The resulting pellet of microtubule-associated material was resuspended in SDS–PAGE buffer and analysed by immunoblotting with antibodies against the proteins indicated on the left. A representative fraction of purified late endosomes (LE) was loaded as a positive control for Rab7 (lane 2). Reactions were carried out in the absence of microtubules (lane 5), the absence of cytosol (lane 6), or with all three components (EE+Cyt +MT, Basal, lane 7), and addition of 2 mM ATP (lane 8), an ATP-depletion system (lane 9), 1 mM RabGDI (lane 10) or 75 nM Rab5–RabGDI complex (lane 11). TfR, transferrin receptor.

endosomes in the microtubule pellet (Fig. 3, lane 10). To test the effect of higher levels of Rab5 on endosomes we added Rab5–RabGDI complex at concentrations that yield saturable binding of Rab5 (75 nM), and which stimulate endosome fusion[21]. This resulted in increased endosome recruitment to microtubules (Fig. 3, lane 11). Rab5 was successfully delivered to endosomes in this assay, as demonstrated by the recruitment of the previously identified Rab5 effector proteins Rabaptin-5 and EEA1 (Fig. 3, lane 11). We conclude that Rab5 regulates the interaction of early endosomes with microtubules.

Rab5 regulates early-endosome motility on microtubules *in vitro*. Next, we investigated the possibility that Rab5 may also control the motility of early endosomes on microtubules. For this, we developed an assay that reconstitutes this process *in vitro*, and analysed the process using time-lapse video microscopy. In this assay, a uniform lawn of taxol-stabilized microtubules was allowed to stick to the glass surfaces of a perfusion chamber. Microtubules were polymerized *in vitro* from purified fluorescently labelled tubulin, and consisted of weakly fluorescent stretches of tubules interspersed with strongly labelled 'seeds'[22]. Purified endosomes labelled with rhodamine–transferrin were mixed with cytosol, ATP and an anti-fade mixture, and perfused into the chamber for analysis by video microscopy. Significant numbers of fluorescently labelled endosomes were observed moving along microtubules (Fig. 4a and Supplementary Information) in a cytosol- and ATP-dependent fashion (data not shown). Interestingly, addition of 1 μM RabGDI resulted in an almost compete inhibition of endosome motility (Fig. 4b), indicating that Rab GTPases are required for this function.

To determine whether Rab5 had a direct role in this process, we used a mutated form of Rab5, Rab5D136N. As this mutant binds xanthine rather than guanine nucleotides, it is possible to specifically modulate the nucleotide-bound state of this protein without interfering with other GTPases[23]. Loading of endosomes with Rab5D136N in the presence of XDP inhibited endosome motility. In contrast, activation of Rab5D136N with XTP-γS led to a ~2.5-fold stimulation of motility events per minute compared with cytosol (Fig. 4b). Neither XTP-γS alone nor the late-endosomal Rab protein Rab7 could stimulate endosome motility in this assay (Fig. 4b). Therefore, Rab5 specifically regulates early-endosome motility along microtubules.

It is possible that the effect of Rab5 on endosome motility may be indirectly caused by enhanced endosome fusion due to an increased number of motor proteins per organelle. To distinguish the role of Rab5 in endosome motility from its role in endosome fusion we assessed whether endosome motility *in vitro* was affected under conditions in which endosome fusion was blocked. In the presence of 5 μM of a mutated form of α-SNAP, α-SNAP L294A[24], early-endosome fusion is completely inhibited[13]. However, in the presence of this inhibitor, early-endosome motility and the stimulatory effect of Rab5 on this process were not significantly reduced (Fig. 4b). Altogether, these results demonstrate that the small GTPase Rab5 directly and specifically regulates early endosome motility along microtubules in a nucleotide-dependent fashion, and that this regulation can be uncoupled from early-endosome fusion.

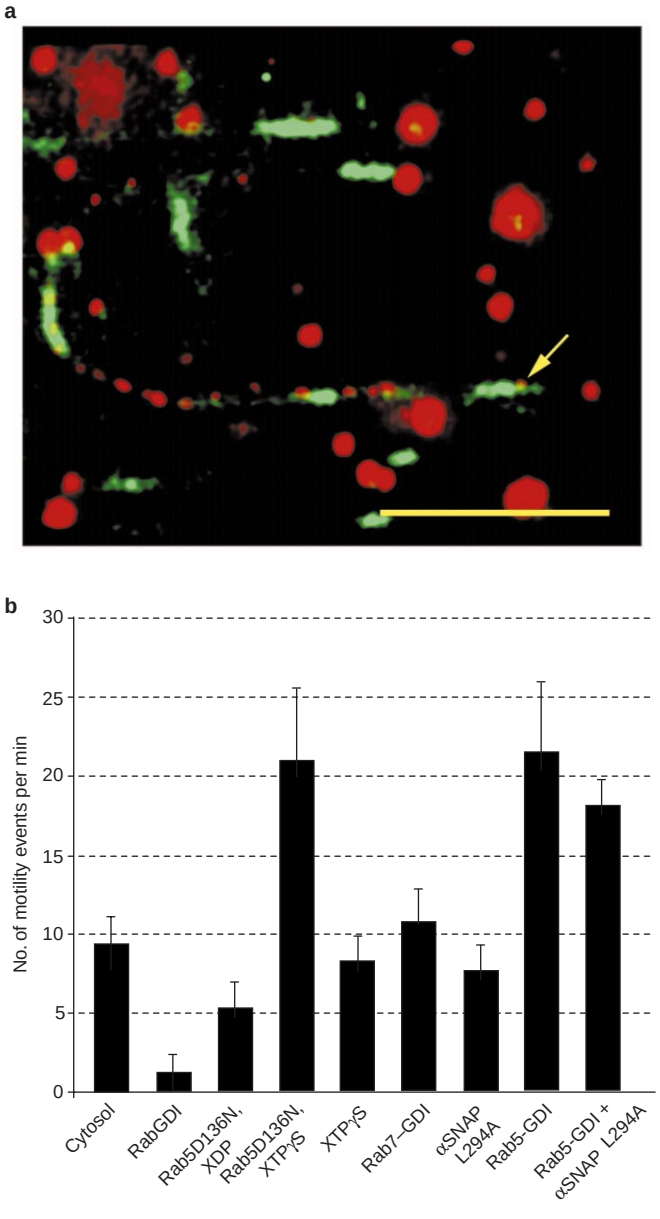

Figure 4 **Early endosomes move on microtubules in a Rab5-dependent fashion *in vitro*.** Purified endosomes labelled with rhodamine–transferrin were mixed with cytosol (2 mg ml^{-1}) and ATP (200 μM) and perfused into a chamber coated with Oregon-green-labelled microtubules. Movement of endosomes along microtubules was observed using time-lapse fluorescence video microscopy. A merged image (**a**) was generated from a stack of overlaid photos collected at 2-s intervals in which first fluorescent microtubules (green), then fluorescent endosomes (red) were visualized. In this example, an endosome associates with a microtubule (arrow indicates beginning of motility event), and moves along the microtubule, resulting in a linear series of overlapping or closely associated spots (see also Supplementary Information). Scale bar represents 5 μm. **b**, Endosome movement depends on Rab5 function. Moving endosomes were counted (see Methods) and motility activity expressed as a function of movement events per field per min. Conditions were as follows: cytosol (2 mg ml^{-1}) and ATP (200 μg ml^{-1}) alone (Cytosol); addition of 1 μM RabGDI (RabGDI); loading of endosomes with 100 nM Rab5D136N–REP1 complex in the presence of XDP (Rab5D136N, XDP), or XTP-γS (Rab5D136N, XTPγS); XTP-γS alone (XTPγS); 100 nM Rab7–RabGDI complex (Rab7–GDI); 5 μM α-SNAP L294A (αSNAP L294A); 75 nM Rab5–RabGDI complex (Rab5–GDI); or both (Rab5–GDI + αSNAP L294A). Each value represents an average of the number of moving endosomes observed from at least three separate fields. Error bars represent population standard deviations.

Rab5 increases minus-end motility of early endosomes along microtubules. Endosomes accumulate in the juxtanuclear region of cells when Rab5 (ref. 16), constitutively active Rab5Q79L[17] or EGFP–Rab5 is overexpressed (Fig. 2d). This suggests that Rab5 stimulates motility of endosomes towards the minus ends of microtubules *in vivo*. We tested this prediction, using a modified *in vitro* motility assay based on microtubules with identifiable plus and minus ends. Fluorescently labelled microtubule asters were grown *in vitro*, generating microtubules with their minus ends attached to purified centrosomes (Fig. 5a; the centrosome is labelled with an asterisk). Because of the large variability in size and extent of microtubule bundling between different asters, this assay did not allow for measurement of stimulation or inhibition of motility, but it allowed us to assess the proportion of plus- versus minus-end motility. We observed endosomes moving towards both plus (~52%, single arrowheads) and minus (~48%, double arrowheads) ends of microtubules on these asters (Fig. 5a and Supplementary Information). Addition of 75 nM Rab5–RabGDI complex increased the proportion of endosomes moving towards minus ends of microtubules to more than 78% (Fig. 5b).

Plus-end-directed motility of organelles on microtubules occurs through the action of motor proteins of the kinesin family, whereas minus-end motility can occur through the action of either dynein motor proteins or specific members of the kinesin family. As we observed bidirectional motility of endosomes, we used function-blocking antibodies to gain insight into which of these motor protein families was responsible for the endosome movement *in vitro*. Dynein-based motility has been reported for several trafficking steps associated with endocytosis[6] and phagocytosis[25]. Surprisingly, addition of antibody 70.1, which blocks dynein function, did not alter the proportion of minus-end versus plus-end endosome motility (Fig. 5b), even at concentrations that completely inhibited dynein-based motility of purified Golgi membranes (data not shown). On the other hand, addition of MC44, an anti-kinesin antibody that recognizes multiple forms of kinesin[26], inhibited motility in both directions. Anti-SUK4 antibody, which specifically inhibits conventional kinesin heavy chain[27], a plus-end motor protein, decreased the proportion of plus-end motility. These results are consistent with kinesin motors being primarily responsible for the Rab5-dependent movement of early endosomes in this assay, but the identification of the motor protein awaits further investigation.

Endosome motility is dependent on phosphatidylinositol-3-OH-kinase activity. The finding that, in addition to regulating endosomal membrane docking and fusion, Rab5 also regulates movement of endosomes along microtubules raises the question of how these functions are coordinated. Phosphatidylinositol-3-OH kinase (PI(3)K) activity is necessary for the Rab5-dependent regulation of membrane transport. Subsequent studies have shown that generation of phosphatidylinositol-3-phosphate (PtdIns(3)P) is required for the membrane recruitment of EEA1 (refs 12,28) and that PI(3)Ks are among the Rab5 effectors[29]. We therefore tested the possibility that PI(3)Ks were also involved in the Rab5 regulation of endosome movement. *In vitro* motility of endosomes on non-polarity-marked microtubules was inhibited by addition of increasing concentrations of the PI(3)K inhibitor wortmannin (Fig. 5c). This pharmacological approach suggests that PI(3)K is indeed necessary for endosome movement. To determine which PI(3)K family member is responsible for this function, we made use of specific function-blocking antibodies[30]. Treatment with antibodies against the PI(3)K hVPS34 significantly inhibited endosome movement, whereas equivalent concentrations of antibodies against the PI(3)Ks p110α and p110β, or addition of control IgG, did not alter the number of motility events observed (Fig. 5d). Interestingly, the proportion of endosomes moving along microtubules towards the plus end increased after addition of wortmannin or anti-hVPS34 (Fig. 5b). Therefore, it is likely that the PI(3)K activity of hVPS34 is required for minus-end-directed motility of endosomes along microtubules.

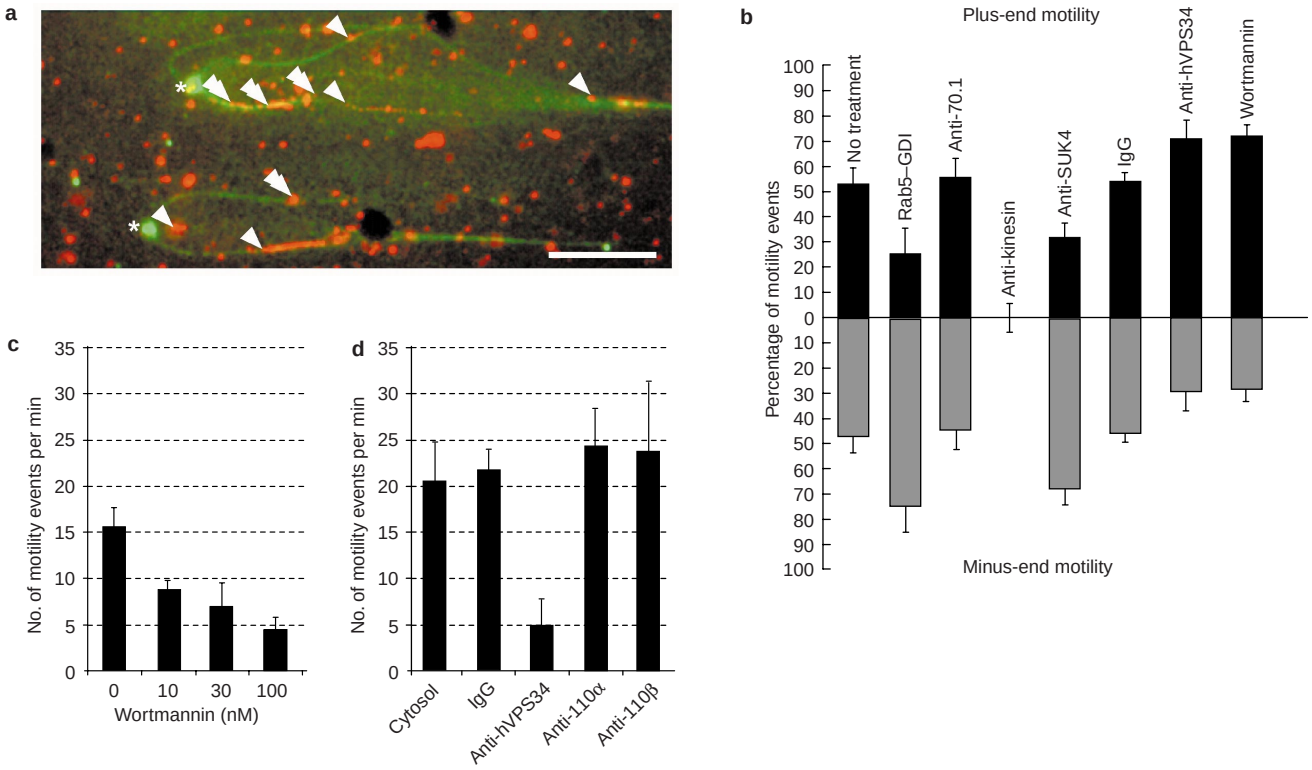

Figure 5 **Rab5 increases the proportion of minus-end-directed endosome movements on microtubules and this effect requires PI(3)K activity.** Purified endosomes labelled with rhodamine–transferrin were mixed with cytosol (2 mg ml^{-1}), and ATP (200 μM), and perfused into a chamber with Oregon-green-labelled microtubule asters. Movement of endosomes along microtubules was observed using time-lapse fluorescence video microscopy. **a**, Merged image consisting of a stack of overlaid photos collected at 2-s intervals in which fluorescent microtubules (green), then fluorescent endosomes (red), are visualized. When represented in this manner, moving endosomes shift position, creating a series of overlapping or closely associated spots overlying a microtubule. Plus-end-directed movements are indicated with single arrowheads, minus-end movements with double arrowheads, and centrosomes with asterisks (see also Supplementary Information). Scale bar represents 10 μm. **b**, The proportion of plus-end versus minus-end endosome movements was evaluated after loading endosomes with 75 nM Rab5–RabGDI complex (Rab5–GDI), and the addition of 0.15 mg ml^{-1} inhibitory antibodies specific for dynein intermediate chain (anti-70.1), or for kinesin motor proteins (anti-kinesin), or for conventional kinesin heavy chain (anti-SUK4), or for hVPS34 (anti-hVPS34), or nonspecific IgG (IgG), or inhibiting PI(3)K activity by addition of 100 nM wortmannin (Wortmannin). Proportions are averages calculated from counting endosome movements from at least three different asters, and error bars represent population standard deviations. **c**, Quantification of inhibition by wortmannin of *in vitro* endosome motility on non-polarity-marked microtubules. **d**, Quantification of the inhibition of *in vitro* endosome motility on non-polarity-marked microtubules by function-blocking anti-PI(3)K antibodies. Error bars represent population standard deviations.

Discussion

Interaction with the microtubule network and movement upon it is essential for maintaining the normal intracellular distribution of endocytic organelles as well as for vesicular trafficking steps between these structures[31]. Here we describe a new role for Rab5 in endosome dynamics—the regulation of endosome association with, and motility along, microtubules. Expression of high levels of EGFP–Rab5 led to a redistribution of early endosomes to the juxtanuclear region of the cell, as noted previously[10,16,17]. This effect can be explained by a Rab5-dependent increase in motility of endosomes towards the centre of the cell as shown by video microscopy (Fig. 2). Consistent with this interpretation, Rab5 also stimulated endosome motility along microtubules, and increased the proportion of minus-end-directed movement on microtubule asters in reconstituted systems *in vitro*. Taken together, these observations suggest that Rab5 regulates endosome motility towards the minus ends of microtubules. A kinesin molecule, Rabkinesin-6, has been shown to interact with Rab6 (ref. 32), although the precise role of Rab6 in Golgi motility has not been established. Together with our observations, this suggests that the control of organelle motility may be a general mechanism shared by different Rab proteins, and that this class of GTPases may participate in regulating the directionality of organelle movements along microtubules.

We have not yet identified the motor protein regulated by Rab5 and so far no motor protein could be detected among the Rab5 effectors[13]. Surprisingly, the minus-end-directed motility of endosomes was not inhibited by function-blocking antibodies against the minus-end motor dynein, unlike other minus-end endocytic organelle movements along microtubules[25,33]. Rather, the motility of endosomes towards both plus and minus ends of microtubules was inhibited by addition of anti-kinesin antibodies, suggesting that a kinesin motor may be responsible for the Rab5-stimulated endosome motility. Clearly, more work is required to establish unambiguously whether a kinesin-related motor protein can propel early endosomes towards microtubule minus ends. So far, minus-end kinesins have not been found in association with endocytic organelles, but KIFC2, a putative minus-end kinesin, has been shown to associate with unidentified multivesicular organelles in neuronal cells and has been proposed to mediate transport of these organelles along microtubules. Although KIFC2 is specifically expressed in neuronal cells, other minus-end kinesins are more ubiquitous, and might therefore mediate endosome movements towards the minus ends of microtubules[34–36].

The effect of Rab5 on endosome motility could be uncoupled from endosome fusion. Although this indicates that the Rab5 regulation of endosome motility does not depend on membrane fusion,

we have nevertheless found that these processes are coordinated through PI(3)K. The observation that endosome motility requires the PI(3)K activity of hVPS34 is of particular interest given the recent advances in the understanding of the role of PtdIns(3)P in membrane trafficking. *In vivo*, inhibition of PI(3)K activity with wortmannin or with function-blocking antibodies to hVPS34 interferes with the accumulation of newly endocytosed proteins in structures at the centre of the cell[30,37]. The Rab5 effector protein EEA1 and several other endosomal proteins are recruited to endosomes through interactions with PtdIns(3)P[38–40]. We have recently demonstrated that Rab5 selectively interacts with the PI(3)Ks p110β and hVPS34 (ref. 29). We propose that the interaction of Rab5–GTP with hVPS34 results in a localized production of PtdIns(3)P which, together with the recruitment of Rab5 effectors, contributes to the formation of a 'microdomain' on the endosomal membrane. This model is supported by recent studies showing that EEA1 forms high-molecular-weight oligomers with the Rabaptin-5 complex and the ATPase NSF (*N*-ethylmaleimide-sensitive factor) upon recruitment to endosomal membranes[41]. In such microdomains Rab5 could directly interact with a microtubule motor, as Rab6 does with Rabkinesin-6, or interact with an accessory protein associated with the motor protein, or recruit a regulatory protein that would stimulate a kinesin motor. In addition, the observation that Rab5 stimulates the stable association of endosomes with microtubules suggests that this GTPase may not only regulate motor activity but also modulate the activity of microtubule-associated proteins. In this way the multiplicity of effectors recruited by Rab5–GTP[13] would be coordinated to regulate endosomal docking, fusion and motility on microtubules. □

Methods

Antibodies and other reagents.

Human anti-EEA1 serum (1:1,000) was a gift from B.H. Toh; anti-Giantin serum (1:200) was a gift from B. Soennichsen; affinity purified anti-kinesin antibody (MC44) was a gift from M. McNiven; and anti-tubulin antibody (1:250) was a gift from E. Karsenti. Polyclonal anti-Rab5 (ref. 16; 1:1,000), anti-EEA1 (ref. 12; 1:2,000), anti-Rabaptin-5α (ref. 44; 1:1,000), and anti-Rab7 (ref. 16; 1:10) antibodies have been described previously. Monoclonal anti-transferrin receptor (Zymed; 1:1000), and 70.1 (Sigma) antibodies, rhodamine-conjugated transferrin (Molecular Probes) and nocodazole (Molecular Probes) were obtained from commercial sources. Rhodamine-conjugated phalloidin and cytochalasin D were gifts from M. Way.

Construction and expression of EGFP–Rab5.

pEGFP–Rab5 was constructed by PCR amplification of the human Rab5a complementary DNA from pGEM–Myc–Rab5 (ref. 42) using the primers CCCAAGCTTATGGCTAGTCGAGGCGCAACA and AACTGCAGTTAGTTACTACAACACTGATT followed by cloning of the *Hin*dIII–*Pst*I fragment from the PCR product into a pEGFP–C3 expression vector (Clontech). A431 cells were grown to ~80% confluency in 10-cm Petri dishes and transfected with 20–30 μg of plasmid DNA using a calcium-phosphate-based protocol[43]. Stably transfected clonal lines were isolated after incubation in selective growth medium (0.5 μg ml^{-1} G418) for 7–10 days and checked for GFP fluorescence on endosomal structures. Despite the presence of the selectable marker, we observed variable levels of expression of EGFP–Rab5 in these cells.

Immunofluorescence microscopy.

A431 cells stably expressing EGFP–Rab5 were grown on glass coverslips, fixed in 3% paraformaldehyde and processed for immunofluorescence as previously described[44]. Coverslips were mounted in Moviol and examined on a Zeiss confocal microscope (Microsystems LSM-510) using a Zeiss Axioplan2 microscope with 63×/1.40 plan-Apochromat lens. Fluorescence images were collected at 2× zoom using the Zeiss LSM software package, and processed using Adobe Photoshop 5.0.

Time-lapse fluorescence video microscopy of EGFP–Rab5 *in vivo*.

Cells were grown on glass coverslips and were transferred to custom-built aluminium microscope slide chambers (EMBL workshop, Heidelberg) just before observation. Unless otherwise stated, cells expressing average levels of EGFP–Rab5 were selected and analysed on a Zeiss Axioskop microscope using 100×/1.40 plan-Apochromat lens with a temperature-controlled objective sleeve attached (EMBL workshop, Heidelberg). Time-lapse imaging was performed, collecting images at 2-s intervals using a computer-controlled shutter (Uniblitz) with illumination by a 100-W mercury arc lamp attenuated with two heat reflection filters and a KG-1 heat absorbance filter (Zeiss). GFP fluorescence was visualized with Hi-Q FITC, or GFP filter sets (Chroma Technologies), and images were acquired using a COHU 4913 CCIR video camera with on-chip integration controlled by the NIH-Image v1.60 software package.

Microtubule spin-down assay.

Purified early endosomes (75–100 μg) were incubated at room temperature for 20 min with 100 μg HeLa cytosol protein in a reaction brought to a final volume of 50 μl by addition of BRB-80 (80 mM K-PIPES, 1 mM $MgCl_2$, 1 mM EGTA pH6.8). Taxol-stabilized microtubules (10 μl; 100 μg tubulin equivalent) were added and this mix was incubated for 10 min at room temperature, and then overlaid on 600 μl of a 35% (wt/vol.) sucrose cushion. After sedimentation at 100,000*g* for 20 min at 22°C in a TLA 100.4 rotor, the upper layer was removed, the cushion was washed with 100 μl BRB-80 and then also removed. The remaining pellet was resuspended in 30 μl 2× SDS–PAGE buffer plus 30 μl dH_2O, and analysed by SDS–PAGE and immunoblotting.

In vitro endosome motility assays.

Early endosomes and cytosol were isolated as previously described[44], except that HeLa spinner culture cells were allowed to internalize rhodamine-labelled transferrin for 10 min at 37°C in order to label early endosomes. Oregon-green-labelled tubulin was polymerized *in vitro*, and the resulting microtubules were isolated, stabilized with taxol, and perfused into a microscope slide or glass coverslip chamber as previously described[25]. Alternatively, purified centrosomes[45] were allowed to stick to the glass surfaces of the chamber. Microtubule asters were then polymerized *in situ* by 30 min incubation of the chamber at 37°C with fluorescently labelled tubulin (4 mg ml^{-1}) and 1 mM GTP in BRB-80. The chamber was then washed with BRB-80 plus 10 μM taxol to stabilize microtubules and remove non-polymerized tubulin. A mixture of HeLa cytosol (2 mg ml^{-1}), MgATP (200 μM), fluorescently labelled endosomes (3 mg ml^{-1}), purified bovine haemoglobin (3 mg ml^{-1}; Sigma), and an anti-fade system[22] was perfused into the chamber. Images of fluorescently labelled microtubules (FITC filter set) or endosomes (rhodamine filter set) were collected at 2-s intervals using the time-lapse fluorescence video microscope set-up described above.

Analysis and quantification of videos.

Image acquisition and processing and analysis of movies was performed using the NIH Image v1.60 software package. Endosome movements were defined as linear, vectorial motions that occurred on fluorescent microtubules over four or more consecutive images. These were distinguished from tethered brownian motions, which generally displayed nonlinear shaking, or flipped randomly back and forth from image to image. Movements were counted from at least three, and in most cases many more, individual movies, and averaged, and significant population differences were calculated using the Excel spreadsheet program (Microsoft).

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