A novel interaction between Rab7b and actomyosin reveals a dual role in intracellular transport and cell migration

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Running title: Roles of Rab7b-myosin II complex
Abstract

Rab proteins are small GTPases that regulate the transport between the different compartments of the endomembrane system in eukaryotic cells. Here, we show that Rab7b, a Rab that controls the transport between late endosomes and the TGN, interacts directly with myosin II. We illustrate the functional significance of this interaction, demonstrating that myosin II mediates the transport of Rab7b endosomes, as Rab7b dynamics are strongly affected after myosin II depletion or inhibition. We also demonstrate that a member of the Rab family regulates actin remodeling and, consequently, influences cell adhesion, polarization and migration. We find the molecular mechanism by which Rab7b influences stress fiber formation, through controlling the activation status of the small GTPase RhoA, and therefore by influencing myosin light chain phosphorylation.

Our findings reveal a novel role for Rab proteins outside their canonical role in intracellular trafficking, identifying Rab7b as a coordinator of cytoskeletal organization.

Keywords: Rab proteins, Rab7b, endosomes, acto-myosin
Introduction

A complex intracellular compartmentalization is a distinctive feature of eukaryotic cells. To ensure a proper communication through the endomembrane system, the transport between all intracellular compartments has to be tightly controlled. Rab proteins are master regulators of intracellular trafficking, controlling all the steps from the formation of vesicles from a donor membrane, through their detachment, transport, tethering, and fusion with the acceptor compartment (Stenmark, 2009). They are small GTPases that cycle between an inactive GDP-bound and an active GTP-bound form. When activated, they are recruited on membranes, where they bind effector proteins to perform their function (Stenmark, 2009).

More than 60 different Rabs have been identified in humans, and each of them controls a different step of the intracellular transport (Stenmark, 2009). We have recently demonstrated that a new member of this family, Rab7b, regulates the transport from late endosomes to the Trans Golgi Network (TGN), (Progida et al., 2010; Progida et al., 2012). Interestingly, we and other groups have observed that Rab7b expression is associated with monocytic and megakaryocytic differentiation (Yang et al., 2004; He et al., 2011; Berg-Larsen et al., 2013), and that Rab7b is able to modulate Toll-like receptor 4 (TLR4) and Toll-like receptor 9 (TLR9) signaling in macrophages (Wang et al., 2007; Yao et al., 2009). However, not much is known regarding the mechanisms by which Rab7b regulates these processes. To obtain more insight into the molecular mechanisms controlled by this small GTPase, we searched for its interacting partners.

In this study, we identify an actin motor, myosin II, as a Rab7b effector by using yeast two-hybrid screening. We confirm biochemically the interaction between myosin II and Rab7b, further proving that it is direct, and show that inhibition of myosin II motor by siRNAs or chemical inhibitors alters Rab7b dynamics.

Myosin II is an actin-binding protein that contracts and cross-links actin. The ability of myosin II to form filaments and regulate actin cytoskeleton is controlled by the phosphorylation state of its light chains. By regulating the remodeling of the actin cytoskeleton, myosin II controls processes that require cellular reshaping and movement, such as cell adhesion, polarization and migration (Amano et al., 1996; Aguilar-Cuenca et al., 2014). Strikingly, we observe that Rab7b is also important for actin organization. Indeed, Rab7b depletion strongly prevents stress fiber formation and cell adhesion on fibronectin and delays cell migration, indicating a role for Rab7b in controlling actin remodeling.
As Rab7b depletion decreases the fraction of GTP-bound RhoA and phosphorylated myosin light chain (MLC), we propose that Rab7b regulates actomyosin dynamics by modulating RhoA activation and consequently the phosphorylation of MLC.

In conclusion, in this work we report the discovery that Rab7b and myosin II interact directly in order to regulate intracellular traffic, and importantly we provide evidence that a member of the Rab family also can control actin organization.
Results

Rab7b interacts directly with myosin II

We recently identified the small GTPase Rab7b as a regulator of the transport from late endosomes to TGN (Progida et al., 2010; Progida et al., 2012). However, not much is known about the mechanisms used by this small GTPase to perform its function. As Rab proteins require specific effectors to regulate membrane transport (Stenmark, 2009), a yeast two-hybrid screening was performed using Rab7b as bait to find its interacting partners (unpublished data). In this way, the actin motor myosin II was identified as a positive hit.

To confirm the results obtained with the two-hybrid screen, we immunoprecipitated Rab7b in monocyte-derived dendritic cells (MDDCs), which express high levels of endogenous Rab7b (Yang et al., 2004; Progida et al., 2010; Berg-Larsen et al., 2013). Endogenous Rab7b was sufficient to co-immunoprecipitate myosin II (Fig. 1A), thus confirming the authenticity of the interaction.

Next, we transfected HeLa cells with HA-tagged Rab7bwt or the constitutively active mutant (Rab7bQ67L), and we verified that myosin II was co-immunoprecipitated by Rab7b (Fig. 1B). Notably, Rab7bQ67L co-immunoprecipitated significantly more myosin II, compared to Rab7bwt, suggesting the importance of the Rab GTP-bound form for the interaction.

We also investigated whether the interaction was specific for Rab7b, testing the ability of different purified Rabs to pull down myosin II from cell extracts. Therefore, His-tagged Rab7b, His-tagged Rab7a, and His-tagged Rab9 were expressed in bacteria, then purified and incubated with total extracts of HeLa cells. As shown in Fig. 1C, only His-tagged Rab7b was able to pull down myosin II from total cell extracts, thus showing that the interaction is specific for Rab7b.

Subsequently, we checked whether this interaction was direct. Purified bacterially expressed GST-tagged myosin II tail was incubated together with His-tagged Rab7bQ67L, and then GST-tagged myosin II tail was precipitated using glutathione resin. Western blot analysis of the precipitated proteins revealed that His-Rab7b was able to specifically bind to GST-myosin II, and no binding of His-Rab7b to GST protein alone was detected (Fig. 1D).

Altogether, our results demonstrate that Rab7b interacts directly with myosin II.
Rab7b transport is dependent on myosin II

As myosin motors are known to interact and regulate the transport of several Rabs (Seabra and Coudrier, 2004; Miserey-Lenkei et al., 2010; Roland et al., 2011; Lindsay et al., 2013), we next investigated whether the physiological relevance of the Rab7b-myosin II interaction is linked to the regulation of intracellular trafficking. First, we studied the dynamics of GFP-myosin II together with mCherry-Rab7b in live cells. Even though GFP-myosin II is mostly associated with actin filaments, as expected a small fraction is also visible in the proximity of the Golgi (Ikonen et al., 1997) (Fig. 2A, Movie 1A). Interestingly, time-lapse imaging revealed small Rab7b vesicles positive for myosin II, moving toward the Golgi region, suggesting that the functional role of the interaction between Rab7b and myosin II is to mediate Rab7b transport along the cytoskeleton (Fig. 2A, Movie 1A). Quantification of more than 100 Rab7b-positive vesicles in cells transfected with mCherry-Rab7b and GFP-myosin II showed that 69% of the Rab7b vesicles colocalize with myosin II (Fig. 2B).

To test for a potential role of myosin II in Rab7b-transport, we followed by time-lapse video-microscopy the dynamics of GFP-Rab7b in HeLa cells depleted for myosin II (Figs. 2C–E, Movie 2C). In cells knocked down for myosin II, GFP-Rab7b-positive endosomes were strongly clustered in the perinuclear region and significantly increased in size, compared to cells transfected with a control siRNA (Fig. 2C, Movie 2C). These enlarged structures were late endosomes, being positive for the late endosomal marker Lamp-1 (Fig. S1). A similar effect was also observed, although at a minor extent, by knocking down Rab7b (Fig. S1). Interestingly, Rab7b-positive endosomes were also less mobile when cells were silenced using two different siRNAs, targeting different regions of myosin II (Figs. 2C–E, Movie 2C).

These results indicate the importance of myosin II for the proper intracellular distribution and dynamics of Rab7b-positive endosomes.

To further confirm the importance of myosin II in Rab7b transport, we used two different myosin II inhibitors that function with diverse mechanisms: 1) a mix containing ML7 (myosin light chain kinase inhibitor) and Y27632 (Rho kinase inhibitor) or 2) blebbistatin (myosin II motor activity inhibitor). In the presence of the first, we observed a remarkable increase in the size of Rab7b-positive endosomes, and concomitantly, a clustering in the perinuclear region, as in cells depleted for myosin II (Fig. 2F). Moreover, we observed a specific and marked reduction in the velocity of Rab7b endosomes (0.19 µm/s) of almost 50% compared to the untreated cells (0.34 µm/s) (Fig. 3A, Movie 5F). These effects were specific for Rab7b, as the inhibitors did not affect Rab5, Rab7a, or Rab9 (Figs. S2 and 3A, Movies 3F–4F).
In cells treated with the second inhibitor, blebbistatin, this reduction in velocity was even stronger, with a specific decrease of velocity for Rab7b-positive endosomes to 0.08 µm/s, almost 4-fold less than the control (Figs. 3B–C, Movie 7C). This effect was reversible, as washing away the inhibitor restored the initial endosomal speed. The effect was also specific for Rab7b, as Rab5, Rab7a, and Rab9 were not affected (Figs. 3B–C, Movie 6C). The reduction in the velocity of Rab7b endosomes is consistent with an active role of myosin II as a molecular motor that mediates Rab7b movement along the actin cytoskeleton.

In the presence of blebbistatin, we again observed clustering of Rab7b endosomes in the perinuclear region, but no significant differences in the endosomal size. This difference in the effect of the two inhibitors on endosomal size might, however, be explained by the different mechanism of action of the drugs, as ML7 and Y27632, but not blebbistatin, interfere with the myosin II phosphorylation (Wang et al., 2008; Shutova et al., 2012).

To gain more insights into the mechanisms that cause these altered Rab7b phenotypes in the absence of functional myosin II, we examined the actin cytoskeleton in the presence of these drugs. After blebbistatin treatment, a fraction of stress fibers was still visible, while the mix of ML7 and Y27632 completely destroyed actin filaments (Katoh, Kazuo et al., 2001), (Fig. S3). Notably, in the presence of this mix of inhibitors, we observed actin puncta on Rab7b endosomes (Fig. S3). As actin puncta on endosomes are important for carrier formation (Morel et al., 2009), this suggests that the enlarged size of Rab7b endosomes is caused by a defect in fission due to the lack of actomyosin force to efficiently drive membrane scission.

Together, all these observations show that myosin II is required for the correct dynamics of Rab7b-positive endosomes.

**Rab7b regulates stress fiber organization**

As myosin is an actin motor, we depleted HeLa cells for Rab7b by using siRNA and we analyzed the actin cytoskeleton by confocal microscopy (Fig. 4). Strikingly, in Rab7b-depleted cells we observed a significant reduction in the number of stress fibers, together with a simultaneous increase in the number of actin foci. Indeed, only in 38% of the cells silenced for Rab7b was it possible to detect intact actin fibers, while they were present in 78% of the control cells (Fig. 4B). The specificity of the effect was further supported by the finding that
stress fibers recovered in cells first silenced for Rab7b and then transfected with GFP-Rab7b, and the percentage of cells with normal actin fibers went up to 65% (Figs. 4A–B).

As expected, we observed stress fiber disruption also after silencing of myosin II (Figs 4 A, E). Furthermore, by knocking down Rab9, which regulates the late endosome-to-TGN transport similarly to Rab7b, we were still able to detect intact stress fibers in more than 70% of the cells (Fig. 4), indicating that the disruption of stress fibers is a specific consequence of Rab7b depletion.

These results suggest that Rab7b controls actin cytoskeleton organization through the association with myosin II.

**Loss of Rab7b reduces cell spreading and delays wound closure**

Having observed that Rab7b interferes with actin organization, we next investigated whether, by regulating actomyosin organization, Rab7b influences processes that require actin cytoskeleton rearrangements such as cell adhesion and migration.

We therefore measured the ability of the cells to spread out on fibronectin-coated dishes. While the average area of control cells 1 hour after plating was 407 µm² (and 75% of the cells had an area > 200 µm²), Rab7b-depleted cells showed impairment in their ability to spread. Indeed, only 15% had an area > 200 µm², with an average area of 129 µm², meaning 3.4-fold less than the control (Figs. 5A–C).

To validate that the inability of cells knocked down for Rab7b to spread onto fibronectin was due to a specific role of Rab7b, we performed a rescue experiment. The transient expression of GFP-Rab7b on Rab7b knocked-down cells was sufficient to re-induce cell spreading onto fibronectin (63.5% of cells had an area > 200 µm², with a total average area of 249 µm²), confirming the involvement of Rab7b in cell adhesion (Figs. 5A-C).

F-actin staining revealed that cells knocked down for Rab7b had reduced membrane protrusions at the cell periphery in response to adhesion on fibronectin (Fig. 5A). To check whether this effect was the consequence of a slower spreading rate (cells at an earlier stage of spreading are characterized by fewer membrane protrusions), we next used live imaging to follow cell adhesion and spreading (Figs 5D–E, Movie 8D). Interestingly, cells depleted for Rab7b showed not only less membrane ruffling even at later time points, but also a constantly reduced spreading compared to control cells (Figs 5D–E, Movie 8D), indicating that Rab7b is necessary for the correct cell spreading.
As cell adhesion is an important step in the process of cell migration, we performed a wound-healing assay to investigate whether Rab7b depletion also affects cell motility. A confluent monolayer of HeLa cells transfected with a control siRNA or a siRNA against Rab7b was scratched, and the migration of the cells toward the wound area was imaged and quantified. Interestingly, Rab7b-depleted cells were slower compared to control cells, showing a 40% reduction in the average single-cell speed (Figs. 6A–C, Movie 9A).

We confirmed that the delayed cell migration was a consequence of Rab7b depletion on actin cytoskeleton dynamics, as we could observe in parallel a significant decrease of filamentous actin structures (lamellipodia) at the leading edge after staining migrating cells for F-actin. Indeed, while almost 60% of control cells extended broad lamellipodia at the leading edge 2 hours after the wound, only 32% of the Rab7b-depleted cells showed membrane protrusions into the open wound area (Figs. 6D–E).

To further test whether Rab7b knock down also interferes with cell polarization, we measured the cell polarity during migration, based on the orientation of the Golgi apparatus into the direction of the wound. When cells polarize for migration, the Golgi re-orientates from a random perinuclear position to the third of the cell area that occupies the space between the nucleus and the wound edge area (Kupfer et al., 1982; Bisel et al., 2008). The Golgi re-orientation was strongly affected in Rab7b-depleted cells, where only 44% of the cells showed an orientation toward the wound edge area, compared to control cells, where 68% of the cells responded positively to Golgi reorientation (Figs. 6D–E). This, along with the results of decreased cell migration in Rab7b-depleted cells, indicates that Rab7b plays a role in cell polarization in response to a wound.

All together, these data show that, by acting on actin cytoskeleton, Rab7b is important for the establishment of cell polarity to induce cell adhesion and migration.

**Rab7b regulates actin organization by controlling myosin phosphorylation**

Stress fiber formation, cell adhesion, polarization, and migration are all processes where the reorganization of the actin cytoskeleton and actomyosin contractility is required (Naumanen et al., 2008; Parsons et al., 2010; Rottner and Stradal, 2011). As we demonstrated that these cellular functions are all affected by Rab7b depletion, and they are dependent on MLC phosphorylation (Watanabe et al., 2007; Hirata et al., 2009), we next investigated whether Rab7b knock down altered MLC phosphorylation status. Ser19 is the primary
phosphorylation site of MLC, and phosphorylation of MLC at Ser19 increases both the actin-activated Mg-ATPase activity and the stability of myosin II filaments (Amano et al., 1996; Bresnick, 1999). Using an antibody that specifically recognizes phosphorylated MLC on Ser19, we found that MLC phosphorylation was strongly downregulated in Rab7b-depleted cells, whereas the levels of total MLC remained unchanged (Figs. 7A–B). As expected, we observed the same effect after myosin II depletion, using siRNAs targeting the myosin heavy chain (Fig. S4A–B).

MLC phosphorylation is regulated by several kinases, with ROCK (Rho kinase) as one the most important (Amano et al., 1996; Katoh, K. et al., 2001). ROCK is a direct target of RhoA, a small GTPase that works as a molecular switch cycling between an inactive GDP-bound and an active GTP-bound form (Matsui et al., 1996). In its active state, RhoA stimulates the formation of stress fibers and cell migration through effectors such as ROCK (Amano et al., 1997; Riento and Ridley, 2003; Worthylake and Burridge, 2003). The Rho GTPase and its downstream targets are therefore responsible, by phosphorylating MLC, for controlling these cytoskeleton dynamics.

To determine whether Rab7b influences MLC phosphorylation by acting on the RhoA–ROCK pathway, we measured RhoA activity by a pull-down assay. Lysates from control cells or Rab7b-depleted cells were incubated with glutathione S-transferase (GST)–Rhotekin–Rho-binding domain (RBD) bound to glutathione agarose beads, and affinity-precipitated proteins were probed for RhoA-GTP by immunoblot analysis. As shown in Figs. 7C–D, the activity of RhoA was strongly decreased (of more than 60%) in Rab7b-depleted cells. Depletion of myosin II instead decreased RhoA activity of maximum 30% (Fig. S4C–D).

In conclusion, our results reveal that, by interfering with RhoA activation, Rab7b can control the phosphorylation of MLC and thereby actin cytoskeleton organization (Fig. 8).
Discussion

We have recently demonstrated that the small GTPase Rab7b regulates the endosome-to-TGN transport (Progida et al., 2010; Progida et al., 2012). Depletion of Rab7b or the expression of the dominant-negative or constitutively active mutants disturbs this transport pathway (Progida et al., 2010; Progida et al., 2012). However, little is known about the function of this Rab or its interaction partners.

In an attempt to identify Rab7b interactors, we discovered myosin II as a direct effector. Rab proteins, in their active state, bind effectors, and several Rabs are known to bind cytoskeletal motors that ensure their directional transport (Seabra and Coudrier, 2004; Horgan and McCaffrey, 2011). In agreement with this, here we provide evidence that myosin II behaves as an effector that regulates Rab7b dynamics. Indeed, we show that, by interfering with this molecular motor by either siRNA transfection or chemical inhibitors, myosin II is important for both Rab7b-endosomal fission (as its inhibition causes enlargement of Rab7b endosomes) and movement (visualized by a block of Rab7b endosome motility).

In particular, after destroying actin stress fibers by inhibition of MLC phosphorylation, we observed enlarged endosomes with actin puncta on the membrane (Fig. S3). This suggests that myosin II anchors Rab7b-positive membranes onto the actin cytoskeleton and that the phosphorylation inhibition prevents the actin polymerization necessary to generate the force needed to drive membrane fission.

It is known that actomyosin filament contraction is involved in carrier scission and that the presence of actin patches on endosomal membranes is important for driving the biogenesis of transport intermediate (Morel et al., 2009; Anitei and Hoflack, 2012). Thus, our data indicate that the interaction between Rab7b and myosin II is important for both the fission and the transport of Rab7b endosomes.

The Rab family of proteins belongs to the Ras superfamily of small GTPases. This superfamily consists of five main families, each of them characterized by a different function despite a conserved structure and similar biochemical proprieties (Goitre et al., 2014). Among them, the Rho family members are key regulators of actin cytoskeleton and cell migration, while the Arf and Rab families are important regulators of the intracellular transport (Stenmark, 2009; Donaldson and Jackson, 2011; Hall, 2012). Interestingly, an additional function for the Arf family has been recently identified. Indeed, members of this family are
also regulators of actin remodeling and cell migration (Myers and Casanova, 2008; Lewis-Saravalli et al., 2013).

Here, we have shown that, similar to Arfs, also a member of the Rab family of small GTPases, Rab7b, by interacting with myosin II, is able to control not only intracellular trafficking but also actin cytoskeleton dynamics. Specifically, silencing of Rab7b by RNAi reduces stress fiber formation, cell adhesion, and cell migration. In addition, formation of lamellipodia at the leading edge is attenuated in migrating Rab7b-depleted cells.

In line with the requirement of actin contractility for the Golgi positioning during cell migration (Gomes et al., 2005), a decrease in the reorientation of the Golgi toward the wound was observed in cells knocked down for Rab7b (Fig. 6D–E). However, as Rab7b and myosin II are involved in a plethora of cellular functions, we cannot exclude that the effects on cell spreading and migration could also be the outcome of a more complex signaling network.

As the processes dependent on actomyosin contractility are regulated by MLC phosphorylation, we investigated whether Rab7b, by interacting with myosin II, influences this phosphorylation. Importantly, the levels of phosphorylated MLC were strongly downregulated in Rab7b-depleted cells (Fig. 7A–B), indicating that the mechanism used by Rab7b to regulate actin cytoskeleton remodeling is through the control of MLC phosphorylation.

ROCK is one of the main kinases that regulate MLC phosphorylation, and its inhibition results in phenotypes similar to those observed upon Rab7b depletion, such as disrupted stress fibers and reduced cell adhesion (Amano et al., 1997; Katoh et al., 2001). ROCK is activated by another member of the Ras superfamily of small GTPases, RhoA. To activate the kinase, RhoA has to be in an active, GTP-bound state (Matsui et al., 1996). Strikingly, the fraction of GTP-bound RhoA was strongly reduced upon Rab7b depletion (Fig. 7C–D). This suggests that Rab7b regulates RhoA, and explains the decrease in MLC phosphorylation in Rab7b-depleted cells. In sum, our results support a role for Rab7b in the regulation of actin cytoskeleton organization by mediating MLC phosphorylation through RhoA activation (Fig. 8).

In line with our data, a very recent report suggests that Rab7b may associate with calpain-myosin in platelets, small cytoplasmic fragments where the actin cytoskeleton remodeling is crucial (Tsai et al., 2014). We now unequivocally demonstrate that Rab7b directly interacts with myosin II, and we characterize the functional role of this interaction in both intracellular trafficking and actin cytoskeleton organization. We show that Rab7b triggers myosin II
activation by stimulating phosphorylation of Ser19 of MLC, through the activation of RhoA, revealing that a Rab protein modulates the activity of another family of small GTP-binding proteins, the Rho family. Interestingly, other small GTPases, Arf1 and Arf6, known to regulate endosomal transport (Kobayashi and Fukuda, 2013; Nakai et al., 2013; Hongu and Kanaho, 2014), also control cytoskeletal organization through crosstalk with Rho family GTPases (Lewis-Saravalli et al., 2013; Schlienger et al., 2014). Although the exact mechanism of Rho activation remains to be elucidated, it could occur on several ways, such as the recruitment of GEFs or the downregulation of Rho GAPs.

Other Rabs that interact with members of the myosin family also interfere with the process of cell migration (Seabra and Coudrier, 2004; Allaire et al., 2013). However, their effects on cell migration have so far been investigated only in connection to the well-established role of Rabs in intracellular trafficking (Kawauchi et al., 2010; Mai et al., 2011; Linford et al., 2012; Allaire et al., 2013; Wiesner et al., 2013).

By illuminating the role of a Rab protein in controlling actin remodeling, our results also open new possibilities for those Rabs that have been found to have a role in cell migration.

In conclusion, by demonstrating that Rab7b interacts directly with myosin II to regulate actin organization and endosomal dynamics, we propose the novel mechanism by which a Rab protein controls both intracellular trafficking and cytoskeleton remodeling, revealing an unexpected role for this protein family.
Materials and methods

Cell culture
HeLa cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin.
Mononuclear cells were isolated from buffy-coats from healthy blood donors through density gradient centrifugation by using Lymphoprep (Axis Shield). Blood components (buffy-coats) from anonymous blood donors were obtained from the local blood bank (Section for Immunology and Blood Transfusion, Ullevål University Hospital, Oslo, Norway) according to the guidelines of the local blood bank approved by the Norwegian Regional Committee for Medical Research Ethics. Monocyte-derived dendritic cells (MDDCs) were generated from plastic adherent or directly isolated monocytes (Monocyte Isolation Kit II, Miltenyi Biotec) by culture for 6 days in RPMI media containing 100 ng/ml GM-CSF (Immunotools) and 20 ng/ml IL-4 (Invitrogen, Life Technologies) supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. GM-CSF and IL-4 were replenished every 2–3 days.

Constructs, antibodies and inhibitors
pEGFP-Rab7bwt, pcDNA-2XHA-Rab7bwt, pcDNA-2XHA-HA-Rab7bQ67L, pcDNA-mCherry-Rab7b, pET16b His-Rab7a, pET16b His-Rab7b, pET16b His-Rab7bQ67L, and pET16b His-Rab9 have been described before (Spinosa et al., 2008; Progida et al., 2010; Progida et al., 2012). pEGFP-myosin II A was obtained from Robert Adelstein through Addgene (plasmid #11347) (Wei and Adelstein, 2000). pEGFP-Rab5 and pEGFP-Rab7a were a gift from Cecilia Bucci (Bucci et al., 2000). pEGFP Rab9 was constructed by amplifying Rab9 by PCR using the following primers containing respectively a BglII and a PstI restriction site: Rab9-for 5’-AGAGAGATTCTATGGCAGGAAAATCTTCATTT-3’ and Rab9-rev 5’-AGAGACTGCAGTCAACAGCAAGATGAGCTAGGCT-3’. The fragment was then inserted into pEGFP-C1 cut with BglII and PstI. For the construction of pGBK7T7 Rab7bΔC, where the two C-terminal cysteine residues were deleted to avoid post-translational prenylation, the coding sequence of Rab7b was amplified by PCR using the following primers containing respectively an EcoRI and a SalI restriction site: Rab7b-for 5’-AGAGAGATTCTATGGCAGGAAAATCTTCATTT-3’ and Rab7b-rev 5’-
AGAGAGACGTCGACTCTGCTCCTTGACTGGTCTG-3’. Rab7bΔC fragment was then cloned into pGBK7 plasmid cut with EcoRI and SalI.

Myosin II A heavy chain tail (aa 1795–1960) was amplified by PCR using the following oligonucleotides: 5’-AGAGAGGATCCCAGGAGATGGAGGGCACTGTC-3’ and 5’-TTCTCTGAATTCTTTATTCGGCAGGTTTGGCCTCA-3’, and cloned in frame in the pGEX2T vector using BamHI–EcoRI restriction sites to obtain a plasmid for the bacterial expression of GST-myosin II tail. All the newly made constructs were sequence verified.

Primary antibodies used in this study were anti-HA (1:1500, catalogue number ab9110), anti-giantin (1:1000, catalogue number ab24586), and anti-non-muscle myosin IIA (1:3000, catalogue number ab24762) from Abcam; anti-GST (1:1000, catalogue number G1160), anti-MLC (1:40, catalogue number M4401), and anti-vinculin (1:100, catalogue number V9131) from Sigma-Aldrich; anti-phospho-myosin light chain-Ser19 (1:300, catalogue number 3671, Cell Signaling Technology), Anti-RhoA (1:500, catalogue number ARH03, Cytoskeleton), anti-Rab7b (1:300, catalogue number H00338382-M01, AbNova), anti-His (1:5000, catalogue number MCA1396, AbD Serotec), and anti-tubulin (1:10000, catalogue number 138000, Life Technologies). Mouse IgG2aκ was purchased from BD Pharmingen (catalogue number 555571). Secondary antibodies conjugated with Alexa Fluor 488 or 647 (Life Technologies) were used at dilution 1:200 for immunofluorescence. Secondary antibodies conjugated with horseradish peroxidase (GE Healthcare) were used at 1:5000 for immunoblotting.

ML7 and Y27632 were purchased from Calbiochem, and blebbistatin from Sigma-Aldrich. For the experiments, 30 µM ML7 and 10 µM Y27632, or 25 µM blebbistatin, was added to the cell medium for 40 minutes at 37°C with 5% CO₂.

Transfection and RNA interference

Cells were transiently transfected at 50–70% confluency using FuGENE 6 (ProMega) according to the protocol provided by the manufacturer. The cells were analyzed 24 hours post transfection.

Transfection of HeLa cells with siRNA was performed as described (Progida et al., 2007). Briefly, HeLa cells were plated 1 day before transfection in 6 cm dishes (~4 x 10⁵ cells/dish). Cells were transfected with siRNA using Oligofectamine (Invitrogen) for 72 hours, replated, and left for another 48 hours before further experiments were carried out.
For RNA interference (RNAi) we used the following oligonucleotides: Rab7b siRNA, sense sequence 5’-GUAGCUCAAGGCUGGUATT-3’, and antisense sequence 5’-UACACCAGCCUUAGCUACTT-3’. For Myosin II siRNA 1, sense sequence 5’-GAACAGCAUGGCAAGTTT-3’, and antisense sequence 5’-CAUUGCCAUUGCUUGUUCTT-3’. For Myosin II siRNA 2, sense sequence 5’-CAAGGACUUAAGGCUAAGTT-3’, and antisense sequence 5’-CUUGACCUUAAGCUCCUGTT-3’. For Rab9 siRNA, sense sequence 5’-GUUUGAUACCCAGCUUUCTT-3’, and antisense sequence 5’-GAAGAGCUGGGUAUCCACTT-3’.

As a negative control we used a scrambled sequence: control siRNA, sense sequence 5’-ACUUCGAGCGUGCAUGGUTT-3’, and antisense sequence 5’-AGCCAUGACGCUCGAAGUTT-3’.

All chemically synthesized oligonucleotides were purchased from a commercial supplier (Eurofins MWG Operon).

RNA procedures and quantitative Real-Time RT-PCR

Total RNA was extracted from HeLa cells by using the PerfectPure RNA Cell Kit (5Prime), according to the manufacturer’s instructions.

cDNA was synthesized with use of the First Strand cDNA Synthesis Kit (Roche). 1 µg of cytosolic RNA from cells and 60 µM Random Hexamer Primers were mixed and heated at 65° C for 10 minutes before immediate cooling on ice. First strand cDNA synthesis was then carried out with 10 U Reverse Transcriptase, 20 U Ribonuclease inhibitor, and 1 mM dNTPs for 10 minutes at 25° C, followed by 30 minutes at 55° C. The reactions were stopped by heat inactivation at 85° C for 5 minutes.

Following each reverse transcription, cDNA was amplified and quantified by Real Time RT-PCR, using LightCycler 480 SYBR Green I Master mix and LightCycler 480 Real-Time PCR system (Roche). Primers for Rab7b (forward primer, 5’-GGCCAGCATCTCCTCAAGATTAC-3’, and reverse primer, 5’-GATGCAGCCATCGGAGCCCTGT-3’) and for human actin (forward primer, 5’-CTGACTGACTACCTCATGAAGATCCT -3’, and reverse primer, 5’-CTTAATGTCACCGACAGTATTCC -3’) were purchased from Eurofins MWG Operon.

The PCR program was as follows: 1 cycle 3 minutes at 94° C; 35 cycles 30 seconds at 94° C, 30 seconds at 60° C, and 30 seconds at 72° C; 1 cycle 6 seconds at 75° C. The specificity and
the identity of the PCR product was checked by performing a melting curve test. Actin transcript levels were used for the normalization of the samples.

**Yeast two-hybrid screening**

A yeast two-hybrid screen of a human cDNA library from hearth using human Rab7bΔC cloned in pGBKT7 as a bait was performed by DFKZ (German Cancer Research Center).

**Co-immunoprecipitation and western blot experiments**

For immunoprecipitation, Dynabeads Protein G (Invitrogen) were used according to the manufacturer's instructions. Briefly, 0.6 µg Dynabeads were washed with RIPA buffer and incubated with anti-Rab7b or IgG2aK (negative control) for 60 minutes at room temperature, with end-over-end rotation. After washing, precleared cell lysates were added to the Dynabeads and incubated for 90 additional minutes with end-over-end rotation. Immunoprecipitated samples were loaded on SDS-PAGE and analyzed by western blotting.

For western blot experiments, proteins were separated on SDS-PAGE, blotted onto a Immobilon Polyvinylidene fluoride (PVDF) membrane (Millipore) and probed with the specific primary antibodies diluted in 2% blotting grade non-fat dry milk (Bio-Rad) followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000, GE Healthcare). Bands were visualized using the ECL system (GE Healthcare). Protein levels were quantified by densitometry using ImageQuant TL software (GE Healthcare).

**Protein purification, pull-down, and direct interaction experiments**

GST, GST-myosin II, and His-tagged Rabs were expressed in *E.coli* BL21 (DE3) (Agilent Technologies) after induction with 0.5 mM IPTG for 4 hours at 22° C. Expressed His-Rabs fusion proteins were purified from bacterial soluble fraction using His-tagged isolation Dynabeads (Invitrogen) in the presence of 50 mM Na-phosphate, pH 8, 300 mM NaCl, and 0.01% Tween 20, according to the manufacturer’s protocol. To activate Rab GTPases, purified His-tagged Rabs bound to Dynabeads were loaded with 0.1 mM GTPγS.

Purification of GST and GST-myosin II was performed according to the manufacturer’s protocol (GE Healthcare).

For pull-down experiments, 20 µg of GTPγS-His-Rab fusion proteins bound to Dynabeads were incubated with precleared HeLa cell lysates for 30 minutes at 4° C and then washed six times with buffer containing 3.25 mM Na-phosphate, pH 7.4, 70 mM NaCl, and 0.01%
Tween 20. Bound proteins were eluted with elution buffer (50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 0.01% Tween 20, 300 mM imidazole). Samples were analyzed by SDS–PAGE and immunoblotting.

For testing the direct interaction, His-Rab7bQ67L was incubated with GST or GST-myosin II in PBS with 2 mM MgCl2 and GTP 0.8 mM for 1 hour at 4° C on a rotating wheel. Subsequently, pull-down was performed using a glutathione resin. Samples were then subjected to SDS-PAGE and western blotting.

**RhoA activity assay**

RhoA activity was assessed by a pull-down assay according to the manufacturer’s instructions for a RhoA Activation Assay Biochem Kit (Cytoskeleton). Briefly, GTP-bound RhoA was immunoprecipitated from cell lysates with GST-Rhotekin-RBD bound to glutathione-agarose beads. After washing, the beads were subjected to western blot analysis using anti-RhoA antibody to detect GTP-bound RhoA. Total proteins were detected by immunoblotting of whole cell lysates.

**Cell spreading assay**

Control cells and Rab7b-depleted cells (5x10^4 cells/ml) were seeded onto fibronectin-coated coverslips (10 µg/ml) and fixed 1 hour later. After staining with rhodamine-conjugated phalloidin, coverslips were mounted and examined using an Olympus FluoView FV1000 microscope. For real-time imaging of cell spreading, 5x10^4 cells/ml were seeded onto 10 µg/ml fibronectin-coated MatTek glass-bottom dishes and imaged every 15 minutes at 37° C and 5% CO2. Live cells were imaged with a 40x PlanApo NA 0.90 objective on an Olympus Fluoview 1000 IX-81 inverted confocal laser scanning microscope. The cell area was determined by using ImageJ software (NIH).

**Cell migration assay**

Wound-healing assays were performed by scratching confluent monolayers of HeLa cells with a pipette tip. Cells were imaged for a period of 24 hours with a 10× PlanApo NA 0.75 objective on a Scan®R imaging platform (Olympus) equipped with a Hamamatsu C8484-05G camera. Images were acquired every 3 hours, using the Olympus Scan®R software. Cells were maintained in DMEM without phenol red at 37° C and 5% CO2 throughout the observation.
period. The velocity of migration was determined by using the Manual Tracking plugin of Image J software (NIH) and Chemotaxis and Migration Tool software (Ibidi).

**Golgi reorientation measurements**

A confluent monolayer of cells that had been cultured on glass slides was scratched with a pipette tip and incubated for 4 hours at 37°C and 5% CO2. Cells were fixed and stained with rhodamine-conjugated phalloidin, Hoechst and anti-giantin antibody to visualize the actin cytoskeleton, the nuclei, and Golgi respectively. To measure Golgi reorientation, cells on the wound edge were equally divided into three sectors. The Golgi in the front sector between the nucleus and the leading edge was determined to be in the polarized position.

**Immunofluorescence and live cell microscopy**

HeLa cells grown on coverslips were fixed with 3% paraformaldehyde, permeabilized with 0.25% saponin in PBS and incubated for 20 minutes at room temperature with primary antibodies. After washing with 0.25% saponin, coverslips were incubated with the appropriate secondary antibody for 20 minutes in the dark at room temperature. Mounted coverslips were examined by using a 63× PlanApo NA 1.42 objective on an Olympus FluoView FV1000 microscope with the FV1000 software (version 1.7a).

For live imaging experiments, HeLa cells grown on MatTek glass-bottom dishes were washed and incubated in DMEM without phenol red. Live cells were imaged with a 63× PlanApo NA 1.42 objective on an Olympus IX-71 microscope equipped with a CSU22 spinning-disk confocal unit (Yokogawa), an Ixon EMCCD camera (Andor) and the Andor iQ1.8 software. During imaging, cells were kept at 37°C and 5% CO2 in an incubation chamber (Solent Scientific).

**Image analysis and processing**

Image analysis and processing was carried out with Adobe Photoshop (Adobe Systems Inc.) and ImageJ (NIH).

Cell tracking was conducted using the Manual Tracking plugin of ImageJ software (NIH). The speed of each migrating cell was calculated on the basis of the migration track by using the Chemotaxis and Migration Tool software (Ibidi GmbH). During each experiment, 5 videos were acquired for each condition, and a minimum number of 50 cells were analyzed per condition.
Object-based colocalization analysis was performed using ImageJ software to quantify the degree of colocalization between Rab7b vesicles and myosin II. Specifically, the number of Rab7b positive vesicles with an overlapping signal from myosin II was measured with respect to the total number of Rab7b positive vesicles within each cell.

Tracking of individual endosomes and quantification of velocity was performed with ImageJ Software, using the Manual Tracking plugin. For each experiment, at least 30 different endosomes for each Rab were tracked manually for 1 minute, and the mean velocity was determined by the software.

Imaris Software (Bitplane AG) was used to analyze the length of endosomal movement during blebbistatin experiments. A representative area with regard to size and number of endosomes was chosen for each cell, and endosomal movement was tracked by the software for a 3-minute interval.

Statistical Analysis.

Statistical differences were assessed with the one-tailed unpaired Student's t test (GraphPad Prism4 software). In the figures, statistical significance is indicated as follows: *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$. Unless otherwise stated, data are from assays performed in triplicate, with error bars representing standard deviations or standard errors of the mean.

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Author Contributions

C.P. conceived the project and designed experiments. M.B. and C.P. performed experiments and analyzed data. O.B. and C.P. supervised the project. M.B., O.B., and C.P. wrote the manuscript. The authors have no conflicting financial interests.


Figure Legends

Figure 1. Rab7b interacts with myosin II.
A Lysates of monocyte-derived dendritic cells (MDDCs) were subjected to immunoprecipitation with an anti-Rab7b antibody or an isotype control (IgG2aK). Whole cell lysates (WCL) and immunoprecipitates (IP) were subjected to western blot analysis using anti-myosin II, anti-tubulin or anti-Rab7b specific antibodies.
B Lysates of control HeLa cells (NT) or cells expressing HA-Rab7bwt or HA-Rab7bQ67L were subjected to immunoprecipitation with an anti-Rab7b antibody or an isotype control (IgG2aK). Whole cell lysates (WCL) and immunoprecipitates (IP) were subjected to western blot analysis using anti-myosin II, anti-tubulin or anti-HA specific antibodies.
C Upper panel: Coomassie blue staining of bacterially expressed His-Rab7a, His-Rab7b and His-Rab9 purified using cobalt-coated dynabeads. Lower panel: Bacterially expressed and purified His-Rab7a, His-Rab7b or HisRab9 were loaded with GTPγS, and incubated with lysates from HeLa cells. Proteins were pulled down using cobalt-coated dynabeads and subjected to western blot (WB) analysis using anti-His and anti-myosin II antibodies.
D Left panel: Coomassie blue staining of bacterially expressed GST and GST-myosin II tail purified using glutathione resin. Right panel: Purified GST or GST-myosin II tail was incubated with purified His-Rab7bQ67L. Samples were subjected to affinity chromatography followed by western blot analysis using anti-GST and anti-Rab7b specific antibodies.

Figure 2. Myosin II regulates the transport of Rab7b-positive endosomes.
A HeLa cells co-transfected with GFP-myosin II and mCherry-Rab7b were imaged with a Spinning Disk confocal microscope for the indicated time points. The arrow indicates a vesicle positive for both GFP-myosin II and mCherry-Rab7b moving toward the Golgi. Scale bar, 10 µm. See also Movie 1A.
B Quantification of the overlap between Rab7b and myosin II. HeLa cells were transfected with GFP-myosin II and mCherry-Rab7b. The percentage of Rab7b vesicles positive for myosin II was calculated by using an object-based colocalization analysis with ImageJ software. Total number of objects counted (n) is shown for ten different cells.
C HeLa cells treated with either control siRNA or two different siRNAs against myosin II, and transfected with GFP-Rab7b, were imaged with a Spinning Disk confocal microscope. Magnifications of the boxed areas are shown in the respective lower insets. See also Movie 2C.

D HeLa cells treated with either control siRNA or two different siRNAs against myosin II were subjected to western blot analysis with anti-myosin II and anti-tubulin as a loading control.

E Quantification of myosin II expression. The intensities of the bands were quantified by densitometry, normalized against the amount of tubulin, and plotted relative to the intensities obtained in cells transfected with control siRNA. The values represent the mean ± s.d. of 5 independent experiments.

F HeLa cells transfected with GFP-Rab7b were imaged using a Spinning Disk confocal microscope before addition of the inhibitory mix ML7+Y27632 (no drugs), 40 minutes after addition of 30 µM ML7 and 10 µM Y27632, and after washing away the inhibitory drugs (wash). Scale bar, 10 µM. Magnifications of the boxed areas are shown in the respective lower insets. See also Movies 3F, 4F, and 5F.

Figure 3. Inhibition of myosin II specifically slows down Rab7b endosomes.

A-C HeLa cells were transfected with GFP-Rab5, GFP-Rab7a, GFP-Rab9, or GFP-Rab7b, imaged with a Spinning Disk confocal microscope, and the velocity of single endosomes was quantified using the manual tracking plugin of the program ImageJ. The graphs represent the quantification of the mean velocity of single endosomes (A) both before addition (no drug) and after addition of the inhibitory mix ML7+Y27632, and (B) before addition (no drug), after addition of blebbistatin, and after wash. At each of these conditions, a minimum of 30 endosomes (from 3 independent experiments) were measured over time. Error bars indicate s.e.m. *P < 0.05; **P < 0.01. (C) HeLa cells were transfected with GFP-Rab5, GFP-Rab7a, GFP-Rab9, or GFP-Rab7b, and treated with blebbistatin (25 µM) for 40 minutes at 37° C, before imaging with a Spinning Disk confocal microscope. In the lower insets, magnifications of the respective boxed areas are shown. Red arrows indicate endosomal movement tracked with IMARIS software over 3 minutes. Scale bars, 10 µM. See also Movies 6C–7C.
Figure 4. Rab7b regulates stress fibers formation.

A HeLa cells transfected with control siRNA, siRNA against Rab7b, Rab9, myosin II, or depleted for Rab7b and transfected afterward with GFP-Rab7b were fixed and stained as indicated. The images represent maximum-intensity projections of Z stacks. Magnifications of the boxed areas are shown in the insets. Scale bar, 10 µm.

B Quantification of percentage of cells with intact stress fibers. Data represents the mean ± s.e.m. of 3 independent experiments (n > 100). *P < 0.05; ***P < 0.001.

C Quantification of Rab7b transcript levels after silencing. Rab7b mRNA levels were quantified by real-time RT-PCR in HeLa cells transfected with control siRNA or siRNA targeted toward Rab7b. The level of Rab7b mRNA was normalized to the amount of actin, and plotted relative to the levels of Rab7b mRNA in the control sample. Data represents the average of 10 independent experiments ± s.d. ***P < 0.001.

D HeLa cells treated with either control siRNA or siRNA against Rab9 were subjected to western blot analysis with anti-Rab9 and anti-tubulin as a loading control.

E HeLa cells treated with either control siRNA or siRNA against myosin II were subjected to western blot analysis with anti-myosin II and anti-tubulin as a loading control.

Figure 5. Depletion of Rab7b suppresses cell spreading.

A HeLa cells transfected with control siRNA, siRNA against Rab7b, or depleted for Rab7b and transfected afterward with GFP-Rab7b were plated on fibronectin-coated coverslips and left to adhere for 1 hour. Samples were subsequently fixed and stained with rhodamine-conjugated phalloidin. Scale bars, 10 µm.

B Quantification of the average cell area (in µm²) of HeLa cells transfected with control siRNA, siRNA against Rab7b, or depleted for Rab7b and transfected afterward with GFP-Rab7b after 1 hour of adhesion on fibronectin. Data represents the mean ± s.d. of 3 independent experiments (n > 100). **P < 0.01; ***P < 0.001.

C The graph shows the percentage of full spread cells (area > 200 µm²) 1 hour after plating on fibronectin-coated coverslips. Data represents the mean ± s.d. of 3 independent experiments (n ≥ 100). ***P < 0.001.

D Control cells and Rab7b-depleted cells were plated on fibronectin-coated dishes, and cell spreading was imaged every 15 minutes. Scale bars, 10 µm. See also Movie 8D.
Quantification of the average cell area (in $\mu m^2$) of HeLa cells transfected with control siRNA or siRNA against Rab7b during adhesion on fibronectin-coated dishes at the indicated time points. Data represents the mean ± s.e.m. of 3 independent experiments ($n > 100$).

**Figure 6: Rab7b silencing delays cell migration and polarization in response to wound.**

A  Wound healing assay of HeLa cells transfected with control siRNA or siRNA against Rab7b at time 0 (0 hours) and after 9 hours. Scale bar, 20 µm.

B  Quantification of the migration speed of control cells and Rab7b-depleted cells in wound healing assays. Data represents the mean ± s.d. of 3 independent experiments ($n \geq 100$). ***$P < 0.001$.

C  Representative track plots of the distances of migration of control cells (left plot) or Rab7b-depleted cells (right plot) in a wound healing assay. The movement of single cells into the wound was followed using a manual tracking software. Individual tracks are shown so that each starts at the origin (0).

D  Confluent monolayers of HeLa cells transfected with control siRNA or siRNA against Rab7b were scratched with a pipette tip and fixed 2 hours later. Cells were immunostained with anti-giantin antibody; actin was labeled with rhodamine-conjugated phalloidin and nuclei with Hoechst. White lines indicate the wound. The Golgi is labeled as (+) if its majority lies within a 120° angle facing the wound, or as (-) if not. Scale bar, 10 µm.

E  Quantification of the percentage of cells with Golgi located in the 120° angle facing the wound. The graph shows the mean ± s.d. of 4 independent experiments ($n \geq 100$). ***$P < 0.001$.

F  Quantification of the percentage of the cells emanating lamellipodia into the wound area. The graph shows the mean ± s.d. of 4 independent experiments ($n \geq 100$). ***$P < 0.001$.

**Figure 7. Rab7b regulates MLC phosphorylation by interfering with RhoA activity.**

A  Lysates from HeLa cells transfected with either control siRNA or siRNA against Rab7b were subjected to western blot analysis using antibodies against phosphorylated myosin light chain (P-MLC), total MLC and tubulin (as a loading control).
B Quantification of P-MLC levels in control and Rab7b-depleted cells. The intensities of the bands from the western blot were normalized to the amount of tubulin and plotted relative to the intensities of P-MLC in the control siRNA sample. Quantifications are based on three independent experiments and were determined by using ImageQuant Software (Amersham). Error bars represent ± s.e.m.

C Lysates from HeLa cells transfected with either control siRNA or siRNA against Rab7b were mixed with GST–Rhotekin–RBD bound to glutathion-agarose beads to precipitate the active form (GTP-bound) of RhoA. As a positive control, cells were loaded with GTPγS. The immunoprecipitate samples were subjected to western blot analysis using antibodies against RhoA and tubulin (loading control).

D Quantification of the levels of active RhoA in control and Rab7b-depleted cells. Intensities of the bands from the western blot were quantified with ImageQuant, normalized to the amount of tubulin and plotted relative to the intensities of GTP-bound RhoA in the control sample. The values represent the mean ± s.e.m. of 5 independent experiments.

Figure 8. Model of Rab7b-myosin II regulation of actin remodeling.
Rab7b regulates MLC phosphorylation by modulating RhoA activity, and therefore actin remodeling, including the formation of stress fibers and the actin cytoskeleton reorganization necessary for processes such as cell adhesion and migration.