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Rab11 activity and PtdIns(3)*P* turnover removes recycling cargo from endosomes

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Abstract

Directional transport of recycling cargo from early endosomes (EE) to endocytic recycling compartment (ERC) relies on Phosphatidylinositol 3-phosphate (PtdIns(3)*P*) hydrolysis and activation of the small GTPase Rab11. However, how these events are coordinated is yet unclear. By using a novel genetically-encoded FRET biosensor for Rab11, we report that generation of endosomal PtdIns(3)*P* by the clathrin binding phosphoinositide 3-kinase Class 2 alpha (PI3K-C2α) controls the activation of Rab11. Active Rab11, in turn, prompts the recruitment of the phosphatidylinositol 3-phosphatase myotubularin 1 (MTM1), eventually enabling the release of recycling cargo from the EE and its delivery towards the ERC. Our findings thus define that delivery of recycling cargo towards the ERC requires spatial and sequential coupling of Rab11 activity with PtdIns(3)*P*-turnover.

Introduction

Intracellular trafficking of endocytosed molecules ensures the delivery of plasma membrane components and receptor-associated ligands to several cellular compartments. After internalization, such molecules can be either degraded or re-used by returning to the plasma membrane [1]. This recycling pathway restores the composition of plasma membrane and is mediated by vesicular carriers that transfer endocytosed material from peripherally located early endosomes (PE) to the endocytic recycling compartment (ERC), a juxtanuclear tubulovesicular compartment [2-4]. To be effective, this transport route requires regulated recruitment of molecular motors, membrane tethers, as well as lipid kinases and phosphatases in time and space[5-12]. Such engagement is partly accomplished by key determinants of functional identity of organelles including phosphatidylinositol 3-phosphate (PtdIns(3)*P*) and the small GTPase Rab11 [9, 13-16].

PtdIns(3)*P* is the major phosphoinositide residing on early endosomes where it serves as a membrane recognition site for the recruitment of proteins, thereby mediating endosomal fusion and maturation [17, 18]. PtdIns(3)*P* homeostasis is controlled by the coordinated action of lipid kinases and phosphatases. In particular, while phosphorylation of (PtdIns) to PtdIns(3)*P* requires members of the class II and class III phosphatidylinositol 3-kinase (PI3K) enzymes[10, 19-21], termination of PtdIns(3)*P* signaling relies on Myotubularins (MTMs), lipid phosphatases that convert PtdIns(3)*P* to (PtdIns) [22-25]. Although endosome maturation and recycling of endocytosed cargos requires control of PtdIns(3)*P* levels by the action of lipid kinases and phosphatases [8, 22, 26], the mechanism responsible for the regulated recruitment of these lipid metabolizing enzymes on endosomes remains largely unknown.

Extensive investigations demonstrated that members of the Rab protein family are coordinators of membrane domain formation and vesicle trafficking dynamics controlling the recruitment of endocytic regulators such as lipid kinases or phosphatases [27]. In particular, trafficking of endocytosed cargos toward the ERC is mediated by Rab11, a small GTPase enriched on ERC membranes and activated by signaling downstream of PI3K-C2α-derived PtdIns(3)*P* pool [9, 10, 20, 28]. In its active GTP-bound form, Rab11 mediates recycling and sorting of endocytosed membrane components through the ERC[12-14, 29]. However, whether the changes in Rab11 activity might determine the efficiency of membrane trafficking by controlling the phosphoinositide composition of endosomes is still unclear.

To monitor changes in Rab11 activity in living cells we developed a genetically-encoded FRET biosensor of active Rab11 named Activation Sensor Rab11 (AS-Rab11). Using this

biosensor, we demonstrated that the increase in Rab11 activity on PtdIns(3)P^+ peripheral endosomes is important to control the release of recycling cargoes, via a circuit involving sequential clathrin/PI3K-C2α-mediated PtdIns(3)*P* burst and subsequent Rab11/MTM1-dependent PtdIns(3)*P* hydrolysis.

Results

Development of a Rab11 FRET biosensor.

The activation cycle of Rab11 is essential to mediate the delivery of internalized plasma membrane components from PE to ERC [1, 30]. To monitor the spatial and temporal regulation of Rab11 nucleotide exchange in living cells, a genetically encoded fluorescence resonance energy transfer (FRET)-based probe, named Activation Sensor Rab11 (AS-Rab11), was developed (Figure 1a, Supplementary Figs.1a, b). The probe includes the C-terminal region of FIP3 binding active Rab11 only [31], a circular permuted version of a modified monomeric yellow fluorescent protein (mcpVenus), a proteinase K-sensitive linker, a monomeric cyan fluorescent protein (mECFP) and human Rab11a (Figure 1a). In this probe design, an increase in GTP-loading of Rab11 promotes the binding of the C-terminal region of FIP3 to Rab11a, thus modifying the orientation of the two fluorophores and thereby increasing FRET which is represented by the 525 nm/475 nm (FRET/CFP) emission ratio [32, 33](Figure 1a). The positioning of Rab11 at the C-terminal end of AS-Rab11 allows correct functioning of the Rab11 C-terminal sequences required for membrane insertion (Figure 1a).

To validate the efficiency of the biosensor energy transfer in the presence of either GDP or GTP, the fluorescence emission profiles of AS-Rab11 were monitored using a fluorometric assay (*see Materials and Methods*). In comparison with the wild type form, constitutively active mutant versions of Rab11 lacking GTPase activity (AS-Rab11^{Q70L} and AS-Rab11^{S20V}) showed decreased fluorescence emission intensity at 475 nm and a concomitant increase at 525nm (Figure 1b, Supplementary Fig. 1c red line). Consequently, the FRET/CFP ratio of AS-Rab11^{Q70L} and AS-Rab11^{S20V} were found significantly higher than the wild-type and the nucleotide-free (AS-Rab11^{N124I}) forms (Figure 1b, Supplementary Fig. 1c). In contrast, a dominant negative version of this biosensor (AS-Rab11^{S25N}, Supplementary Fig. 1c blue line) displayed 475 nm and associated 525nm emission higher and lower than the control, respectively (Figure 1b, Supplementary Fig. 1c cyan line, Supplementary Fig. 1d). A similar observation was made after proteinase-K treatment of AS-Rab11 wild-type (Supplementary Fig. 1c black line) that induced the cleavage of the amino acidic linker connecting the two fluorophores required to promote the energy transfer. Consequently, both AS-Rab11^{S25N} and proteinase-K-treated AS-Rab11^{wt} showed decreased FRET emission ratio (Figure 1b, Supplementary Fig. 1c blue and black lines), and similarly to a Rab11-GTP binding mutant (AS-Rab11$^{RBD\ mutant}$) (Figure 1b). In line with these results, increased FRET emission ratio and Rab11-GTP content were detected after co-expression of AS-Rab11^{wt} with SH3BP5, a Rab11-GEF [34] (Figures 1b, Supplementary Fig. 1e). On the contrary, co-expression of

112TBC1D9B, a Rab11-GAP [35] decreased FRET emission ratio and Rab11-GTP content in cells 113(Figures 1b, Supplementary Fig. 1e). A similar emission was obtained by co-expression of RabGDI, 114a Rab11 dissociation inhibitor [36], and was reverted by the use of a GDI-insensitive biosensor mutant 115(AS-Rab11^{N206X})(Figure 1b). This regulation was found specific, as co-expression of AS-Rab11^{wt} 116with either Rac1 or Rab5 GEFs and GAPs had no effect on biosensor response (Figure 1b). Next, 117AS-Rab11 binding to guanine nucleotides was assessed by thin layer chromatography. Equal 118amounts of GDP and GTP associated with the wild-type biosensor form, whereas either GTP or 119GDP bound the constitutively active (Q70L) or the dominant negative (S25N) biosensor forms, 120respectively (Supplementary Fig. 1f). At the same time, AS-Rab11 was able to bind and replace 121GDP with GTP similarly to Rab11 (Supplementary Fig. 1g). The biosensor was found to interact 122with recombinant FLAG-RabGDI, FLAG-SH3BP5, and FLAG-TBC1D9B (Supplementary Figs. 1232a-c). Whereas AS-Rab11$^{RBD\ mutant}$ interacted with endogenous FIP2 and FIP4, AS-Rab11^{WT} did not, 124indicating that the probe in its active conformation is not able to compete for endogenous targets 125(Supplementary Fig. 2d). Finally, As-Rab11 localized with markers of early and recycling 126endosomes but was absent from cis-Golgi and late endosome structures (Supplementary Figs. 3a-e), 127thus showing a pattern consistent with the functions of unmodified, endogenous Rab11.

128 Increased FRET emission ratio was detected both on tubulovesicular structures situated in the 129proximity of the nucleus and on small membrane-bound organelles positioned at the cell periphery 130(Figures 1c, d). To exclude the possibility that such high FRET efficiency was caused by random 131probe accumulation, a correlation plot of the sensitized FRET (i.e. the measure of FRET efficiency 132corrected for excitation and emission crosstalk) versus the CFP intensities was generated. Sensitized 133FRET was higher in endosomes than in cytosol (Figure 1e), as indicated by the 2 different slopes of 134the regression line that correlates the sensitized FRET and the CFP intensities measured in 135endosomes and cytosol, respectively. Moreover, to assess the spatial distribution of active Rab11 in 136cells, the FRET/CFP ratio of structures was measured as a function of the distance from the nucleus 137and FRET emission ratio appeared significantly higher on the ERC than on PE (Figure 1f).

138 Overall, these results demonstrate that this biosensor can monitor the nucleotide binding 139status of Rab11 and that active Rab11 is spatially restricted in both peripheral and juxtanuclear 140endosomal structures.

141***Activated-Rab11 labels PtdIns(3)P^{+} endosomes.***

142 To examine the subcellular distribution and the identity of membrane-bound structures 143displaying active Rab11, AS-Rab11-expressing cells were analyzed by confocal microscopy after

the internalization of fluorescent transferrin (Tf-647), an early-recycling endosome marker [16]. In line with previous studies [13, 14], perinuclear accumulation of active Rab11 (Figure 2a left panel, pseudocolor map) and Tf-647 (Figure 2a left panel, gray scale) was observed. In addition, enlargement of the peri-plasmalemmal region showed overlap between the highest FRET signal (Figure 2a right panel, red line) and Tf-647 (Figure 2a right panel, black line). Accordingly, two-dimensional representation of pixel intensities (Figure 2a right panel, line intensity profile) along a line starting from the nucleus and reaching the plasma membrane (Figure 2a left panel, white line) showed almost perfect overlap between FRET ratio (Figure 2a righ panel, red line) and Tf-647 (Figure 2a right panel, black line) signals in both the perinuclear and peripheral region. In further agreement, analysis of colocalization, as determined by Pearson's coefficient, showed high correlation between FRET ratio and Tf-647 positivity both in the ERC and PE (Figure 2a right panel), indicating that active Rab11 is equally distributed in perinuclear and peripheral Tf-positive compartments.

To gain insight into the localization of active Rab11, early and recycling endosome specific markers were similarly studied by analyzing a red fluorescent tagged versions of either Rab4, Rab5 or the PtdIns(3)*P* probe mCherry-FYVE2X. Identical distribution and strong colocalization were observed by fine mapping of active-Rab11 and mRFP-Rab4-positive structures, in pseudocolor and grayscale, respectively (Figure 2b). Conversely, endosomal membranes labelled by mRFP-Rab5 colocalized with active Rab11 at the cell periphery but not at the perinuclear recycling compartment (Figure 2c). Similarly, active-Rab11 strongly co-localized with the early endosome marker PtdIns(3)*P*, as detected with the mCherry-FYVE2X probe, on peripheral but not on perinuclear membrane-bound structures (Figure 2d). These results indicate that PEs, in which active Rab11 colocalized with PtdIns(3)*P*, correspond to early endosomes.

Exit from endosome relies on Rab11 and PtdIns(3)P

To examine the relationship between Rab11, PtdIns(3)*P* and recycling cargo in peripheral endosomes, the localization of Rab11 and Transferrin receptor (TfR) on PtdIns(3)*P*-positive structures was monitored during the continuous uptake of Tf. Confocal microscopy analysis revealed a frequent growth of tubular Rab11$^+$/mCherry-TfR$^+$ structures from PEs (Figure 3a). Furthermore, Tf uptake increased Rab11 activity (Supplementary Figs. 4a, b) and expression of a Rab11 dominant negative form inhibited Tf recycling and promoted its accumulation (Supplementary Figs. 4c-e), thus indicating that removal of recycling cargo from endosomes requires Rab11 activation. In agreement, increased FRET/CFP signal on the nascent vesicle began 5 seconds before fission, concomitantly with a PtdIns(3)*P* burst, and reached maximal signal at the

time of fission (Figures 3b, c, Supplementary Figs. 4f-h). Such activation kinetics did not rely on biosensor abundance as both temporal assessment and titration of AS-Rab11 level on endosomes showed robust and coherent biosensor response at various probe expression levels (Figure 3c grey line, Supplementary Figs. 4i). Unexpectedly, on the nascent recycling structure, Rab11 activation was initially preceded by the increase of PtdIns(3)*P*-levels but was later followed by a PtdIns(3)*P* decrease, starting at the time of fission (Figures 3b, c, Supplementary Figs. 4f-h). These results show that, on peripheral endosomes, PtdIns(3)*P* peaks concomitantly with Rab11 activation and declines with the fission of recycling cargo-containing vesicles.

To gain insight into this process, COS-7 cells expressing perinuclear localized AS-Rab11 were bleached to avoid contaminating signals from the ERC region and movement of active Rab11$^+$ vesicles was analyzed after Tf addition (Figure 3d). Rab11$^+$ vesicles followed long-range linear movements and then eventually collapsed into ERC membranes (Figure 3d), thus indicating that juxtanuclear AS-Rab11-positive structures derived in part from peripheral endosomes. Accordingly, AS-Rab11-positive endosomal structures accumulated in the perinuclear region during the continuous uptake of Tf and resulted in a steady state after 15 minutes (Supplementary Figs. 5a-e). By interfering with microtubule polymerization using a treatment with Nocodazole, vesicles carrying an active Rab11 failed to appear as a linear series of dots over time (Supplementary Fig. 5f). This indicated that disruption of microtubule-dependent transport abolished long-range movements of AS-Rab11-positive membranes without compromising Tf accumulation (Supplementary Figs. 5f-h). Consistently, displacement of endocytic structures, as well as Rab11 activation were decreased upon Nocodazole treatment (Figures 3e, f). These data suggested that minus end-directed microtubule motor transport is required for endocytic structure movement. To test this hypothesis, acute inactivation of retrograde transport using Ciliobrevin D, a Dynein inhibitor [37], was performed. Ciliobrevin D treatment decreased long-range retrograde motion of active Rab11$^+$ vesicles and had a minor impact on vesicle linear movement/displacement (Figures 3e, g, h) consistently with multiple Rab11/microtubule-dependent trafficking routes [38]. Finally, both Nocodazole and Ciliobrevin D treatments decreased juxtanuclear accumulation of endocytosed transferrin (Figures 3i, j).

Overall, these data indicate that transferrin receptor is removed from PEs and transported to the ERC through a local increase of PtdIns(3)*P*, the activation of Rab11, the hydrolysis of PtdIns(3)*P*, and eventually the dynein-mediated vesicular transport.

PI3K-C2α controls Rab11 activity on PtdIns(3)P$^+$ endosomes

In early endosomes, PtdIns(3)*P* is mainly produced by Class III PI3K. However, a small but significant amount of PtdIns(3)*P,* ranging up to 20%, derives from Class II PI3Ks [39] and is putatively required for Rab11 activation [9, 10]. To further determine whether the activation of Rab11 preferentially depended on either Class II or III PI3K, modulation of the AS-Rab11 probe was studied after either PI3K-C2α or Vps34 knock-down or Vps34 inhibition (Supplementary Figs. 6a-c). As expected, knock-down of PI3K-C2α induced a 20% loss of PtdIns(3)*P* as well as a 50% drop in Rab11 activity (Figures 4a, b). On the contrary, knock-down or inhibition of Vps34 by VPS34-IN1 decreased PtdIns(3)*P* cell content by 80% but failed to significantly reduce the levels of active Rab11 (Figures 4a, b). This highlights the distinct role of PI3K-C2α in controlling Rab11 activity in PE. In addition, PI3K-C2α localization during cargo release from PtdIns(3)*P*$^+$ structures was imaged. GFP-PI3K-C2α co-localized with mCherry-FYVE2X during the fission of mECFP-Rab11 positive structures (Figures 4c, Supplementary Figs. 6d-f), while it was undetected in the newly formed mECFP-Rab11$^+$/mCherry-TfR$^+$ membranes (Supplementary Figs. 6e-g). Interestingly, such PI3K-C2α localization strictly depended on its N-terminal Clathrin binding domain [40], as loss of this domain [41] resulted in a diffuse cytosolic distribution around the PE (Figure 4d).

Number, displacement and direction of Rab11-positive vesicles leaving PEs were analyzed in PI3K-C2α-knock-down cells to further characterize the role of PI3K-C2α in the control of Rab11-mediated intracellular trafficking. Reduction of PI3K-C2α abundance as well as the expression of GFP-Rab11^{S25N} lowered the number of Rab11$^+$ vesicles emerging from PEs (Figure 4e). In further support, increased residence time of Rab11 at the PEs was observed in both PI3K-C2α-KD and Rab11^{S25N} expressing cells (Figure 4f). On the contrary, pharmacological inhibition of Vps34 did not alter either the number of fission events or the residence time of Rab11 on PEs (Figures 4e, f), strengthening the idea that Vps34 and PI3K-C2α present non-redundant functions during the endocytic recycling of transferrin.

Next, to further confirm this evidence, quantitation and localization of labelled Tf were performed. PI3K-C2α-KD and GFP-Rab11^{S25N}-expressing cells displayed increased Tf accumulation and decreased Tf perinuclear storage, which was not affected by either inhibition or RNAi-mediated suppression of Vps34 (Figures 4g, h, Supplementary Figs. 6c, h). Such transferrin recycling delay did not depend on the efficiency of molecular motors, as similar distribution of linearity and vesicle speed between GFP-Rab11^{S25N} expressing cells, PI3K-C2α-KD and controls were measured by tracking of individual Rab11$^+$ vesicles (Supplementary Figs. 6i, j). This Tf delivery defect in PI3K-C2α-KD cells was rescued by expression of a wild-type (PI3K-C2α^{WT}) or a

241PI3P-only producing PI3K-C2α form (PI3K-C2α^{CIII}) (Figure 4i). On the contrary, expression of a 242kinase inactive mutant (PI3K-C2α^{R1251P}) did not restore juxtanuclear Tf localization (Figure 4i), thus 243demonstrating that Tf delivery to perinuclear endosomes is controlled by the PI3K-C2α-dependent 244PI3P production. In line with these results, silencing of PI3K-C2α led to the intracellular 245entrapment of Tf (Supplementary Fig. 6k).

246 Altogether, these results indicated that removal of recycling cargo from early endosomes 247requires PI3K-C2α-mediated PI3P production, necessary for Rab11 activation. Nonetheless, 248PtdIns(3)*P* decreased prior fission and disappeared from the detached Rab11$^+$ vesicle, suggesting 249that removal of recycling cargo from endosomes depends on PtdIns(3)*P* hydrolysis.

250***The PtdIns(3)P phosphatase MTM1 is a Rab11 effector***

251 In order to identify the PtdIns(3)*P* phosphatase that connects the increase in Rab11 activity 252with the concomitant decrease of PtdIns(3)*P*, pull-down of potential PtdIns(3)*P* phosphatases 253working as Rab11 effectors was performed. Five different PtdIns(3)*P* phosphatases, members of the 254Myotubularin protein family, were tested using immobilized Glutathione S-transferase (GST)-255Rab11 as a probe. Among them, MTM1 was found to preferentially bind Rab11:GTP-γS rather than 256Rab11:GDP (Figure 5a). On the contrary, no interactions were detected for MTMR2, MTMR4, 257MTMR6, MTMR9 (Figure 5a). Remarkably, MTM1 was isolated from total cell extracts by pull-258down of Rab11-GTP using a recombinant Rab11-GTP interacting protein (GST-RBD11) as a probe 259[9] (Figure 5b), and by immunoprecipitation of endogenous Rab11 (Supplementary Fig. 7a), thus 260indicating that Rab11 is associated with MTM1 *in vivo*. In further agreement, an *in vitro* binding 261assay using purified GST-Rab11 and His-Flag-MTM1 showed preferential binding of recombinant 262MTM1 with Rab11:GTP-γS compared to Rab11:GDP or other Rabs (Figures 5c, Supplementary 263Fig. 7b). RNA-interference mediated downregulation of MTM1 (MTM1-KD) (Supplementary Fig. 2647c) significantly increased PtdIns(3)*P* levels as well as Rab11 activity (Figures 5d, e). In MTM1-265KD cells, additional silencing of PI3K-C2α but not of Vps34 reduced Rab11 activation (Figure 5e). 266These results indicated that active Rab11 is associated with the PtdIns(3)*P* phosphatase MTM1 267which actively dephosphorylates the PtdIns(3)*P* present on the structures directed towards the ERC. 268In agreement with this view, confocal microscopy analysis showed that Rab11 and MTM1 269colocalized both in PE and ERC membranes as well as in TfR$^+$ vesicles (Figures 5f, g, 270Supplementary Fig. 7d). In line with these results, Rab11 silencing blocked perinuclear and 271peripheral MTM1 localization (Supplementary Fig. 7e).

To further characterize the impact of MTM1 in the control of Rab11-mediated intracellular trafficking, Rab11^{+} vesicles detaching from PEs were analyzed after RNAi-mediated downregulation of MTM1 (MTM1-KD). Loss of MTM1 as well as expression of GFP-Rab11^{S25N} decreased the number of Rab11 positive fission events from PEs (Figure 5h), without affecting vesicle speed (Supplementary Fig. 7f). Furthermore, the residence time of Rab11 positive structures on PEs increased in both conditions (Figure 5i). Therefore, either impaired activation of Rab11 or lack of the phosphatase activity delayed fission. In agreement, MTM1-KD and GFP-Rab11^{S25N}-expressing cells displayed increased Tf content and decreased perinuclear accumulation of the recycling cargo (Figures 5j, k, Supplementary Fig. 7g). To identify the lipid kinase that antagonizes MTM1 activity, the rescue of Tf uptake and Tf accumulation of the recycling cargo at the ERC were performed. Acute perturbation of PtdIns(3)*P* synthesis by Vps34 inhibition partially restored Tf accumulation and perinuclear storage in MTM1-KD cells (Figures 5j, k) without affecting the increase in Rab11 activation due to MTM1 loss (Figure 5e). In agreement, Rab11-mediated fission events appeared more frequent in MTM1-KD/VPS34-IN1 than in MTM1-KD cells (Figure 5h), thus indicating that fission requires a significant reduction of PtdIns(3)*P*. In the absence of MTM1, knock-down of either PI3K-C2α alone or in combination with Vps34 inhibition led to decreased Rab11 activity. Conversely, in the absence of MTM1, Vps34 inhibition alone was not able to restore increased Rab11 activity (Figure 5e). Therefore, PI3K-C2α is the main kinase driving PtdIns(3)*P* production required for Rab11 activation, consequent fission and Tf recycling (Figure 5h, j, k).

Taken together, these results show that removal of recycling cargo from peripheral endosomes depends on subsequent PI3K-C2α-mediated PtdIns(3)*P* production, Rab11 activation and MTM1-dependent PtdIns(3)*P* destruction, leading to fission of vesicles and their eventual dynein-mediated transport to the ERC.

Discussion

Removal of recycling cargo from peripheral PtdIns(3)*P*$^{+}$ endosome requires PtdIns(3)*P* hydrolysis and the activation of the small GTPase Rab11. However, whether these events are linked is unknown. Therefore, a genetically-encoded FRET biosensor for Rab11 was generated to detect spatial and temporal variations of Rab11 activity in endosomes. This biosensor named AS-Rab11 was proven to be effective into limited diffusional space, such as in membrane and vesicular compartments and its activity was found to depend on both positive and negative Rab11 regulators, such as Rab11 GEF, GAP and GDI [34-36]. Using AS-Rab11, we revealed that: (I) Rab11 activation is initiated on PtdIns(3)*P*-positive membranes where sorting of recycling cargo occurs; (II) Rab11 activation level determines the release rate of membranes destined to the ERC; and (III) such release required MTM1, a PtdIns(3)*P* phosphatase, which was found to interact with active Rab11. These results establish that removal of recycling cargo from peripheral PtdIns(3)*P*$^{+}$ endosome requires coupling of Rab11 activity and PtdIns(3)*P* turnover (Figure 6).

Extensive time lapse analyses and biochemical experiments revealed enrichment of active-Rab11 on juxtanuclear positioned ERC and peripheral PtdIns(3)*P*$^{+}$ endocytic structures. In addition, they evidenced a critical role of activated Rab11 in the release of Transferrin receptors (TfR) from PtdIns(3)*P*$^{+}$ membranes. Given that PtdIns(3)*P* is a bona-fide marker [18] of EE, a compartment where recycling cargoes are sorted and directed toward the ERC or plasma membrane [14, 28], our data suggest that Rab11 activation is initiated on EE membranes where sorting of recycling cargoes occurs. In agreement, the direct visualization of active Rab11 patches localizing with TfR on PtdIns(3)*P*$^{+}$ membranes corroborate these evidences. Our experiments show that membranes decorated by active Rab11 are not maintained indefinitely on PtdIns(3)*P*$^{+}$ structures but are delivered from peripheral to juxtanuclear recycling compartment. These observations define that, differently from active Rab5 that mediates the expansion of Rab5 domain on early endosomes, active Rab11 critically affects cargo flow by recruiting the protein machinery involved in vesicle transport. In line with this view, our results evidenced that active Rab11 vesicles detaching from peripheral endosomes accumulate on ERC membranes in a dynein-dependent manner.

Our observations extend the previous identification of PI3K-C2α as a key controller of Rab11 activation on endosomes [9] and define that localization of PI3K-C2α on endosomes strictly depends on its clathrin binding domain [20, 40]. Notably, depletion of PI3K-C2α delays the kinetic of vesicle release from PtdIns(3)*P*$^{+}$ structure where TfR sorting takes place [1], thus linking the role of PI3K-C2α to endosomal sorting. Accordingly, depletion of PI3K-C2α, as well as loss of its catalytic

activity, decreases both activity and fission of Rab11-positive vesicles from PtdIns(3)P^+ structures, thereby mimicking the phenotype observed in cells expressing dominant negative Rab11.

In light of the highly dynamic Rab11 activation on PtdIns(3)P^+ structures, and the distinct phosphoinositide composition of EE and the perinuclear recycling compartment [42], a phosphoinositide conversion can be expected between these two Rab11 positive membrane domains. Our data demonstrated that this transition is controlled by MTM1, which was found to interact with active Rab11. MTM1 was shown to antagonize the Class II and Class III derived PtdIns(3)*P* pools in *D. melanogaster* and *C. elegans* and was demonstrated to be essential in the exit of cargos from PtdIns(3)*P* endosomes [8, 26]. Accordingly, MTM1$^+$/Rab11$^+$ vesicles were observed during removal of recycling cargo from PtdIns(3)*P* compartment, thus indicating that active Rab11 provides a signal to control MTM1 localization. Given that recycling vesicles require dynein to reach the ERC, removal of PI3P can be explained by the fact that the presence of this lipid, a well-known activator of centrifugal kinesin-mediated transport[43], might disturb this centripetal trafficking.

The development and application of AS-Rab11 to quantitatively analyze Rab11 activity in living cells allowed to dissect and analyze the initial step of the PtdIns conversion mechanism [42] required for the exit of recycling cargo from endosomes. Our data indicate that PI3K-C2α provides a spatially localized and temporally controlled PtdIns(3)*P* pool sufficient to activate Rab11 on early endosomes, allowing establishment and maintenance of receptor recycling towards the ERC. Activation of Rab11 eventually contributes to the recruitment of MTM1 and the ensuing reduction of PtdIns(3)*P* level on membranes destined to the perinuclear endosome.

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Author Contribution:

C.C.C., J.P.M., and E.H. conceived and designed the experiments. C.C.C., M.D.S, L.G. and F.C perform in vitro experiments and analyzed the data, J.P.M., C.C.C., A.D, perform in vitro experiments and analyzed the data, M.D.G and C.B. analyzed imaging data, C.C.C. and E.H. wrote the manuscript. All authors contributed to data interpretation. All authors reviewed the paper and provided comments.

Conflict of interest:

EH is a co-founder of Kither Biotech, a company involved in the development of PI3K inhibitors. The other authors declare that the research was conducted in the absence of any commercial and financial relationships that could be construed as a competing financial of interest.

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470

Figure Legends

Fig. 1: The FRET biosensor AS-Rab11 specifically measures Rab11 nucleotide binding status.

a. Tridimensional representation of the genetically encoded fluorescence energy transfer (FRET) probe named AS-Rab11 (Activation Sensor Rab11) in its inactive (bound to GDP, left panel) or active conformation (bound to GTP, right panel). Yellow and light blue β-barrels represents a yellow- and cyan-emitting mutant of fluorescent proteins, respectively. Sea-green α-helix, black line and green β-barrels structures indicate Rab11-GTP binding domain (C-terminal region of FIP3), proteinase K sensitive linker domain and Rab11a protein, respectively. In this probe design, an increase of Rab11 GTP-loading promotes the binding of the C-terminal region of FIP3 to Rab11a, modifying the relative orientation of the two fluorophores and thereby increasing FRET signal (left and right panel).

b. Quantifications of FRET efficiency of AS-Rab11^{wt}, AS-Rab11 mutant forms, proteinase K treated AS-Rab11^{wt}; AS-Rab11^{wt} co-expressed with the indicated GEFs and their target GTPase (SH3BP5, RABEX-5, TIAM1, respectively); AS-Rab11^{wt} co-expressed with the indicated GAPs and their target GTPase (TBC1D9B, RN-3, ARHGAP15, respectively), and AS-Rab11^{wt} or AS-Rab11^{N206X} co-expressed with RabGDI (n=12 independent experiments; data represent mean ± SEM, *** $p< 0.005$, One-way ANOVA).

c. Representative FRET/CFP ratio images of AS-Rab11 biosensor in COS-7 cells (pseudocolor images represent FRET/CFP ratio intensity values). The upper and lower limits of the FRET/CFP ratio are shown on the left side bar (left panel). Magnification of FRET/CFP ratio images and AS-Rab11 localization in juxtanuclear (upper) and peripheral endocytic structures (lower) [right panel; pseudocolor images represent FRET/CFP ratio intensity values; grayscale image indicates emission of mcpVenus (AS-Rab11) after its direct excitation]. The scale bars represent 10 μm.

d. Representative line intensity profile of FRET sensitized (magenta) and CFP (cyan) signal detected in juxtanuclear (upper panel) and peripheral (lower panel) endocytic structures. The magenta and cyan image represent FRET sensitized and CFP signals, respectively. The scale bars represent 1 μm.

e. Scatter plot of sensitized FRET intensities as a function of CFP intensities in AS-Rab11 expressing cells (black and red dots represent the sensitized FRET and CFP intensity value of cytosolic and membrane-bound structures, respectively; regression line is in black) (upper panel) (n=4 independent experiments). Quantification of FRET/CFP ratio between cytosolic and endocytic structure labelled by AS-Rab11 (lower panel) (n=50 independent experiments; data represent mean ± SEM, *** $p< 0.005$, t-test).

504f. Scatter plot of relative FRET efficiency as a function of distance from the nucleus for 505endocytic structures labelled by AS-Rab11 (upper panel) (n=4 independent experiments; black line 506represents mean ± SEM). Quantification of FRET/CFP ratio between juxtanuclear (ERC, from 0 to 5073 μm from nucleus) and peripheral (PE, from 3 to 12 μm from the nucleus) endocytic structures 508labelled by AS-Rab11 (lower panel) (n=50 independent experiments; data represent mean ± SEM, 509*** $p < 0.005$, t-test).

510

Fig. 2: Juxtanuclear and peripheral localization of active Rab11 on distinct endosome populations.

a. Representative localization of active Rab11 on transferrin-positive endosomes. FRET/CFP ratio images (left panel, pseudocolor mode) of AS-Rab11 biosensor on endosomes labelled by fluorescent transferrin (left panel, gray scale). White line defines the region over which FRET/CFP ratio and fluorescent-transferrin signal were measured. The scale bar represents 1 μm.

Line intensity profile of FRET/CFP ratio (red line), labelled-transferrin (black line) and nuclei (blue line) (right upper panel)

Quantification in juxtanuclear (ERC, black bar) and peripheral (PE, red bar) endosomes of colocalization percentage (Pearson's correlation coefficient) between FRET/CFP signal and transferrin labelled endocytic structures (n=15 independent experiments; data represent mean ± SEM, t-test) (right lower panel)

b. Representative localization of active Rab11 on Rab4-positive endosomes. FRET/CFP ratio images (left panel, pseudocolor mode) of AS-Rab11 biosensor on endosomes labelled by mRFP-Rab4 (left panel, gray scale). White line defines the region over which FRET/CFP ratio and mRFP-Rab4 signal were measured. The scale bar represents 1 μm.

Line intensity profile of FRET/CFP ratio (red line), mRFP-Rab4 (black line) and nuclei (blue line) (right upper panel).

Quantification in juxtanuclear (ERC, black bar) and peripheral (PE, red bar) endosomes of colocalization percentage (Pearson's correlation coefficient) between FRET/CFP signal and Rab4 labelled endocytic structures (n=15 independent experiments; data represent mean ± SEM, t-test) (right lower panel).

c. Representative localization of active Rab11 on Rab5-positive endosomes. FRET/CFP ratio images (left panel, pseudocolor mode) of AS-Rab11 biosensor on endosomes labelled by mCherry-Rab5 (left panel, gray scale). White line defines the region over which FRET/CFP ratio and mCherry-Rab5 signal were measured. The scale bar represents 1 μm.

Line intensity profile of FRET/CFP ratio (red line), mCherry-Rab5 (black line) and nuclei (blue line) (right upper panel).

Quantification in juxtanuclear (ERC, black bar) and peripheral (PE, red bar) endosomes of colocalization percentage (Pearson's correlation coefficient) between FRET/CFP signal and Rab5 labelled endocytic structure (n=15 independent experiments; data represent mean ± SEM, *** p< 0.005, t-test) (right lower panel).

d. Representative localization of active Rab11 on PtdIns(3)*P*-positive endosomes. FRET/CFP ratio images (left panel, pseudocolor mode) of AS-Rab11 biosensor on endosomes labelled by mCherry-FYVE2X (left panel, gray scale). White line defines the region over which FRET/CFP ratio and mCherry-FYVE2X signal were measured. The scale bar represents 1 μm.

Line intensity profile of FRET/CFP ratio (red line), mCherry-FYVE2X (black line) and nuclei (blue line) (right upper panel)

Quantification in juxtanuclear (ERC, black bar) and peripheral (PE, red bar) endosomes of colocalization percentage (Pearson's correlation coefficient) between FRET/CFP signal and PtdIns(3)*P* labelled endocytic structures (n=15 independent experiments; data represent mean ± SEM, *** p< 0.005, t-test) (right lower panel)

Fig. 3: Rab11 activation kinetics on PtdIns(3)P-positive endosomes.

a. Representative time-lapse series of cells co-expressing mCherry-Transferrin Receptor (TfR), mECFP-Rab11 and GFP- FYVE2X. White circles represent membrane bound structures. The scale bar represents 1 μm.

b. Representative time-lapse series of cells co-expressing AS-Rab11 and mCherry-FYVE2X. White circles represent membrane bound structures. The pseudocolor mode represents the FRET/CFP ratio; the gray scale indicates the emission of mcpVenus after its direct excitation. The scale bar represents 1 μm.

c. Quantification of FRET/CFP ratio (green line), mCherry-FYVE2X fluorescent emission (orange line) and AS-Rab11 mcpVenus emission (gray line) as a function of time in 28 individual vesicle tracks directed towards the ERC. The time point of detachment from early endosomes was recorded and used to shift the time courses so that all 28 detachment events were synchronized at the chosen time point of 0 s. The normalized FYVE2X is shown on primary vertical axis. The normalized value of FRET/CFP ratio is shown on secondary vertical axis (right)(n=4 independent experiments).

d. Representative time-lapse series of long range transport of active Rab11 vesicle towards the ERC. Gray scale represents mcpVenus fluorescence emission intensities before and after bleaching (left panel, the scale bar represents 5 μm). Magnification of juxtanuclear region and time-projection (right panel, the scale bar represents 1 μm). Pseudocolor mode represents FRET/CFP ratio (n=10 independent experiments).

e. Frequency distribution of $Rab11^{+}$ vesicle displacement from the origin in cells expressing GFP-$Rab11^{S25N}$ (green line) or GFP-Rab11 treated with either vehicle (DMSO, black line), Nocodazole, a microtubule depolymerizing drug (red line) or the dynein inhibitor, CiliobrevinD (blue line) (n=4 independent experiments, *** $p< 0.005$, * $p< 0.05$, two-way ANOVA).

f. Quantification of Rab11 activation in the perinuclear area. Cells were treated with either vehicle (DMSO, black line) or Nocodazole (red line) (n=14 independent experiments; data represent mean ± SEM, ** $p< 0.01$, t-test).

g. Quantification of Rab11 activation in the perinuclear area. Cells were treated with either vehicle (DMSO, black line) or Ciliobrevin D (red line) (n=14 independent experiments; data represent mean ± SEM, ** $p< 0.01$, t-test).

h. Frequency distribution of linearity of movement of $Rab11^{+}$ vesicles in cells expressing GFP-$Rab11^{S25N}$ (green line) or GFP-Rab11 treated with either vehicle (DMSO, black line), Nocodazole (red line) or Ciliobrevin D (blue line) (n=4 independent experiments; data represent mean ± SEM, *** $p< 0.005$, two-way ANOVA).

i. Representative image of endocytosed transferrin localization in cells expressing GFP-$Rab11^{S25N}$ or GFP-Rab11 treated with either vehicle (DMSO), Nocodazole or Ciliobrevin D (n=4 independent experiments). The scale bar represents 10 μm.

j. Quantification of perinuclear localization of fluorescent transferrin in cells expressing GFP-$Rab11^{S25N}$ (green bar) or treated with either DMSO/scramble siRNA (DMSO, black bar), Rab11 siRNA 1 ($RAB11\text{-}KD_1$, gray bar), Rab11 siRNA 2 ($RAB11\text{-}KD_2$, light blue bar), Nocodazole (red bar) or the dynein inhibitor, Ciliobrevin D (blue bar) (n=12 independent experiments; data represent mean ± SEM, *** $p< 0.005$, One-way ANOVA).

Fig. 4: PI3K-C2α-dependent Rab11 activation on PtdIns(3)P-positive endosomes.

a. Quantification of PtdIns(3)*P* abundance in COS-7 cells treated with either DMSO/Scramble siRNA (Control, black bar), VPS34 inhibitor (VPS34-IN1, red bar) or PI3K-C2α siRNA (PI3KC2α-KD, blue bar) (n=15 independent experiments; data represent mean ± SEM, *** p< 0.005, * p< 0.05, One-way ANOVA).

b. Quantification of Rab11 activity in COS-7 cells treated with either DMSO/Scramble siRNA (Control, black bar), VPS34 inhibitor (VPS34-IN1, red bar) or PI3K-C2α siRNA (PI3KC2α-KD, blue bar). (n=12 independent experiments; data represent mean ± SEM, * p< 0.05, One-way ANOVA).

c. Representative time-lapse series of cells co-expressing mCherry-FYVE2X, GFP-PI3K-C2α and mECFP-Rab11 (gray scale). White circles represent membrane-bound structures (n=6 independent experiments). The scale bar represents 1 μm.

d. Representative image of cells co-expressing mCherry-FYVE2X, mECFP-Rab11 (gray scale) and GFP-PI3K-C2α (PI3K-C2α) or its mutant version GFP-PI3K-C2α-ΔClathrin (PI3K-C2α-ΔClath). White circles represent membrane bound structures (n=6 independent experiments). The scale bar represents 1 μm.

e. Quantification of the number of Rab11-associated fission events generated from mCherry-FYVE2X positive membranes. COS-7 cells expressing GFP-Rab11^{S25N} (green bar) or GFP-Rab11 treated with either DMSO/Scramble siRNA (Control, black bar), VPS34 inhibitor (VPS34-IN1, red bar), PI3K-C2α siRNA (PI3KC2α-KD, blue bar). (n=10 independent experiments; data represent mean ± SEM, * p< 0.05, One-way ANOVA).

f. Residence time of GFP-Rab11^{S25N} (green bars) or GFP-Rab11 structures on mCherry-FYVE2X-positive membranes (black, blue, red bars). GFP-Rab11 expressing cells were treated with either DMSO/Scramble siRNA (Control, black bar), VPS34 inhibitor (VPS34-IN1, red bar), PI3K-C2α siRNA (PI3KC2α-KD, blue bar). (n=10 independent experiments; data represent mean ± SEM, *** p< 0.005, ** p< 0.01, Two-way ANOVA).

g. Quantification of internal transferrin percentage in cells expressing GFP-Rab11^{S25N} (green bar) or GFP-Rab11 treated with either DMSO/Scramble siRNA (Control, black bar), VPS34 inhibitor (VPS34-IN1, red bar), PI3K-C2α siRNA (PI3KC2α-KD, blue bar). (n=12 independent experiments; data represent mean ± SEM, ** p< 0.01, *** p< 0.005, One-way ANOVA).

626h. Quantification of perinuclear localization of fluorescent transferrin in cells expressing GFP-627Rab11^{S25N} (green bar) or GFP-Rab11 treated with either DMSO/Scramble siRNA (Control, black 628bar), VPS34 inhibitor (VPS34-IN1, red bar), PI3K-C2α siRNA (PI3KC2α-KD, blue bar) (n=12 629independent experiments; data represent mean ± SEM, *** p< 0.005, One-way ANOVA).

630i. Quantification of perinuclear localization of fluorescent transferrin in cells expressing GFP-631Rab11 treated with either DMSO/Scramble siRNA (Control, black bar), PI3K-C2α siRNA 632(PI3KC2α-KD, red bar), PI3K-C2α siRNA and PI3K-C2α^{wt} siRNA resistant (PI3KC2α-KD/ 633PI3KC2α^{wt}, blue bar), PI3K-C2α siRNA and PI3K-C2α^{R1251P} siRNA resistant (PI3KC2α-KD/ 634PI3KC2α^{1251}, green bar), PI3K-C2α siRNA and PI3K-C2α^{CIII} siRNA resistant (PI3KC2α-KD/ 635PI3KC2α^{CIII}, purple bar) (n=12 independent experiments; data represent mean ± SEM, *** p< 6360.005, One-way ANOVA).

Fig. 5: The PtdIns(3)P phosphatase MTM1 is a Rab11 effector.

a. Affinity chromatography of Rab11-GTP effectors. Representative western blot of both Rab11-GDP and Rab11-GTPγS column eluate probed with anti-MTM1, anti-MTMR2, anti-MTMR4, anti-MTMR6, and anti-MTMR9 antibodies (n=5 independent experiments) (uncropped blots are shown in Supplementary Figure 8).

b. Pull-down of endogenous Rab11-GTP and MTM1 complex. Representative western blot of Rab11-GTP pull-down assay probed with anti-MTM1 antibody (n=5 independent experiments). Quantification of endogenous MTM1 (central panel) and Rab11-GTP (right panel) pulled-down by GST or GST-RBD11 probe (n=5 independent experiments, data represent mean ± SEM, ** p< 0.01, t-test))(uncropped blots are shown in Supplementary Figure 8).

c. In vitro assessment of the association between recombinant Rab11-GTP and MTM1. Representative western blot of recombinant Rab11 loaded with GDP or GTPγS and probed for MTM1 interaction. Quantification of recombinant MTM1 pulled-down by recombinant Rab11 loaded with GDP or GTPγS (n=4 independent experiments, data represent mean ± SEM, ** p< 0.01, t-test)(uncropped blots are shown in Supplementary Figure 8).

d. Quantification of PtdIns(3)*P* abundance in COS-7 cells treated with either Scramble siRNA (Control, black bar) or MTM1 siRNA (MTM1-KD, blue bar). (n=12 independent experiments; data represent mean ± SEM, ** p< 0.01, t-test).

e. Quantification of active Rab11 levels in COS-7 cells treated with either Scramble siRNA (Control, black bar), MTM1 siRNA (MTM1-KD, blue bar), MTM1 siRNA and PI3K-C2α siRNA (MTM1-KD/PI3K-C2α-KD, dotted blue bar), MTM1 siRNA and VPS34 inhibitor (MTM1-KD/VPS34-IN1, dashed blue bar), MTM1 siRNA in combination with PI3K-C2α siRNA and VPS34 inhibitor (MTM1-KD/PI3K-C2α-KD/VPS34-IN1, red bar). (n=12 independent experiments; data represent mean ± SEM, ** p< 0.01, One-way ANOVA).

f. Representative immunofluorescence of COS-7 cells, showing peripheral and perinuclear colocalization of MTM1 with Rab11. Peripheral (left) and perinuclear (right) magnification are shown in the bottom part of the panel (n=6 independent experiments). White arrows highlight colocalization. The scale bar represents 15 μm.

g. Representative time-lapse series of cells co-expressing mCherry-Transferrin receptor (TfR), mECFP-Rab11 (gray scale) and GFP-MTM1. White circles represent membrane-bound structures (n=6 independent experiments). The scale bar represents 1 μm.

669h. Quantification of the number of Rab11-associated fission events generated from mCherry-
670FYVE2X-positive membranes. Cells expressing GFP-Rab11^{S25N} (green bar) or GFP-Rab11 treated
671with either DMSO/Scramble siRNA (Control, black bar), MTM1 siRNA (MTM1-KD, blue bar),
672MTM1 siRNA and PI3K-C2α siRNA (MTM1-KD/PI3K-C2α-KD, dotted blue bar), MTM1 siRNA
673and VPS34 inhibitor (MTM1-KD/VPS34-IN1, dashed blue bar), MTM1 siRNA in combination
674with PI3K-C2α siRNA and VPS34 inhibitor (MTM1-KD/PI3K-C2α-KD/VPS34-IN1, red bar)
675(n=12 independent experiments; data represent mean ± SEM, ** p< 0.01, * p< 0.05, One-way
676ANOVA).

677i. Residence time of GFP-Rab11^{S25N} (green bars) or GFP-Rab11 structures on mCherry-
678FYVE2X-positive membranes (black, blue bars). Cells expressing GFP-Rab11 were treated with
679either Scramble siRNA (Control, black bar) or MTM1 siRNA (MTM1-KD, blue bar). (n=12
680independent experiments; data represent mean ± SEM, *** p< 0.005, ** p< 0.01, Two-way
681ANOVA).

682j. Quantification of internal transferrin percentage in COS-7 cells expressing GFP-Rab11^{S25N}
683(green bar) or GFP-Rab11 treated with either DMSO/Scramble siRNA (Control, black bar), MTM1
684siRNA (MTM1-KD, blue bar), MTM1 siRNA and PI3K-C2α siRNA (MTM1-KD/PI3K-C2α-KD,
685dotted blue bar), MTM1 siRNA and VPS34 inhibitor (MTM1-KD/VPS34-IN1, dashed blue bar),
686MTM1 siRNA in combination with PI3K-C2α siRNA and VPS34 inhibitor (MTM1-KD/PI3K-C2α-
687KD/VPS34-IN1, red bar) (n=12 independent experiments; data represent mean ± SEM, *** p<
6880.005, ** p< 0.01, * p< 0.05, One-way ANOVA).

689k. Quantification of perinuclear localization of fluorescent transferrin in COS-7 cells
690expressing GFP-Rab11^{S25N} (green bar) or GFP-Rab11 treated with either DMSO/Scramble siRNA
691(Control, black bar), MTM1 siRNA (MTM1-KD, blue bar), MTM1 siRNA and PI3K-C2α siRNA
692(MTM1-KD/PI3K-C2α-KD, dotted blue bar), MTM1 siRNA and VPS34 inhibitor (MTM1-
693KD/VPS34-IN1, dashed blue bar), MTM1 siRNA in combination with PI3K-C2α siRNA and
694VPS34 inhibitor (MTM1-KD/PI3K-C2α-KD/VPS34-IN1, red bar) (n=12 independent experiments;
695data represent mean ± SEM, *** p< 0.005, ** p< 0.01, One-way ANOVA).

Fig.6: Trafficking of recycling cargo from peripheral endosome to ERC requires Rab11 activation and PtdIns(3)P turnover.

On a peripheral PtdIns(3)*P* membrane a transient and local burst of PI3K-C2α-derived PtdIns(3)*P* triggers Rab11 activation (first and second panel from the lefts). Active Rab11 (Rab11-GTP) recruits MTM1, a PtdIns(3)*P* phosphatase, that catalyzes PtdIns(3)*P* hydrolysis (third panel from the left). PtdIns(3)*P* reduction allows vesicle fission and trafficking of cargo towards the ERC (rightmost panel).

Materials and Methods:

Antibodies

The following antibodies were used in this study: mouse-anti-PI3K-C2α (BD Biosciences 611046, western blotting (WB) 1:500), mouse-anti-Rab11 (BD Biosciences 610656, WB 1:1000), rabbit-anti-MTM1 (SIGMA HPA010008, WB 1:1000), mouse-anti-FLAG (SIGMA clone M2, WB 1:2000), mouse-anti-GFP (ABCAM ab127417, WB 1:1000), rabbit-anti-VPS34 (Novus Biologicals NB110-87320SS, WB 1:1000), mouse-anti-MTMR4 (Santa Cruz sc-373922, WB 1:500), mouse-anti-MTMR6 (ABCAM ab69875, WB 1:1000), mouse-anti-MTMR9 (Santa Cruz sc-514366, WB 1:1000), rabbit-anti-FIP2 (ABCAM ab76892, WB 1:1000), rabbit-anti-FIP4 (Biorbyt orb215321, WB 1:1000). Anti-mouse IgG (ab131368, WB 1:5000) Anti-Rabbit IgG (A0545 SIGMA, 1:5000). anti mouse/rabbit IgG Alexa fluor 488/568 (IF 1:1000).

SiRNA and plasmid transfection

All siRNAs used in this study were 21-, 23-, or 27-base oligonucleotides including 3′-dTdT overhangs. For silencing, the following siRNAs were used targeting the human isoform: PI3K-C2α 5’-GGCAAGATATGTTAGCTTT-3’, MTM1 5’-GATGCAAGACCCAGCGTAA-3′. The scrambled control siRNA used throughout this study corresponded to the sequence 5’-ATGAGTTAGATGCGTTCTA-3’.

COS-7 cells were transfected with siRNA using Lipofectamin 2000 (Invitrogen) according to the manufacturer’s protocol. To achieve optimal knockdown efficiency, two rounds of silencing were performed. Cells were transfected on day 1, expanded on day 2, seeded for the experiment on day 3, and the experiment was performed on day 4.

For transient overexpression of proteins in silenced cells, plasmids were transfected on day 4 12 h before analysis using Lipofectamin 2000 (Invitrogen). For transient overexpression of proteins in untreated cells, plasmids were transfected 12 h before analysis using Lipofectamin 2000 (Invitrogen).

Recombinant protein production

GST-Rab11a recombinant protein was generated by cloning Rab11a cdna in pGex vector. Protein expression was induced by addition of isopropyl β-D–thiogalactoside (IPTG, 0.1 mM) at room temperature for 6 hours.

735 Recombinant proteins (GST-Rab11a, GST-Rab5a, GST-Rab7a) were purified (elution 10 mM
736glutathione, PBS), dialyzed, frozen in liquid nitrogen, and stocked (50% glycerol in Tris-HCl
73750 mM 5 mM MgCl2, 100 mM NaCl) at −80°C. His-Flag-tagged MTM1 was generated according
738to previously established protocol [25]. In brief, Flagged MTM1 was clone in Pqe vector and bacteria
739were grown in 2X-YT (1% Yeast extract, 221 1% bactotryptone, 2,5mM NaOH and 0.5% NaCl)
740enriched medium until mid-log phase. Induction was performed with 1mM IPTG at 16°C for 12 hr.
741Soluble protein fraction was purified, dyalized, and stocked (50% glycerol in Tris-HCl 50 mM
7425 mM MgCl2, 100 mM NaCl, 0.5% Triton).

743***Plasmids***

744The biosensor was built in sequential cloning steps using monomeric version of fluorescent proteins
745(A206K mutation) to avoid signal artifacts during FRET quantitation caused by multimerization of
746biosensor molecules into limited diffusional space, such as in membrane and vesicular
747compartments. Rab11 binding domain (RBD11) was fused with RBD11-circularly permutated
748Venus (mcpVenus) at residue 195, while cyan fluorescent protein (mECFP)-Rab11a fusions were
749first constructed. Two repetition of a linker encoding for a 17-mer unstructured soluble and
750proteinase-K sensitive polypeptide (GSTSGSGKPGSGEGSTK) [44] was then cloned by PCR that
751allow to maximize the FRET change between the active and inactive state. To construct RBD11-
752cpVenus, polymerase chain reaction (PCR) was used to amplify amino-acids 649-756 of FIP3 using
753the primers: 5'–CTAGCTAGCATGGGCCTGCAGGAGTACCACA–3' and 5'–
754GCTCTAGAATGGGCACCCGCGACG–3', and pGEX- FIP3 RBD11 as a template [9]. mcpVenus
755was amplified using the primers: 5'–GGTAGTGGTGAATTCATGCTCGGAGCAGTCCTGA–3'
756and 5'–ATCCCCTCGAGAGCACGGGGCCGTCGCCGAT–3' using ICUE3 FRET probe[45] as a
757template. Both fragments were then digested, gel purified, and subcloned in PGEM 3tEasy vector
758(Promega). The resulting fragment contained, from the 5'-end: a NheI site, RBD11, a EcoRI site, a
759linker (GGSG), and mcpVenus. This was cloned. To construct mECFP-Rab11a, a construct
760encoding mECFP was amplified with the primers: 5'–
761AAGCGGCCGCATGGTGAGCAAGGGCGAGGAGCTG–3' and 5'–
762GGTGCCCATTCTAGAAGTTCCCACGGGGGTACCAGCCTTGTACAGCTCGT–3'. Rab11a
763was amplified with the primers: 5'–GCTCTAGAATGGGCACCCGCGACG–3' and 5'–
764GCGGATCCAATGCCTTAGATGTTCTGACAGCACTGC–3' using a Rab11a expression
765construct as a template. Both fragments were then digested gel purified and subcloned in PGEM
7663tEasy vector (Promega). The resulting fragment contained, from the 5'-end: a NotI site, a mECFP,
767a linker (GTPVGT), XbaI site, Rab11a and a BamHI site. In the next step, the 3' end of RBD11-

768mcpVenus was flanked with zero, one or two copies of a sequence encoding a 17-mer,
769(GSTSGSGKPGSGEGSTK) generated by polymerase chain reaction (PCR). For that purpose,
770seven annealed 5' phopsorilated oligos: 5'–TCGAGGGGAGGCAGC–3', 5'–
771GGCCGCTGCCTCCCC–3' and 5'-
772TCGAGGGGATCAACTTCAGGATCAGGAAAACCCGGCTCCGGCGAGGGATCAACTAAA
773AGC–3' and 5'–
774GGCCGCTTTTAGTTGATCCCTCGCCGGAGCCGGGTTTTCCTGATCCTGAAGTTGATCCC
775C–3' and 5'–
776TATATATATATATATACTCGAGGGATCAACTTCAGGATCAGGAAAACCCGGCTCCGGC
777GAGGG–3'and 5'–
778CCGGGCTTGCCGCTGCCGGAAGTAGAGCCTTTAGTTGATCCCTCGCCGGAGCCGGG–3'
779and 5'–
780TATATATATATGCGGCCGCTTTTAGTTGATCCTTCTCCTGATCCGGGCTTGCCGCTGCCG
781-3' that encode the linker sequence flanked at the 5' by a XhoI and at the 3' by a NotI restriction
782site were ligated and subcloned in PGEM 3tEasy vector (Promega). To assemble the biosensor all
783the subcloned fragments were digested with the single cutter enzyme inserted at 5' and 3'-end, gel
784purified and cloned in pcDNA3.1(-myc/His) vector (Invitrogen), thus originating the following
785fusion protein containing from the N-terminus RBD11-cpVenu-2x17-mer linker-mECFP-Rab11a.
786The constructs were fully sequenced to ensure fidelity of the PCR reactions. Constituvely active
787(AS-Rab11^{Q70L}), dominant negative (AS-Rab11^{S25N}), a second constitutive active form (AS-
788Rab11^{S20V}) , an RBD mutant (AS-Rab11$^{RBD\ mutant}$ in which the "RBD domain" of FIP3 carries a 3
789aminoacids mutation abrogating binding of active Rab11)[31], a nucleotide free form (AS-Rab11^{N124I})
790and a mutant lacking GDI interaction (AS-Rab11^{N206X}, in which Asn-206 was changed to a stop
791codon, eliminating Rab11 prenylation/GDI binding site) versions of this biosensor were then
792engineered by site directed mutagenesis (Quikchange kit, Stratagene) using the following primers:
7935'-GATATGGGACACAGCAGGGCTAGAGCGATATCGAGC-3', 5'-
794GCTCGATATCGCTCTAGCCCTGCTGTGTCCCATATC-3' and 5'-
795GATTCTGGTGTTGGAAAGAATAATCTCCTGTCTCG-3', 5'-
796CGAGACAGGAGATTATTCTTTCCAACACCAGAATC-3' and 5'-
797GTTGTCCTTATTGGAGATGTTGGTGTTGGAAAGAGTA-3', 5'-
798TACTCTTTCCAACACCAACATCTCCAATAAGGACAAC-3' and 5'-
799CAACTTCCGCCTGCAGGACGCCGCCGCCAGGATCATCGTGGCCATCAT-3', 5'-
800ATGATGGCCACGATGATCCTGGCGGCGGCGTCCTGCAGGCGGAAGTTG-3' and 5'-
801GTTATCATGCTTGTGGGCATTAAGAGTGATCTACGTCATCTC-3', 5'-

802GAGATGACGTAGATCACTCTTAATGCCCACAAGCATGATAAC-3’ and 5’-
803ATGTTCCACCAACCACTGAATAAAAGCCAAAGGTGCAGTGCTG-3’, 5’-
804CAGCACTGCACCTTTGGCTTTTATTCAGTGGTTGGTGGAACAT-3’. Red fluorescent tagged
805version of Rab5, Rab4, Rab11 and Rab7 was generated by PCR and cloned into pmRFP-c1 plasmid.

806 The plasmid encoding RabGDI and GST-Rab7 were kindly provided by Cecilia Bucci from
807University of Salento. To allow the expression in mammalian cells the DNA sequence ecoding
808RabGDI and flanked by EcoRI site at 5’ –end and BamHI site at 3’-end was subcloned in
809pcDNA3.1(-myc/His) vector (Invitrogen). A FLAG tag (DYKDDDDK) was inserted by digestion
810of pcDNA3.1(-myc/His)-RabGDI with EcoRI followed by calf intestinal phosphatase (CIP)
811treatment and gel purification. The opened plasmid was ligated with a linker sequence encoding the
812FLAG epitope obtained by annealing two 5’phosphorylated oligos with the following sequence: 5’–
813AATTCATGGACTACAAAGACGATGACGACAAGC-3’ and 5’–
814AATTGCTTGTCGTCATCGTCTTTGTAGTCCATG–3’. The plasmid encoding hSH3BP5 was
815kindly gifted by Ken Sato from University of Gunma. A FLAG- N-terminal tag was added by PCR
816and the product of this reaction was cloned in pcDNA3.1(-myc/His) vector, thus generating a
817FLAG-hSH3BP5. The plasmid encoding TBC1D9B was kindly provided by Gerard Apodaca from
818University of Pittsburgh. A FLAG- N-terminal tag was added by PCR and the product of this
819reaction was cloned in pcDNA3.1(-myc/His) vector, thus generating a FLAG-TBC1D9B. The
820plasmid encoding mCherry-FYVE(2X) (pCI-neo-mCh-2XFYVE) was kindly gift by Matteo
821Bonazzi from University of Montpellier. The plasmid encoding TIAM1 was kindly gifted by
822Giorgio Scita and Andrea Palamidessi from IFOM in Milan. The plasmid encoding Rabex-5 was
823kindly gifted by Sara Sigismund from IFOM in Milan. The plasmid encoding RN-3 was kindly
824gifted by Letizia Lanzetti from IRCC in Candiolo.

825***Fluorometry assay***

826 $2*10^5$ HEK 293T cells were plated in a 6-well plate and transfected using Lipofectamine 2000
827(Invitrogen) according to manufacturer’s instructions. In experiments in which the biosensor was
828co-transfected with a negative or positive regulator, the biosensor/regulator DNA ratio was 1/4. The
829total amount of transfected DNA was kept to 500 ng. 36 hours post-transfection, cells were lysed in
830lysis buffer (50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 100 mM NaCl, 1% Tritorn X-100,
831proteinase inhibitors) and clarified lysate was placed in a fluorometer cuvette. The lysates were
832analyzed using a Fluoromax-4 Horiba fluorometer. The lysates were excited at 433 nm and an
833emission scan was acquired from 450 to 550 nm. To normalize for biosensor concentration a second
834measurement was made by directly exciting YFP at 505 nm and measuring its emission at 525 nm.

Immunoprecipitation assay for interaction of AS-Rab11 with SH3BP5 or AS-Rab11 with TBC1D9B

HEK 293T cells growing in 10-cm dishes were transfected with DNA mixtures containing 10 µg of pcDNA3.1(-myc/His)-Flag- SH3BP5 or 10 µg of pcDNA3.1(-myc/His)-AS-Rab11 or both, using calcium phosphate method. 48 h after transfection, cultures were harvested and homogenized in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 0.2 mM GDP, 10 mM $MgCl_2$, 100 mM NaCl, 1% Tritorn X-100, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride). Cytosol was obtained by centrifuging the lysates at 20,000g for 30 min at 4 °C and protein concentration was determined by Bradford method. 1 mg of cytosol was incubated with FLAG M2 antibody (SIGMA, S.Louis, Missouri, USA) or with 1 µg of anti-GFP antibody (ABCAM, Cambridge, UK) for 2 hours and incubated on a rotating rack for 1 hour at 4 °C with Protein-G sepharose beads (GE, Buckinghamshire, UK). Samples were collected by centrifugation and washed six-times with phosphate wash buffer (10 mM NaH_2Po_4, 137 mM NaCl, and 2.7 mM KCl). Bound FLAG-SH3BP5, FLAG-TBC1D9B or GFP-AS-Rab11 protein complexes were then eluted by adding Laemmli sample buffer. SDS-PAGE and western blotting followed standard procedures. Similar approach was employed for the RabGAP TBC1D9B using the following lysis buffer: 50 mM Tris-HCl, pH 7.4, 0.2 mM GTP, 10 mM $MgCl_2$, 100 mM NaCl, 1% Triton X-100, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride).

Immunoprecipitation assay for interaction of AS-Rab11 with GDI

HEK 293T cells growing in 10-cm dishes were transfected with DNA mixtures containing 10 µg of pcDNA3.1(-myc/His)-Flag-RabGDI or 10 µg of pcDNA3.1(-myc/His)-AS-Rab11 or both, using calcium phosphate method. 48 h after transfection, cultures were harvested and gently homogenized in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 0.2 mM GDP, 10 mM $MgCl_2$, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride). Cytosol was obtained by centrifuging the lysates at 20,000g for 30 min at 4 °C and protein concentration was determined by Bradford method. 1 mg of cytsol was incubated with FLAG M2 antibody (SIGMA, S.Louis, Missouri, USA) or with 1 µg of anti-GFP antibody (ABCAM, Cambridge, UK) for 2 hours and incubated on a rotating rack for 1 hour at 4 °C with Protein-G sepharose beads (GE, Buckinghamshire, UK). Samples was collected by centrifugation and washed six-times with phosphate wash buffer (10 mM NaH_2Po_4, 137 mM NaCl, and 2.7 mM KCl). Bound FLAG-GDI or GFP-AS-Rab11 protein complexes were then eluted by adding Laemmli sample buffer. SDS-PAGE and western blotting followed standard procedures.

Radiolabeling of intracellular nucleotides and identification of the nucleotide-bound forms of AS-Rab11.

HEK293T cells cultured in 6 well plate dishes and transfected for 48 h were radiolabeled for 4 h with ^{32}P (6.0 MBq per dish) in phosphate-free DMEM (Invitrogen, Cat. Number 11971025). The expression levels of AS-Rab11 proteins and mutant forms were assessed by immunoblot analysis with the anti-GFP antibody (ABCAM, Cambridge, UK). The labeled cells ($7x10^5$ cells) were lysed with 0.3 ml of an ice-cold solubilizing buffer consisting of 40 mM Tris–HCl (pH 7.5), 100 mM NaCl, 20 mM $MgCl_2$, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1% (w/v) Triton X-100, and 2 μg/ml aprotinin and clarified. The precleared lysates were incubated with anti-GFP antibody-immobilized Protein G–Sepharose beads (GE Healthcare) at 4 C for 30 min. After extensive washing of the immunocomplexes, associated nucleotides were separated by thin layer chromatography and quantified with a Amersham Hyperfilm MP (GE Healthcare).

Guanine nucleotide exchange assay

HEK 293T cells growing in 10-cm dishes were transfected with DNA mixtures containing 10 μg of pEGFP-Rab11a or 10 μg of pcDNA3.1(-myc/His)-AS-Rab11, using calcium phosphate method. At 48 h after transfection, cultures were harvested and homogenized in 1 ml of lysis buffer (50 mM HEPES, pH 7.6, and 1% (v/v) Triton-x100, 100 mM NaCl, protease inhibitors). Cytosol was obtained by centrifuging the lysates at 20,000g for 20 min at 4 °C and protein concentration of clarified lysates was determined by Bradford method. 1 mg of protein was immunoprecipitated using 1 μg of anti-GFP antibody (ABCAM, Cambridge, UK) for 1 hours and incubated on a rotating rack for 1 hour at 4 °C with Protein-G sepharose beads (GE, Buckinghamshire, UK). Samples were collected by centrifugation and washed six-times with buffer A containing 50 mM HEPES, pH 7.6, 1 mM DTT and 20 mM EDTA and incubated for 20 min at 25 °C, to remove Mg^{2+} and nucleotide bound to the Rab11 GTPases. The treatments of the samples with buffer A were repeated three times more. To determine the GDP binding affinities to Rab11 GTPases, [3H]GDP at a specific activity of 6000 cpm/μM was incubated with the respective apo-GTPases at 25 °C for 1 h in buffer B containing 50 mM HEPES, pH 7.6, 100 mM NaCl, 2.5 mM $MgCl_2$ and 1 mM DTT. Samples were collected by centrifugation and washed six-times with buffer C containing 50 mM HEPES, pH 7.6, 100 mM NaCl, 10 mM $MgCl_2$ to stop the binding reaction, and the radionucleotides remaining bound to the Rab GTPases were quantified by scintillation counting. To measure the GDP/GTP exchange from Rab11 GTPases, the immunoprecipitated apo-GTPases were first complexed with [3H]GDP or in buffer B. After 60 min a binding equilibrium was reached, the dissociation reactions were initiated by the addition of 500 μM GTPγS to the incubation mixtures.

900At the indicated time intervals, samples were collected by centrifugation and washed six-times with
901buffer C to stop the exchange reaction. The radionucleotides remaining bound to the Rab GTPases
902were quantified by scintillation counting.

903***Rab11-activity pull down assay***

904 Cells were washed in ice-cold PBS and lysed in 1 ml of MLB buffer (25 mM HEPES [pH
9057.5], 150 mM NaCl, 1% Igepal CA-630, 10% glycerol, 25 mM NAF, 10 mM MgCl2, 1 mM EDTA,
9061mM sodium orthovanadate, and protease inhibitor cocktail). Supernatant was collected after 15
907min centrifugation at 13,000 rpm. A total of 1 mg of protein extract was incubated with 30 µg of
908recombinant protein coupled with glutathione S-transferase agarose (GE, Buckinghamshire, UK).
909The reaction mixture was gently rocked for 1 hr at 4°C. Beads were washed four times with lysis
910buffer. Samples were resuspended in Laemmli buffer for SDS-PAGE and immunoblot analysis.
911Endogenous content of total Rab11 in cell lysates was measured by loading 50 µg of total extracts
912in a different gel followed by immunoblot and used to normalize measurements of active Rab11.
913For quantification analysis, pictures were taken ensuring that intensity was within the linear range
914and the Quantity One 1-D analysis software (Bio-Rad) was used.

915***Rab11-effectors pull-down assay***

916 50 µg of GST-Rab11 and corresponding molar amount of GST recombinant proteins were
917coupled to with glutathione S-transferase agarose (GE, Buckinghamshire, UK) and gently rocked
918for 1 hr at 4°C. Samples were collected by centrifugation and washed six-times with buffer A
919containing 50 mM HEPES, pH 7.6, 1 mM DTT and 20 mM EDTA and incubated for 20 min at 25
920°C, to remove Mg^{2+} and nucleotide bound to the Rab11 GTPases. The treatments of the samples
921with buffer A were repeated three more times. GDP or GTPγS was added at a final concentration of
9222mM and incubated with the respective apo-GTPases at 25 °C for 1 h in buffer B containing 50 mM
923HEPES, pH 7.6, 100 mM NaCl, 2.5 mM $MgCl_2$ and 1 mM DTT. Samples were collected by
924centrifugation and washed six-times with buffer C containing 50 mM HEPES, pH 7.6, 100 mM
925NaCl, 10 mM $MgCl_2$ to stop the binding reaction. Proteins on beads were incubated with either 1 ml
926of cell lysate made from a confluent dish of COS-7 cells lysed in 1 ml of MLB buffer (25 mM
927HEPES [pH 7.5], 150 mM NaCl, 1% Igepal CA-630, 10% glycerol, 25 mM NAF, 10 mM MgCl2, 1
928mM EDTA, 1mM sodium orthovanadate, and protease inhibitor cocktail), or with recombinant
929purified protein diluted in GST-binding buffer (20 mM Tris-HCl [pH 7.4], 100 mM NaCl, 5 mM
930MgCl2, 0.5% Triton X-100, 5 mg/ml BSA). After 1 hr, beads were washed with MLB or GST-
931binding buffer and proteins solubilized by boiling in LDS sample buffer.

Transferrin recycling assay

2 * 10^5 COS-7 cells were plated in a 6-well plate. After 24 hours, cells were starved for two hours in serum-free DMEM containing 0.1% BSA at 37°C, 5% CO_2, and then, where required, pretreated with 0.1% DMSO as control or with Vps34-IN1 1 uM for 30 min. 20 ug/ml of of alexa Fluor 647- conjugated human Transferrin (Invitrogen) were added for 30 min. After 37°C PBS washing, DMEM containing 0.1% BSA at 37°C, 5% CO_2 was added at various length times. Cells were then washed twice with cold PBS and acid stripping solution (150 mM NaCl, 2 mM $CaCl_2$ and 25 mM CH_3COONa, pH 4.5) was added for 4 min. For FACS analysis cells were detached with PBS 0.5 mM EDTA and fixed in 4% paraformaldehyde for 10 min. After resuspension in PBS, fluorescence flow cytometry was performed using a FACScalibur instrument. 20,000 cells were collected for each sample. The MFI of the cell population was recorded for each time point. Data were normalized to the time 0 MFI. For immunofluorescence analysis, cells were fixed in 4% paraformaldehyde for 10 min and imaged by confocal microscopy.

Internal Transferrin quantitation

2* 10^5 COS-7 wild-type (or interfered) cells were plated in a 6-well plate. After 24 hours, cells were starved for two hours in serum-free DMEM containing 0.1% BSA at 37°C, 5% CO_2, and then, where required, pretreated with 0.001% DMSO as control or with Vps34-IN1 1 uM for 30 min. 20 ug/ml of alexa Fluor 647- conjugated human Transferrin (Invitrogen) were added for 30 minutes to allow continuous uptake and recycling of labelled ligands. Cells were then washed twice with cold PBS and acid stripping solution (150 mM NaCl, 2 mM $CaCl_2$ and 25 mM CH_3COONa, pH 4.5) was added for 4 min. Cells were detached with PBS 0.5 mM EDTA and fixed in 4% paraformaldehyde for 10 min. After resuspension in PBS, fluorescence flow cytometry was performed using a FACScalibur instrument. 20,000 cells were collected for each sample. The MFI of the cell population was recorded for each time point. Data were normalized to control MFI values. For immunofluorescence analysis, cells were fixed in 4% paraformaldehyde for 10 min and imaged by confocal microscopy.

Cell Imaging

Cells were grown on μ-Dish $^{35mm,\ high}$ imaging dishes (Ibidi). Imaging was performed in CO_2 independent medium, Dulbecco's modified Eagle's medium without fetal bovine serum (GIBCO). Time-lapse series were acquired at 37°C on an inverted confocal Leica SP8 microscope with AOBS, equipped with 40X O2/Oil immersion objective, NA 1.30. The temperature was controlled

963by a climate box covering the set up. Hyd detectors (Leica) allowed the simultaneous detection of
964mECFP and mcpVenus or/and mCherry/mRFP, respectively. Fluorescent dyes were imaged
965sequentially in frame-interlace mode to eliminate cross talk between the channels. mECFP was
966excited with a 458-nm laser line and imaged at 470–500-nm bandpass emission filters. mcpVenus
967was exited with the 514-nm Argon laser line and imaged through a 525-550-nm bandpass emission
968filter. mCherry/mRFP was excited with the 568-nm Helium Neon laser line and imaged through a
969580-650-nm bandpass emission filter. Alexa 647 dye was excited with the 633 nm Helium Neon
970laser line and imaged through a 650-700-nm bandpass emission filter. Serial sections were acquired
971satisfying the Nyquist criteria for sampling and processed using Matlab (MathWorks, MA, USA)
972and ICY software (http://icy.bioimageanalysis.org). Signals were referred to as individual structures
973if they comprised of a continuous patch of intensity values 50 (in a range of 0–255). At least two
974sections per cell were counted, ensuring that peripheral and perinuclear structures were equally
975taken into account. mECFP was bleached 5–10 times (2 s/scan) with zoom (x 15) with 100% laser
976power of the 458 nm Argon laser line. At the beginning of each experiment the number of bleaching
977steps that were sufficient to bleach mECFP was assessed and was kept constant all through.
978Acquisition was performed at zoom (x 11), in a region of 26 μm in side. The ROI has been chosen
979in order to contain the photobleached ERC and the surrounding intracellular region, and over a
980sufficiently large and homogeneous region to be able to visualize moving vesicles towards it.
981Exposure times and readout were fixed as follows: 200–300 ms for each channel followed by a 60-
982ms readout delay for the experiment in Figures 3a-f, i, 4b-g), resulting in timelapse sequences of
983roughly one frames per second. The timelapse sequences of roughly one frame per 30 seconds was
984used in Figures 3g, h, Supplementary Fig. 4a, 5a, b, d. Images obtained were merged and exported
985as a single TIFF file.

986***Image/video processing and data analysis***

987 Image processing and analysis for total FRET activation in the cell were carried out with the
988Matlab software (MathWorks, MA, USA) integrated with Image Processing and Bioformats
989Toolbox. Following Gaussian smoothing, the image was converted to binary through thresholding,
990then median filtering, morphological closing and holes filling were applied to eliminate noisy pixels
991and smooth the images. The final mask was obtained computing the distance transform of the
992binary image and using it as the input for a Watershed transform, thus enabling to discriminate and
993separate different contiguous endosomes from one to another. The threshold mask was then applied
994to sensitized FRET and CFP images and background subtraction was performed according to
995previous published protocol [46]. Finally, FRET activity ratio was calculated by dividing the

unsaturated sensitized FRET pixels by the CFP pixels[47, 48]. To measure the dependence of FRET ratio on the distance from the nucleus, endosomes present in each frame included in the threshold mask were binned according to the distance of their centroid from the nuclear membrane.

Video processing and analysis for particle tracking and vesicle intensity profile were carried out with ImageJ, ICY [49] and R studio. Following background subtraction and Gaussian smoothing the CFP, FRET sensitized, YFP and red fluorescence were treated to eliminate noisy pixels and smooth the images. The FRET ratio for each frame was computed by dividing FRET sensitized signal by CFP signal. The videos were then imported in ICY for spot detection and particle tracking procedure performed on the YFP signal [50]. Intensity profile for FRET ratio, YFP and FYVE2X were exported together with trajectory for each detected vesicle. Vesicle intensity profiles were then aligned in R studio according to their speed profile and direction. The vesicle mean FRET ratio was adjusted by substracting cytoplasmic mean FRET ratio.

MatLab code is fully available on GitHub.

Statistical analysis

For biochemichal, immunocytochemistry and microscopy-based experiments a minimum of three independent experiments (n) was performed and statistically significant estimates for each sample were obtained. For microscopy based quantification, cells were chosen arbitrarily according to the fluorescent signal in a separate channel, which was not used for quantification where it was possible. Values were presented as means ± SEM. *P* values were calculated using two-tailed Student's *t* test and one- or two-way ANOVA followed by Bonferroni's multiple comparison posttest (GraphPad Software). Statistical significance is indicated as follows: $*P < 0.05$, $**P < 0.01$, and $***P < 0.005$.

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