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1Rab11 activity and PtdIns(3)P turnover removes recycling cargo from 2endosomes

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30Abstract

31 Directional transport of recycling cargo from early endosomes (EE) to endocytic recycling
32compartment (ERC) relies on Phosphatidylinositol 3-phosphate (PtdIns(3)*P*) hydrolysis and
33activation of the small GTPase Rab11. However, how these events are coordinated is yet unclear.
34By using a novel genetically-encoded FRET biosensor for Rab11, we report that generation of
35endosomal PtdIns(3)*P* by the clathrin binding phosphoinositide 3-kinase Class 2 alpha (PI3K-C2 α)
36controls the activation of Rab11. Active Rab11, in turn, prompts the recruitment of the
37phosphatidylinositol 3-phosphatase myotubularin 1 (MTM1), eventually enabling the release of
38recycling cargo from the EE and its delivery towards the ERC. Our findings thus define that
39delivery of recycling cargo towards the ERC requires spatial and sequential coupling of Rab11
40activity with PtdIns(3)*P*-turnover.

41

42Introduction

43 Intracellular trafficking of endocytosed molecules ensures the delivery of plasma membrane
44components and receptor-associated ligands to several cellular compartments. After internalization,
45such molecules can be either degraded or re-used by returning to the plasma membrane ¹. This
46recycling pathway restores the composition of plasma membrane and is mediated by vesicular
47carriers that transfer endocytosed material from peripherally located early endosomes (PE) to the
48endocytic recycling compartment (ERC), a juxtannuclear tubulovesicular compartment ²⁻⁴. To be
49effective, this transport route requires regulated recruitment of molecular motors, membrane tethers,
50as well as lipid kinases and phosphatases in time and space⁵⁻¹². Such engagement is partly
51accomplished by key determinants of functional identity of organelles including
52phosphatidylinositol 3-phosphate (PtdIns(3)*P*) and the small GTPase Rab11 ^{9, 13-16}.

53 PtdIns(3)*P* is the major phosphoinositide residing on early endosomes where it serves as a
54membrane recognition site for the recruitment of proteins, thereby mediating endosomal fusion and
55maturation ^{17, 18}. PtdIns(3)*P* homeostasis is controlled by the coordinated action of lipid kinases and
56phosphatases. In particular, while phosphorylation of (PtdIns) to PtdIns(3)*P* requires members of
57the class II and class III phosphatidylinositol 3-kinase (PI3K) enzymes^{10, 19-21}, termination of
58PtdIns(3)*P* signaling relies on Myotubularins (MTMs), lipid phosphatases that convert PtdIns(3)*P*
59to (PtdIns) ²²⁻²⁵. Although endosome maturation and recycling of endocytosed cargos requires
60control of PtdIns(3)*P* levels by the action of lipid kinases and phosphatases ^{8, 22, 26}, the mechanism
61responsible for the regulated recruitment of these lipid metabolizing enzymes on endosomes
62remains largely unknown.

63 Extensive investigations demonstrated that members of the Rab protein family are
64coordinators of membrane domain formation and vesicle trafficking dynamics controlling the
65recruitment of endocytic regulators such as lipid kinases or phosphatases ²⁷. In particular, trafficking
66of endocytosed cargos toward the ERC is mediated by Rab11, a small GTPase enriched on ERC
67membranes and activated by signaling downstream of PI3K-C2 α -derived PtdIns(3)*P* pool ^{9, 10, 20, 28}.
68In its active GTP-bound form, Rab11 mediates recycling and sorting of endocytosed membrane
69components through the ERC^{12-14, 29}. However, whether the changes in Rab11 activity might
70determine the efficiency of membrane trafficking by controlling the phosphoinositide composition
71of endosomes is still unclear.

72 To monitor changes in Rab11 activity in living cells we developed a genetically-encoded
73FRET biosensor of active Rab11 named Activation Sensor Rab11 (AS-Rab11). Using this

74biosensor, we demonstrated that the increase in Rab11 activity on PtdIns(3) P^+ peripheral
75endosomes is important to control the release of recycling cargoes, via a circuit involving sequential
76clathrin/PI3K-C2 α -mediated PtdIns(3) P burst and subsequent Rab11/MTM1-dependent PtdIns(3) P
77hydrolysis.

79 Results

80 Development of a Rab11 FRET biosensor.

81 The activation cycle of Rab11 is essential to mediate the delivery of internalized plasma
 82 membrane components from PE to ERC^{1, 30}. To monitor the spatial and temporal regulation of
 83 Rab11 nucleotide exchange in living cells, a genetically encoded fluorescence resonance energy
 84 transfer (FRET)-based probe, named Activation Sensor Rab11 (AS-Rab11), was developed (Figure
 85 1a, Supplementary Figs. 1a, b). The probe includes the C-terminal region of FIP3 binding active
 86 Rab11 only³¹, a circularly permuted version of a modified monomeric yellow fluorescent protein
 87 (mcpVenus), a proteinase K-sensitive linker, a monomeric cyan fluorescent protein (mECFP) and
 88 human Rab11a (Figure 1a). In this probe design, an increase in GTP-loading of Rab11 promotes the
 89 binding of the C-terminal region of FIP3 to Rab11a, thus modifying the orientation of the two
 90 fluorophores and thereby increasing FRET which is represented by the 525 nm/475 nm
 91 (FRET/CFP) emission ratio^{32, 33} (Figure 1a). The positioning of Rab11 at the C-terminal end of AS-
 92 Rab11 allows correct functioning of the Rab11 C-terminal sequences required for membrane
 93 insertion (Figure 1a).

94 To validate the efficiency of the biosensor energy transfer in the presence of either GDP or
 95 GTP, the fluorescence emission profiles of AS-Rab11 were monitored using a fluorometric assay
 96 (see *Materials and Methods*). In comparison with the wild type form, constitutively active mutant
 97 versions of Rab11 lacking GTPase activity (AS-Rab11^{Q70L} and AS-Rab11^{S20V}) showed decreased
 98 fluorescence emission intensity at 475 nm and a concomitant increase at 525 nm (Figure 1b,
 99 Supplementary Fig. 1c red line). Consequently, the FRET/CFP ratio of AS-Rab11^{Q70L} and AS-
 100 Rab11^{S20V} were found significantly higher than the wild-type and the nucleotide-free (AS-
 101 Rab11^{N124I}) forms (Figure 1b, Supplementary Fig. 1c). In contrast, a dominant negative version of
 102 this biosensor (AS-Rab11^{S25N}, Supplementary Fig. 1c blue line) displayed 475 nm and associated
 103 525 nm emission higher and lower than the control, respectively (Figure 1b, Supplementary Fig. 1c
 104 cyan line, Supplementary Fig. 1d). A similar observation was made after proteinase-K treatment of
 105 AS-Rab11 wild-type (Supplementary Fig. 1c black line) that induced the cleavage of the amino
 106 acidic linker connecting the two fluorophores required to promote the energy transfer.
 107 Consequently, both AS-Rab11^{S25N} and proteinase-K-treated AS-Rab11^{wt} showed decreased FRET
 108 emission ratio (Figure 1b, Supplementary Fig. 1c blue and black lines), and similarly to a Rab11-
 109 GTP binding mutant (AS-Rab11^{RBD mutant}) (Figure 1b). In line with these results, increased FRET
 110 emission ratio and Rab11-GTP content were detected after co-expression of AS-Rab11^{wt} with
 111 SH3BP5, a Rab11-GEF³⁴ (Figures 1b, Supplementary Fig. 1e). On the contrary, co-expression of

112TBC1D9B, a Rab11-GAP³⁵ 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 inhibitor³⁶, 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 juxtannuclear
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

144the internalization of fluorescent transferrin (Tf-647), an early-recycling endosome marker¹⁶. In line
145with previous studies^{13, 14}, perinuclear accumulation of active Rab11 (Figure 2a left panel,
146pseudocolor map) and Tf-647 (Figure 2a left panel, gray scale) was observed. In addition,
147enlargement of the peri-plasmalemmal region showed overlap between the highest FRET signal
148(Figure 2a right panel, red line) and Tf-647 (Figure 2a right panel, black line). Accordingly, two-
149dimensional representation of pixel intensities (Figure 2a right panel, line intensity profile) along a
150line starting from the nucleus and reaching the plasma membrane (Figure 2a left panel, white line)
151showed almost perfect overlap between FRET ratio (Figure 2a right panel, red line) and Tf-647
152(Figure 2a right panel, black line) signals in both the perinuclear and peripheral region. In further
153agreement, analysis of colocalization, as determined by Pearson's coefficient, showed high
154correlation between FRET ratio and Tf-647 positivity both in the ERC and PE (Figure 2a right
155panel), indicating that active Rab11 is equally distributed in perinuclear and peripheral Tf-positive
156compartments.

157 To gain insight into the localization of active Rab11, early and recycling endosome specific
158markers were similarly studied by analyzing a red fluorescent tagged versions of either Rab4, Rab5
159or the PtdIns(3)*P* probe mCherry-FYVE2X. Identical distribution and strong colocalization were
160observed by fine mapping of active-Rab11 and mRFP-Rab4-positive structures, in pseudocolor and
161grayscale, respectively (Figure 2b). Conversely, endosomal membranes labelled by mRFP-Rab5
162colocalized with active Rab11 at the cell periphery but not at the perinuclear recycling compartment
163(Figure 2c). Similarly, active-Rab11 strongly co-localized with the early endosome marker
164PtdIns(3)*P*, as detected with the mCherry-FYVE2X probe, on peripheral but not on perinuclear
165membrane-bound structures (Figure 2d). These results indicate that PEs, in which active Rab11
166colocalized with PtdIns(3)*P*, correspond to early endosomes.

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

168 To examine the relationship between Rab11, PtdIns(3)*P* and recycling cargo in peripheral
169endosomes, the localization of Rab11 and Transferrin receptor (TfR) on PtdIns(3)*P*-positive
170structures was monitored during the continuous uptake of Tf. Confocal microscopy analysis
171revealed a frequent growth of tubular Rab11⁺/mCherry-TfR⁺ structures from PEs (Figure 3a).
172Furthermore, Tf uptake increased Rab11 activity (Supplementary Figs. 4a, b) and expression of a
173Rab11 dominant negative form inhibited Tf recycling and promoted its accumulation
174(Supplementary Figs. 4c-e), thus indicating that removal of recycling cargo from endosomes
175requires Rab11 activation. In agreement, increased FRET/CFP signal on the nascent vesicle began 5
176seconds before fission, concomitantly with a PtdIns(3)*P* burst, and reached maximal signal at the

177time of fission (Figures 3b, c, Supplementary Figs. 4f-h). Such activation kinetics did not rely on
178biosensor abundance as both temporal assessment and titration of AS-Rab11 level on endosomes
179showed robust and coherent biosensor response at various probe expression levels (Figure 3c grey
180line, Supplementary Figs. 4i). Unexpectedly, on the nascent recycling structure, Rab11 activation
181was initially preceded by the increase of PtdIns(3)*P*-levels but was later followed by a PtdIns(3)*P*
182decrease, starting at the time of fission (Figures 3b, c, Supplementary Figs. 4f-h). These results
183show that, on peripheral endosomes, PtdIns(3)*P* peaks concomitantly with Rab11 activation and
184declines with the fission of recycling cargo-containing vesicles.

185 To gain insight into this process, COS-7 cells expressing perinuclear localized AS-Rab11
186were bleached to avoid contaminating signals from the ERC region and movement of active Rab11⁺
187vesicles was analyzed after Tf addition (Figure 3d). Rab11⁺ vesicles followed long-range linear
188movements and then eventually collapsed into ERC membranes (Figure 3d), thus indicating that
189juxtannuclear AS-Rab11-positive structures derived in part from peripheral endosomes. Accordingly,
190AS-Rab11-positive endosomal structures accumulated in the perinuclear region during the
191continuous uptake of Tf and resulted in a steady state after 15 minutes (Supplementary Figs. 5a-e).
192By interfering with microtubule polymerization using a treatment with Nocodazole, vesicles
193carrying an active Rab11 failed to appear as a linear series of dots over time (Supplementary Fig.
1945f). This indicated that disruption of microtubule-dependent transport abolished long-range
195movements of AS-Rab11-positive membranes without compromising Tf accumulation
196(Supplementary Figs. 5f-h). Consistently, displacement of endocytic structures, as well as Rab11
197activation were decreased upon Nocodazole treatment (Figures 3e, f). These data suggested that
198minus end-directed microtubule motor transport is required for endocytic structure movement. To
199test this hypothesis, acute inactivation of retrograde transport using Ciliobrevin D, a Dynein
200inhibitor³⁷, was performed. Ciliobrevin D treatment decreased long-range retrograde motion of
201active Rab11⁺ vesicles and had a minor impact on vesicle linear movement/displacement (Figures
2023e, g, h) consistently with multiple Rab11/microtubule-dependent trafficking routes³⁸. Finally, both
203Nocodazole and Ciliobrevin D treatments decreased juxtannuclear accumulation of endocytosed
204transferrin (Figures 3i, j).

205 Overall, these data indicate that transferrin receptor is removed from PEs and transported to
206the ERC through a local increase of PtdIns(3)*P*, the activation of Rab11, the hydrolysis of
207PtdIns(3)*P*, and eventually the dynein-mediated vesicular transport.

208 *PI3K-C2α controls Rab11 activity on PtdIns(3)P⁺ endosomes*

209 In early endosomes, PtdIns(3)P is mainly produced by Class III PI3K. However, a small but
 210 significant amount of PtdIns(3)P, ranging up to 20%, derives from Class II PI3Ks ³⁹ and is
 211 putatively required for Rab11 activation ^{9, 10}. To further determine whether the activation of Rab11
 212 preferentially depended on either Class II or III PI3K, modulation of the AS-Rab11 probe was
 213 studied after either PI3K-C2α or Vps34 knock-down or Vps34 inhibition (Supplementary Figs. 6a-
 214c). As expected, knock-down of PI3K-C2α induced a 20% loss of PtdIns(3)P as well as a 50% drop
 215 in Rab11 activity (Figures 4a, b). On the contrary, knock-down or inhibition of Vps34 by VPS34-
 216 IN1 decreased PtdIns(3)P cell content by 80% but failed to significantly reduce the levels of active
 217 Rab11 (Figures 4a, b). This highlights the distinct role of PI3K-C2α in controlling Rab11 activity in
 218 PE. In addition, PI3K-C2α localization during cargo release from PtdIns(3)P⁺ structures was
 219 imaged. GFP-PI3K-C2α co-localized with mCherry-FYVE2X during the fission of mECFP-Rab11
 220 positive structures (Figures 4c, Supplementary Figs. 6d-f), while it was undetected in the newly
 221 formed mECFP-Rab11⁺/mCherry-TfR⁺ membranes (Supplementary Figs. 6e-g). Interestingly, such
 222 PI3K-C2α localization strictly depended on its N-terminal Clathrin binding domain ⁴⁰, as loss of this
 223 domain ⁴¹ resulted in a diffuse cytosolic distribution around the PE (Figure 4d).

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

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

241PI3P-only producing PI3K-C2 α form (PI3K-C2 α^{CIII}) (Figure 4i). On the contrary, expression of a
 242kinase inactive mutant (PI3K-C2 α^{R1251P}) did not restore juxtannuclear Tf localization (Figure 4i), thus
 243demonstrating that Tf delivery to perinuclear endosomes is controlled by the PI3K-C2 α -dependent
 244PI3P production. In line with these results, silencing of PI3K-C2 α 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-C2 α -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- γ S 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
 259⁹ (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- γ S 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-C2 α 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).

272 To further characterize the impact of MTM1 in the control of Rab11-mediated intracellular
273trafficking, Rab11⁺ vesicles detaching from PEs were analyzed after RNAi-mediated
274downregulation of MTM1 (MTM1-KD). Loss of MTM1 as well as expression of GFP-Rab11^{S25N}
275decreased the number of Rab11 positive fission events from PEs (Figure 5h), without affecting
276vesicle speed (Supplementary Fig. 7f). Furthermore, the residence time of Rab11 positive structures
277on PEs increased in both conditions (Figure 5i). Therefore, either impaired activation of Rab11 or
278lack of the phosphatase activity delayed fission. In agreement, MTM1-KD and GFP-Rab11^{S25N}-
279expressing cells displayed increased Tf content and decreased perinuclear accumulation of the
280recycling cargo (Figures 5j, k, Supplementary Fig. 7g). To identify the lipid kinase that antagonizes
281MTM1 activity, the rescue of Tf uptake and Tf accumulation of the recycling cargo at the ERC
282were performed. Acute perturbation of PtdIns(3)*P* synthesis by Vps34 inhibition partially restored
283Tf accumulation and perinuclear storage in MTM1-KD cells (Figures 5j, k) without affecting the
284increase in Rab11 activation due to MTM1 loss (Figure 5e). In agreement, Rab11-mediated fission
285events appeared more frequent in MTM1-KD/VPS34-IN1 than in MTM1-KD cells (Figure 5h),
286thus indicating that fission requires a significant reduction of PtdIns(3)*P*. In the absence of MTM1,
287knock-down of either PI3K-C2 α alone or in combination with Vps34 inhibition led to decreased
288Rab11 activity. Conversely, in the absence of MTM1, Vps34 inhibition alone was not able to
289restore increased Rab11 activity (Figure 5e). Therefore, PI3K-C2 α is the main kinase driving
290PtdIns(3)*P* production required for Rab11 activation, consequent fission and Tf recycling (Figure
2915h, j, k).

292 Taken together, these results show that removal of recycling cargo from peripheral endosomes
293depends on subsequent PI3K-C2 α -mediated PtdIns(3)*P* production, Rab11 activation and MTM1-
294dependent PtdIns(3)*P* destruction, leading to fission of vesicles and their eventual dynein-mediated
295transport to the ERC.

296 Discussion

297 Removal of recycling cargo from peripheral PtdIns(3) P^+ endosome requires PtdIns(3) P
298 hydrolysis and the activation of the small GTPase Rab11. However, whether these events are linked
299 is unknown. Therefore, a genetically-encoded FRET biosensor for Rab11 was generated to detect
300 spatial and temporal variations of Rab11 activity in endosomes. This biosensor named AS-Rab11
301 was proven to be effective into limited diffusional space, such as in membrane and vesicular
302 compartments and its activity was found to depend on both positive and negative Rab11 regulators,
303 such as Rab11 GEF, GAP and GDI³⁴⁻³⁶. Using AS-Rab11, we revealed that: (I) Rab11 activation is
304 initiated on PtdIns(3) P -positive membranes where sorting of recycling cargo occurs; (II) Rab11
305 activation level determines the release rate of membranes destined to the ERC; and (III) such
306 release required MTM1, a PtdIns(3) P phosphatase, which was found to interact with active Rab11.
307 These results establish that removal of recycling cargo from peripheral PtdIns(3) P^+ endosome
308 requires coupling of Rab11 activity and PtdIns(3) P turnover (Figure 6).

309 Extensive time lapse analyses and biochemical experiments revealed enrichment of active-
310 Rab11 on juxtannuclear positioned ERC and peripheral PtdIns(3) P^+ endocytic structures. In addition,
311 they evidenced a critical role of activated Rab11 in the release of Transferrin receptors (TfR) from
312 PtdIns(3) P^+ membranes. Given that PtdIns(3) P is a bona-fide marker¹⁸ of EE, a compartment where
313 recycling cargoes are sorted and directed toward the ERC or plasma membrane^{14, 28}, our data
314 suggest that Rab11 activation is initiated on EE membranes where sorting of recycling cargoes
315 occurs. In agreement, the direct visualization of active Rab11 patches localizing with TfR on
316 PtdIns(3) P^+ membranes corroborate these evidences. Our experiments show that membranes
317 decorated by active Rab11 are not maintained indefinitely on PtdIns(3) P^+ structures but are
318 delivered from peripheral to juxtannuclear recycling compartment. These observations define that,
319 differently from active Rab5 that mediates the expansion of Rab5 domain on early endosomes,
320 active Rab11 critically affects cargo flow by recruiting the protein machinery involved in vesicle
321 transport. In line with this view, our results evidenced that active Rab11 vesicles detaching from
322 peripheral endosomes accumulate on ERC membranes in a dynein-dependent manner.

323 Our observations extend the previous identification of PI3K-C2 α as a key controller of Rab11
324 activation on endosomes⁹ and define that localization of PI3K-C2 α on endosomes strictly depends
325 on its clathrin binding domain^{20, 40}. Notably, depletion of PI3K-C2 α delays the kinetic of vesicle
326 release from PtdIns(3) P^+ structure where TfR sorting takes place¹, thus linking the role of PI3K-
327 C2 α to endosomal sorting. Accordingly, depletion of PI3K-C2 α , as well as loss of its catalytic

328activity, decreases both activity and fission of Rab11-positive vesicles from PtdIns(3)*P*⁺ structures,
329thereby mimicking the phenotype observed in cells expressing dominant negative Rab11.

330 In light of the highly dynamic Rab11 activation on PtdIns(3)*P*⁺ structures, and the distinct
331phosphoinositide composition of EE and the perinuclear recycling compartment ⁴², a
332phosphoinositide conversion can be expected between these two Rab11 positive membrane
333domains. Our data demonstrated that this transition is controlled by MTM1, which was found to
334interact with active Rab11. MTM1 was shown to antagonize the Class II and Class III derived
335PtdIns(3)*P* pools in *D. melanogaster* and *C. elegans* and was demonstrated to be essential in the
336exit of cargos from PtdIns(3)*P* endosomes ^{8, 26}. Accordingly, MTM1⁺/Rab11⁺ vesicles were
337observed during removal of recycling cargo from PtdIns(3)*P* compartment, thus indicating that
338active Rab11 provides a signal to control MTM1 localization. Given that recycling vesicles require
339dynein to reach the ERC, removal of PI3P can be explained by the fact that the presence of this
340lipid, a well-known activator of centrifugal kinesin-mediated transport⁴³, might disturb this
341centripetal trafficking.

342 The development and application of AS-Rab11 to quantitatively analyze Rab11 activity in
343living cells allowed to dissect and analyze the initial step of the PtdIns conversion mechanism ⁴²
344required for the exit of recycling cargo from endosomes. Our data indicate that PI3K-C2α provides
345a spatially localized and temporally controlled PtdIns(3)*P* pool sufficient to activate Rab11 on early
346endosomes, allowing establishment and maintenance of receptor recycling towards the ERC.
347Activation of Rab11 eventually contributes to the recruitment of MTM1 and the ensuing reduction
348of PtdIns(3)*P* level on membranes destined to the perinuclear endosome.

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357 Author Contribution:

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

363 Conflict of interest:

364 EH is a co-founder of Kither Biotech, a company involved in the development of PI3K
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- 470

471 **Figure Legends**

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

473a. Tridimensional representation of the genetically encoded fluorescence energy transfer
474(FRET) probe named AS-Rab11 (Activation Sensor Rab11) in its inactive (bound to GDP, left
475panel) or active conformation (bound to GTP, right panel). Yellow and light blue β -barrels
476represents a yellow- and cyan-emitting mutant of fluorescent proteins, respectively. Sea-green α -
477helix, black line and green β -barrels structures indicate Rab11-GTP binding domain (C-terminal
478region of FIP3), proteinase K sensitive linker domain and Rab11a protein, respectively. In this
479probe design, an increase of Rab11 GTP-loading promotes the binding of the C-terminal region of
480FIP3 to Rab11a, modifying the relative orientation of the two fluorophores and thereby increasing
481FRET signal (left and right panel).

482b. Quantifications of FRET efficiency of AS-Rab11^{wt}, AS-Rab11 mutant forms, proteinase K
483treated AS-Rab11^{wt}; AS-Rab11^{wt} co-expressed with the indicated GEFs and their target GTPase
484(SH3BP5, RABEX-5, TIAM1, respectively); AS-Rab11^{wt} co-expressed with the indicated GAPs
485and their target GTPase (TBC1D9B, RN-3, ARHGAP15, respectively), and AS-Rab11^{wt} or AS-
486Rab11^{N206X} co-expressed with RabGDI (n=12 independent experiments; data represent mean \pm
487SEM, *** p< 0.005, One-way ANOVA).

488c. Representative FRET/CFP ratio images of AS-Rab11 biosensor in COS-7 cells (pseudocolor
489images represent FRET/CFP ratio intensity values). The upper and lower limits of the FRET/CFP
490ratio are shown on the left side bar (left panel). Magnification of FRET/CFP ratio images and AS-
491Rab11 localization in juxtannuclear (upper) and peripheral endocytic structures (lower) [right panel;
492pseudocolor images represent FRET/CFP ratio intensity values; grayscale image indicates emission
493of mcpVenus (AS-Rab11) after its direct excitation]. The scale bars represent 10 μ m.

494d. Representative line intensity profile of FRET sensitized (magenta) and CFP (cyan) signal
495detected in juxtannuclear (upper panel) and peripheral (lower panel) endocytic structures. The
496magenta and cyan image represent FRET sensitized and CFP signals, respectively. The scale bars
497represent 1 μ m.

498e. Scatter plot of sensitized FRET intensities as a function of CFP intensities in AS-Rab11
499expressing cells (black and red dots represent the sensitized FRET and CFP intensity value of
500cytosolic and membrane-bound structures, respectively; regression line is in black) (upper panel)
501(n=4 independent experiments). Quantification of FRET/CFP ratio between cytosolic and endocytic
502structure labelled by AS-Rab11 (lower panel) (n=50 independent experiments; data represent mean
503 \pm 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 \pm SEM). Quantification of FRET/CFP ratio between juxtannuclear (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 \pm SEM,
509*** $p < 0.005$, t-test).

510

511 **Fig. 2: Juxtannuclear and peripheral localization of active Rab11 on distinct endosome**
 512 **populations.**

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

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

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

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

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

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

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

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

540 Quantification in juxtannuclear (ERC, black bar) and peripheral (PE, red bar) endosomes of
541 colocalization percentage (Pearson's correlation coefficient) between FRET/CFP signal and Rab5
542 labelled endocytic structure (n=15 independent experiments; data represent mean \pm SEM, *** p<
543 0.005, t-test) (right lower panel).

544d. Representative localization of active Rab11 on PtdIns(3)P-positive endosomes. FRET/CFP
545 ratio images (left panel, pseudocolor mode) of AS-Rab11 biosensor on endosomes labelled by
546 mCherry-FYVE2X (left panel, gray scale). White line defines the region over which FRET/CFP
547 ratio and mCherry-FYVE2X signal were measured. The scale bar represents 1 μ m.

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

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

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

555a. Representative time-lapse series of cells co-expressing mCherry-Transferrin Receptor (TfR),
556 mECFP-Rab11 and GFP- FYVE2X. White circles represent membrane bound structures. The scale
557 bar represents 1 μm .

558b. Representative time-lapse series of cells co-expressing AS-Rab11 and mCherry-FYVE2X.
559 White circles represent membrane bound structures. The pseudocolor mode represents the
560 FRET/CFP ratio; the gray scale indicates the emission of mcpVenus after its direct excitation. The
561 scale bar represents 1 μm .

562c. Quantification of FRET/CFP ratio (green line), mCherry-FYVE2X fluorescent emission
563 (orange line) and AS-Rab11 mcpVenus emission (gray line) as a function of time in 28 individual
564 vesicle tracks directed towards the ERC. The time point of detachment from early endosomes was
565 recorded and used to shift the time courses so that all 28 detachment events were synchronized at
566 the chosen time point of 0 s. The normalized FYVE2X is shown on primary vertical axis. The
567 normalized value of FRET/CFP ratio is shown on secondary vertical axis (right) (n=4 independent
568 experiments).

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

574e. Frequency distribution of Rab11⁺ vesicle displacement from the origin in cells expressing
575 GFP-Rab11^{S25N} (green line) or GFP-Rab11 treated with either vehicle (DMSO, black line),
576 Nocodazole, a microtubule depolymerizing drug (red line) or the dynein inhibitor, CiliobrevinD
577 (blue line) (n=4 independent experiments, *** p< 0.005, * p< 0.05, two-way ANOVA).

578f. Quantification of Rab11 activation in the perinuclear area. Cells were treated with either
579 vehicle (DMSO, black line) or Nocodazole (red line) (n=14 independent experiments; data
580 represent mean \pm SEM, ** p< 0.01, t-test).

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

584h. Frequency distribution of linearity of movement of Rab11⁺ vesicles in cells expressing GFP-
585 Rab11^{S25N} (green line) or GFP-Rab11 treated with either vehicle (DMSO, black line), Nocodazole
586 (red line) or Ciliobrevin D (blue line) (n=4 independent experiments; data represent mean \pm SEM,
587 *** p< 0.005, two-way ANOVA).

588i. Representative image of endocytosed transferrin localization in cells expressing GFP-
589 Rab11^{S25N} or GFP-Rab11 treated with either vehicle (DMSO), Nocodazole or Ciliobrevin D (n=4
590 independent experiments). The scale bar represents 10 μ m.

591 j. Quantification of perinuclear localization of fluorescent transferrin in cells expressing GFP-
592 Rab11^{S25N} (green bar) or treated with either DMSO/scramble siRNA (DMSO, black bar),
593 Rab11 siRNA 1 (RAB11-KD₁, gray bar), Rab11 siRNA 2 (RAB11-KD₂, light blue bar),
594 Nocodazole (red bar) or the dynein inhibitor, Ciliobrevin D (blue bar) (n=12 independent
595 experiments; data represent mean \pm SEM, *** p< 0.005, One-way ANOVA).

596 **Fig. 4: PI3K-C2 α -dependent Rab11 activation on PtdIns(3)P-positive endosomes.**

597a. Quantification of PtdIns(3)P abundance in COS-7 cells treated with either DMSO/Scramble
598siRNA (Control, black bar), VPS34 inhibitor (VPS34-IN1, red bar) or PI3K-C2 α siRNA (PI3KC2 α -
599KD, blue bar) (n=15 independent experiments; data represent mean \pm SEM, *** p< 0.005, * p<
6000.05, One-way ANOVA).

601b. Quantification of Rab11 activity in COS-7 cells treated with either DMSO/Scramble siRNA
602(Control, black bar), VPS34 inhibitor (VPS34-IN1, red bar) or PI3K-C2 α siRNA (PI3KC2 α -KD,
603blue bar). (n=12 independent experiments; data represent mean \pm SEM, * p< 0.05, One-way
604ANOVA).

605c. Representative time-lapse series of cells co-expressing mCherry-FYVE2X, GFP-PI3K-C2 α
606and mECFP-Rab11 (gray scale). White circles represent membrane-bound structures (n=6
607independent experiments). The scale bar represents 1 μ m.

608d. Representative image of cells co-expressing mCherry-FYVE2X, mECFP-Rab11 (gray scale)
609and GFP-PI3K-C2 α (PI3K-C2 α) or its mutant version GFP-PI3K-C2 α - Δ Clathrin (PI3K-C2 α -
610 Δ Clath). White circles represent membrane bound structures (n=6 independent experiments). The
611scale bar represents 1 μ m.

612e. Quantification of the number of Rab11-associated fission events generated from mCherry-
613FYVE2X positive membranes. COS-7 cells expressing GFP-Rab11^{S25N} (green bar) or GFP-Rab11
614treated with either DMSO/Scramble siRNA (Control, black bar), VPS34 inhibitor (VPS34-IN1, red
615bar), PI3K-C2 α siRNA (PI3KC2 α -KD, blue bar). (n=10 independent experiments; data represent
616mean \pm SEM, * p< 0.05, One-way ANOVA).

617f. Residence time of GFP-Rab11^{S25N} (green bars) or GFP-Rab11 structures on mCherry-
618FYVE2X-positive membranes (black, blue, red bars). GFP-Rab11 expressing cells were treated
619with either DMSO/Scramble siRNA (Control, black bar), VPS34 inhibitor (VPS34-IN1, red bar),
620PI3K-C2 α siRNA (PI3KC2 α -KD, blue bar). (n=10 independent experiments; data represent mean \pm
621SEM, *** p< 0.005, ** p< 0.01, Two-way ANOVA).

622g. Quantification of internal transferrin percentage in cells expressing GFP-Rab11^{S25N} (green
623bar) or GFP-Rab11 treated with either DMSO/Scramble siRNA (Control, black bar), VPS34
624inhibitor (VPS34-IN1, red bar), PI3K-C2 α siRNA (PI3KC2 α -KD, blue bar). (n=12 independent
625experiments; data represent mean \pm 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-C2 α siRNA (PI3KC2 α -KD, blue bar) (n=12 629independent experiments; data represent mean \pm 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-C2 α siRNA 632(PI3KC2 α -KD, red bar), PI3K-C2 α siRNA and PI3K-C2 α ^{wt} siRNA resistant (PI3KC2 α -KD/ 633PI3KC2 α ^{wt}, blue bar), PI3K-C2 α siRNA and PI3K-C2 α ^{R1251P} siRNA resistant (PI3KC2 α -KD/ 634PI3KC2 α ¹²⁵¹, green bar), PI3K-C2 α siRNA and PI3K-C2 α ^{CIII} siRNA resistant (PI3KC2 α -KD/ 635PI3KC2 α ^{CIII}, purple bar) (n=12 independent experiments; data represent mean \pm SEM, *** p< 6360.005, One-way ANOVA).

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

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

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

648c. In vitro assessment of the association between recombinant Rab11-GTP and MTM1.
649 Representative western blot of recombinant Rab11 loaded with GDP or GTP γ S and probed for
650 MTM1 interaction. Quantification of recombinant MTM1 pulled-down by recombinant Rab11
651 loaded with GDP or GTP γ S (n=4 independent experiments, data represent mean \pm SEM, ** p<
652 0.01, t-test) (uncropped blots are shown in Supplementary Figure 8).

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

656e. Quantification of active Rab11 levels in COS-7 cells treated with either Scramble siRNA
657 (Control, black bar), MTM1 siRNA (MTM1-KD, blue bar), MTM1 siRNA and PI3K-C2 α siRNA
658 (MTM1-KD/PI3K-C2 α -KD, dotted blue bar), MTM1 siRNA and VPS34 inhibitor (MTM1-
659 KD/VPS34-IN1, dashed blue bar), MTM1 siRNA in combination with PI3K-C2 α siRNA and
660 VPS34 inhibitor (MTM1-KD/PI3K-C2 α -KD/VPS34-IN1, red bar). (n=12 independent experiments;
661 data represent mean \pm SEM, ** p< 0.01, One-way ANOVA).

662f. Representative immunofluorescence of COS-7 cells, showing peripheral and perinuclear
663 colocalization of MTM1 with Rab11. Peripheral (left) and perinuclear (right) magnification are
664 shown in the bottom part of the panel (n=6 independent experiments). White arrows highlight
665 colocalization. The scale bar represents 15 μ m.

666g. Representative time-lapse series of cells co-expressing mCherry-Transferrin receptor (TfR),
667 mECFP-Rab11 (gray scale) and GFP-MTM1. White circles represent membrane-bound structures
668 (n=6 independent experiments). The scale bar represents 1 μ m.

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-C2 α siRNA (MTM1-KD/PI3K-C2 α -KD, dotted blue bar), MTM1 siRNA 673and VPS34 inhibitor (MTM1-KD/VPS34-IN1, dashed blue bar), MTM1 siRNA in combination 674with PI3K-C2 α siRNA and VPS34 inhibitor (MTM1-KD/PI3K-C2 α -KD/VPS34-IN1, red bar) 675(n=12 independent experiments; data represent mean \pm 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 \pm 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-C2 α siRNA (MTM1-KD/PI3K-C2 α -KD, 685dotted blue bar), MTM1 siRNA and VPS34 inhibitor (MTM1-KD/VPS34-IN1, dashed blue bar), 686MTM1 siRNA in combination with PI3K-C2 α siRNA and VPS34 inhibitor (MTM1-KD/PI3K-C2 α - 687KD/VPS34-IN1, red bar) (n=12 independent experiments; data represent mean \pm 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-C2 α siRNA 692(MTM1-KD/PI3K-C2 α -KD, dotted blue bar), MTM1 siRNA and VPS34 inhibitor (MTM1- 693KD/VPS34-IN1, dashed blue bar), MTM1 siRNA in combination with PI3K-C2 α siRNA and 694VPS34 inhibitor (MTM1-KD/PI3K-C2 α -KD/VPS34-IN1, red bar) (n=12 independent experiments; 695data represent mean \pm SEM, *** p< 0.005, ** p< 0.01, One-way ANOVA).

696 **Fig.6: Trafficking of recycling cargo from peripheral endosome to ERC requires Rab11 activa-**
697 **tion and PtdIns(3)P turnover.**

698 On a peripheral PtdIns(3)P membrane a transient and local burst of PI3K-C2 α -derived
699 PtdIns(3)P triggers Rab11 activation (first and second panel from the left). Active Rab11 (Rab11-
700 GTP) recruits MTM1, a PtdIns(3)P phosphatase, that catalyzes PtdIns(3)P hydrolysis (third panel
701 from the left). PtdIns(3)P reduction allows vesicle fission and trafficking of cargo towards the ERC
702 (rightmost panel).

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705

706 **Materials and Methods:**

707 *Antibodies*

708 The following antibodies were used in this study: mouse-anti-PI3K-C2 α (BD Biosciences
709 611046, western blotting (WB) 1:500), mouse-anti-Rab11 (BD Biosciences 610656, WB 1:1000),
710 rabbit-anti-MTM1 (SIGMA HPA010008, WB 1:1000), mouse-anti-FLAG (SIGMA clone M2, WB
711 1:2000), mouse-anti-GFP (ABCAM ab127417, WB 1:1000), rabbit-anti-VPS34 (Novus Biologicals
712 NB110-87320SS, WB 1:1000), mouse-anti-MTMR4 (Santa Cruz sc-373922, WB 1:500), mouse-
713 anti-MTMR6 (ABCAM ab69875, WB 1:1000), mouse-anti-MTMR9 (Santa Cruz sc-514366, WB
714 1:1000), rabbit-anti-FIP2 (ABCAM ab76892, WB 1:1000), rabbit-anti-FIP4 (Biorbyt orb215321,
715 WB 1:1000). Anti-mouse IgG (ab131368, WB 1:5000) Anti-Rabbit IgG (A0545 SIGMA, 1:5000).
716 anti mouse/rabbit IgG Alexa fluor 488/568 (IF 1:1000).

717 *SiRNA and plasmid transfection*

718 All siRNAs used in this study were 21-, 23-, or 27-base oligonucleotides including 3'-dTdT
719 overhangs. For silencing, the following siRNAs were used targeting the human isoform: PI3K-C2 α
720 5'-GGCAAGATATGTTAGCTTT-3', MTM1 5'-GATGCAAGACCCAGCGTAA-3'. The
721 scrambled control siRNA used throughout this study corresponded to the sequence 5'-
722 ATGAGTTAGATGCGTTCTA-3'.

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

727 For transient overexpression of proteins in silenced cells, plasmids were transfected on day 4
728 12 h before analysis using Lipofectamin 2000 (Invitrogen). For transient overexpression of proteins
729 in untreated cells, plasmids were transfected 12 h before analysis using Lipofectamin 2000
730 (Invitrogen).

731 *Recombinant protein production*

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

735 Recombinant proteins (GST-Rab11a, GST-Rab5a, GST-Rab7a) were purified (elution 10 mM
 736 glutathione, PBS), dialyzed, frozen in liquid nitrogen, and stocked (50% glycerol in Tris-HCl
 737 50 mM 5 mM MgCl₂, 100 mM NaCl) at -80°C. His-Flag-tagged MTM1 was generated according
 738 to previously established protocol²⁵. In brief, Flagged MTM1 was clone in Pqe vector and bacteria
 739 were grown in 2X-YT (1% Yeast extract, 2% 1% bactotryptone, 2.5mM NaOH and 0.5% NaCl)
 740 enriched medium until mid-log phase. Induction was performed with 1mM IPTG at 16°C for 12 hr.
 741 Soluble protein fraction was purified, dialyzed, and stocked (50% glycerol in Tris-HCl 50 mM
 742 5 mM MgCl₂, 100 mM NaCl, 0.5% Triton).

743 *Plasmids*

744 The 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
 746 biosensor molecules into limited diffusional space, such as in membrane and vesicular
 747 compartments. Rab11 binding domain (RBD11) was fused with RBD11-circularly permuted
 748 Venus (mcpVenus) at residue 195, while cyan fluorescent protein (mECFP)-Rab11a fusions were
 749 first constructed. Two repetition of a linker encoding for a 17-mer unstructured soluble and
 750 proteinase-K sensitive polypeptide (GSTSGSGKPGSGEGSTK)⁴⁴ was then cloned by PCR that
 751 allow to maximize the FRET change between the active and inactive state. To construct RBD11-
 752 cpVenus, polymerase chain reaction (PCR) was used to amplify amino-acids 649-756 of FIP3 using
 753 the primers: 5'-CTAGCTAGCATGGGCCTGCAGGAGTACCACA-3' and 5'-
 754 GCTCTAGAATGGGCACCCGCGACG-3', and pGEX- FIP3 RBD11 as a template⁹. mcpVenus
 755 was amplified using the primers: 5'-GGTAGTGGTGAATTCATGCTCGGAGCAGTCCTGA-3'
 756 and 5'-ATCCCCTCGAGAGCACGGGGCCGTCGCCGAT-3' using ICUE3 FRET probe⁴⁵ as a
 757 template. 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
 759 linker (GGSG), and mcpVenus. This was cloned. To construct mECFP-Rab11a, a construct
 760 encoding mECFP was amplified with the primers: 5'-
 761 AAGCGGCCGCATGGTGAGCAAGGGCGAGGAGCTG-3' and 5'-
 762 GGTGCCCATTTCTAGAAGTTCCACGGGGGTACCAGCCTTGACAGCTCGT-3'. Rab11a
 763 was amplified with the primers: 5'-GCTCTAGAATGGGCACCCGCGACG-3' and 5'-
 764 GCGGATCCAATGCCTTAGATGTTCTGACAGCACTGC-3' using a Rab11a expression
 765 construct as a template. Both fragments were then digested gel purified and subcloned in PGEM
 766 3tEasy vector (Promega). The resulting fragment contained, from the 5'-end: a NotI site, a mECFP,
 767 a 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'-
774GGCCGCTTTTAGTTGATCCCTCGCCGGAGCCGGGTTTTCTGATCCTGAAGTTGATCCC
775C-3' and 5'-
776TATATATATATATACTCGAGGGATCAACTTCAGGATCAGGAAAACCCGGCTCCGGC
777GAGGG-3'and 5'-
778CCGGGCTTGCCGCTGCCGGAAGTAGAGCCTTTAGTTGATCCCTCGCCGGAGCCGGG-3'
779and 5'-
780TATATATATATATGCGGCCGCTTTTAGTTGATCCTTCTCCTGATCCGGGCTTGCCGCTGCCG
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. Constitutively 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'-
795GATTCTGGTGTGGAAAGAATAATCTCCTGTCTCG-3', 5'-
796CGAGACAGGAGATTATTCTTTCCAACACCAGAATC-3' and 5'-
797GTTGTCCTTATTGGAGATGTTGGTGTGGAAAGAGTA-3', 5'-
798TACTCTTTCCAACACCAACATCTCCAATAAGGACAAC-3' and 5'-
799CAACTCCGCCTGCAGGACGCCGCCAGGATCATCGTGGCCATCAT-3', 5'-
800ATGATGGCCACGATGATCCTGGCGGCGGCGTCTGCGAGGCGGAAGTTG-3' and 5'-
801GTTATCATGCTTGTGGGCATTAAGAGTGATCTACGTCATCTC-3', 5'-

802GAGATGACGTAGATCACTCTTAATGCCACAAGCATGATAAC-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 encoding
 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% Triton 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.

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

837 HEK 293T cells growing in 10-cm dishes were transfected with DNA mixtures containing 10
 838 μg of pcDNA3.1(-myc/His)-Flag- SH3BP5 or 10 μg of pcDNA3.1(-myc/His)-AS-Rab11 or both,
 839 using calcium phosphate method. 48 h after transfection, cultures were harvested and homogenized
 840 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,
 841 1% Triton X-100, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride). Cytosol was
 842 obtained by centrifuging the lysates at 20,000g for 30 min at 4 °C and protein concentration was
 843 determined by Bradford method. 1 mg of cytosol was incubated with FLAG M2 antibody (SIGMA,
 844 S.Louis, Missouri, USA) or with 1 μg of anti-GFP antibody (ABCAM, Cambridge, UK) for 2 hours
 845 and incubated on a rotating rack for 1 hour at 4 °C with Protein-G sepharose beads (GE,
 846 Buckinghamshire, UK). Samples were collected by centrifugation and washed six-times with
 847 phosphate wash buffer (10 mM NaH_2PO_4 , 137 mM NaCl, and 2.7 mM KCl). Bound FLAG-
 848 SH3BP5, FLAG-TBC1D9B or GFP-AS-Rab11 protein complexes were then eluted by adding
 849 Laemmli sample buffer. SDS-PAGE and western blotting followed standard procedures. Similar
 850 approach was employed for the RabGAP TBC1D9B using the following lysis buffer: 50 mM Tris-
 851 HCl, pH 7.4, 0.2 mM GTP, 10 mM MgCl_2 , 100 mM NaCl, 1% Triton X-100, 50 mM sodium
 852 fluoride, 1 mM phenylmethylsulfonyl fluoride).

853 ***Immunoprecipitation assay for interaction of AS-Rab11 with GDI***

854 HEK 293T cells growing in 10-cm dishes were transfected with DNA mixtures containing 10
 855 μg of pcDNA3.1(-myc/His)-Flag-RabGDI or 10 μg of pcDNA3.1(-myc/His)-AS-Rab11 or both,
 856 using calcium phosphate method. 48 h after transfection, cultures were harvested and gently
 857 homogenized in 0.5 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, 0.2 mM GDP, 10 mM MgCl_2 , 50
 858 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride). Cytosol was obtained by centrifuging
 859 the lysates at 20,000g for 30 min at 4 °C and protein concentration was determined by Bradford
 860 method. 1 mg of cytosol was incubated with FLAG M2 antibody (SIGMA, S.Louis, Missouri, USA)
 861 or with 1 μg of anti-GFP antibody (ABCAM, Cambridge, UK) for 2 hours and incubated on a
 862 rotating rack for 1 hour at 4 °C with Protein-G sepharose beads (GE, Buckinghamshire, UK).
 863 Samples were collected by centrifugation and washed six-times with phosphate wash buffer (10 mM
 864 NaH_2PO_4 , 137 mM NaCl, and 2.7 mM KCl). Bound FLAG-GDI or GFP-AS-Rab11 protein
 865 complexes were then eluted by adding Laemmli sample buffer. SDS-PAGE and western blotting
 866 followed standard procedures.

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

869 HEK293T cells cultured in 6 well plate dishes and transfected for 48 h were radiolabeled for 4
 870 h with ^{32}P (6.0 MBq per dish) in phosphate-free DMEM (Invitrogen, Cat. Number 11971025). The
 871 expression levels of AS-Rab11 proteins and mutant forms were assessed by immunoblot analysis
 872 with the anti-GFP antibody (ABCAM, Cambridge, UK). The labeled cells (7×10^5 cells) were lysed
 873 with 0.3 ml of an ice-cold solubilizing buffer consisting of 40 mM Tris-HCl (pH 7.5), 100 mM
 874 NaCl, 20 mM MgCl_2 , 1 mM Na_3VO_4 , 1 mM dithiothreitol, 1% (w/v) Triton X-100, and 2 $\mu\text{g}/\text{ml}$
 875 aprotinin and clarified. The precleared lysates were incubated with anti-GFP antibody-immobilized
 876 Protein G-Sepharose beads (GE Healthcare) at 4 °C for 30 min. After extensive washing of the
 877 immunocomplexes, associated nucleotides were separated by thin layer chromatography and
 878 quantified with a Amersham Hyperfilm MP (GE Healthcare).

879 ***Guanine nucleotide exchange assay***

880 HEK 293T cells growing in 10-cm dishes were transfected with DNA mixtures containing 10
 881 μg of pEGFP-Rab11a or 10 μg of pcDNA3.1(-myc/His)-AS-Rab11, using calcium phosphate
 882 method. At 48 h after transfection, cultures were harvested and homogenized in 1 ml of lysis buffer
 883 (50 mM HEPES, pH 7.6, and 1% (v/v) Triton-x100, 100 mM NaCl, protease inhibitors). Cytosol
 884 was obtained by centrifuging the lysates at 20,000g for 20 min at 4 °C and protein concentration of
 885 clarified lysates was determined by Bradford method. 1 mg of protein was immunoprecipitated
 886 using 1 μg of anti-GFP antibody (ABCAM, Cambridge, UK) for 1 hours and incubated on a
 887 rotating rack for 1 hour at 4 °C with Protein-G sepharose beads (GE, Buckinghamshire, UK).
 888 Samples were collected by centrifugation and washed six-times with buffer A containing 50 mM
 889 HEPES, pH 7.6, 1 mM DTT and 20 mM EDTA and incubated for 20 min at 25 °C, to remove Mg^{2+}
 890 and nucleotide bound to the Rab11 GTPases. The treatments of the samples with buffer A were
 891 repeated three times more. To determine the GDP binding affinities to Rab11 GTPases, $[\text{3H}]\text{GDP}$ at
 892 a specific activity of 6000 cpm/ μM was incubated with the respective apo-GTPases at 25 °C for 1 h
 893 in buffer B containing 50 mM HEPES, pH 7.6, 100 mM NaCl, 2.5 mM MgCl_2 and 1 mM DTT.
 894 Samples were collected by centrifugation and washed six-times with buffer C containing 50 mM
 895 HEPES, pH 7.6, 100 mM NaCl, 10 mM MgCl_2 to stop the binding reaction, and the
 896 radionucleotides remaining bound to the Rab GTPases were quantified by scintillation counting. To
 897 measure the GDP/GTP exchange from Rab11 GTPases, the immunoprecipitated apo-GTPases were
 898 first complexed with $[\text{3H}]\text{GDP}$ or in buffer B. After 60 min a binding equilibrium was reached, the
 899 dissociation reactions were initiated by the addition of 500 μM $\text{GTP}\gamma\text{S}$ 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 Mg²⁺ 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 MgCl₂ 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 MgCl₂ 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.

932 *Transferrin recycling assay*

933 2 * 10⁵ COS-7 cells were plated in a 6-well plate. After 24 hours, cells were starved for two
 934 hours in serum-free DMEM containing 0.1% BSA at 37°C, 5% CO₂, and then, where required,
 935 pretreated with 0.1% DMSO as control or with Vps34-IN1 1 uM for 30 min. 20 ug/ml of alexa
 936 Fluor 647- conjugated human Transferrin (Invitrogen) were added for 30 min. After 37°C PBS
 937 washing, DMEM containing 0.1% BSA at 37°C, 5% CO₂ was added at various length times. Cells
 938 were then washed twice with cold PBS and acid stripping solution (150 mM NaCl, 2 mM CaCl₂ and
 939 25 mM CH₃COONa, pH 4.5) was added for 4 min. For FACS analysis cells were detached with
 940 PBS 0.5 mM EDTA and fixed in 4% paraformaldehyde for 10 min. After resuspension in PBS,
 941 fluorescence flow cytometry was performed using a FACScalibur instrument. 20,000 cells were
 942 collected for each sample. The MFI of the cell population was recorded for each time point. Data
 943 were normalized to the time 0 MFI. For immunofluorescence analysis, cells were fixed in 4%
 944 paraformaldehyde for 10 min and imaged by confocal microscopy.

945 *Internal Transferrin quantitation*

946 2* 10⁵ COS-7 wild-type (or interfered) cells were plated in a 6-well plate. After 24 hours,
 947 cells were starved for two hours in serum-free DMEM containing 0.1% BSA at 37°C, 5% CO₂, and
 948 then, where required, pretreated with 0.001% DMSO as control or with Vps34-IN1 1 uM for 30
 949 min. 20 ug/ml of alexa Fluor 647- conjugated human Transferrin (Invitrogen) were added for 30
 950 minutes to allow continuous uptake and recycling of labelled ligands. Cells were then washed twice
 951 with cold PBS and acid stripping solution (150 mM NaCl, 2 mM CaCl₂ and 25 mM CH₃COONa,
 952 pH 4.5) was added for 4 min. Cells were detached with PBS 0.5 mM EDTA and fixed in 4%
 953 paraformaldehyde for 10 min. After resuspension in PBS, fluorescence flow cytometry was
 954 performed using a FACScalibur instrument. 20,000 cells were collected for each sample. The MFI
 955 of the cell population was recorded for each time point. Data were normalized to control MFI
 956 values. For immunofluorescence analysis, cells were fixed in 4% paraformaldehyde for 10 min and
 957 imaged by confocal microscopy.

958 *Cell Imaging*

959 Cells were grown on μ -Dish^{35mm, high} imaging dishes (Ibidi). Imaging was performed in CO₂
 960 independent medium, Dulbecco's modified Eagle's medium without fetal bovine serum (GIBCO).
 961 Time-lapse series were acquired at 37°C on an inverted confocal Leica SP8 microscope with
 962 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 excited 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

996unsaturated sensitized FRET pixels by the CFP pixels^{47, 48}. To measure the dependence of FRET
997ratio on the distance from the nucleus, endosomes present in each frame included in the threshold
998mask were binned according to the distance of their centroid from the nuclear membrane.

999Video processing and analysis for particle tracking and vesicle intensity profile were carried out
1000with ImageJ, ICY⁴⁹ and R studio. Following background subtraction and Gaussian smoothing the
1001CFP, FRET sensitized, YFP and red fluorescence were treated to eliminate noisy pixels and smooth
1002the images. The FRET ratio for each frame was computed by dividing FRET sensitized signal by
1003CFP signal. The videos were then imported in ICY for spot detection and particle tracking
1004procedure performed on the YFP signal⁵⁰. Intensity profile for FRET ratio, YFP and FYVE2X were
1005exported together with trajectory for each detected vesicle. Vesicle intensity profiles were then
1006aligned in R studio according to their speed profile and direction. The vesicle mean FRET ratio was
1007adjusted by subtracting cytoplasmic mean FRET ratio.

1008MatLab code is fully available on GitHub.

1009

1010*Statistical analysis*

1011 For biochemical, immunocytochemistry and microscopy-based experiments a minimum of
1012three independent experiments (n) was performed and statistically significant estimates for each
1013sample were obtained. For microscopy based quantification, cells were chosen arbitrarily according
1014to the fluorescent signal in a separate channel, which was not used for quantification where it was
1015possible. Values were presented as means \pm SEM. *P* values were calculated using two-tailed
1016Student's *t* test and one- or two-way ANOVA followed by Bonferroni's multiple comparison
1017posttest (GraphPad Software). Statistical significance is indicated as follows: **P* < 0.05, ***P* < 0.01,
1018and ****P* < 0.005.

1019 **Methods references**

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