

1 **CRISPR-based bioengineering of the Transferrin Receptor revealed**
2 **a role for Rab7 in the biosynthetic secretory pathway**

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30 **Abstract**

31 The regulated secretory trafficking of neosynthesized transmembrane receptors is
32 particularly challenging to investigate as it is under-represented at steady state compared to
33 the abundance of the other trafficking routes. Here, we combined the retention using
34 selective hook (RUSH) system to a CRISPR/Cas9 gene editing approach (eRUSH) to
35 identify molecular players involved in the trafficking of neosynthesized Transferrin Receptor
36 (TfR) *en route* to the plasma membrane (PM). TfR-eRUSH monoclonal cells expressing
37 endogenous, ER-retainable and fluorescent TfR were engineered and characterized.
38 Spatiotemporal quantitative proteomics of TfR-eRUSH cells allowed the identification of
39 molecular partners associated with TfR-containing membranes and provided a
40 comprehensive list of potential regulators, co-trafficking cargos, and enriched pathways.
41 Furthermore, we chose to focus our attention on the Rab GTPase family members for their
42 function as vesicle trafficking regulators and performed a Rab-targeted siRNA screen that we
43 correlated to our proteomics data. Unexpectedly, we identified Rab7-harboring vesicles as
44 an intermediate compartment of the Golgi-to-PM transport of the neosynthetic TfR. These
45 vesicles did not exhibit degradative properties and were not associated to Rab6A-harboring
46 vesicles, also involved in Golgi-to-PM transport. However, Rab6A-TfR vesicles delivered TfR
47 directly to the PM, while in contrast, Rab7A was transiently associated to neosynthetic TfR-
48 containing post-Golgi vesicles but dissociated before PM vesicle fusion. Together, our study
49 proposes the eRUSH as a powerful tool to further study the secretory pathway and reveals
50 an unforeseen role for Rab7 in the neosynthetic transport of the TfR, highlighting the diversity
51 of the secretory vesicles' nature for a given cargo.

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54 **Introduction**

55 Cells sense environmental changes and adapt accordingly by exposing a variety of
56 transmembrane receptors at their cell surface. Post-translational modifications and
57 final localization of these transmembrane receptors at the plasma membrane (PM)
58 are first occurring through the membrane dynamics along the secretory pathway. The
59 secretory pathway is a constitutive or regulated process (1) carrying neosynthesized
60 proteins from the endoplasmic reticulum (ER) to the PM. Characterizing the
61 molecular mechanisms involved in this cellular process may be useful for the
62 development of inhibitors targeting general or cargo-specific secretion (2).

63 Transmembrane receptors are synthesized and folded in the ER. After synthesis,
64 coatamer protein complex II (COP-II) vesicles export the incorporated receptors to
65 the *cis*-Golgi cisternae (3). The transit of these cargoes through the Golgi stacks is
66 still debated (4,5), although it is well established that proteins undergo successive
67 post-translational modifications during their trafficking from the *cis*-Golgi to the TGN.
68 Upon protein arrival at the TGN, cargoes are specifically packaged and sorted to be
69 delivered to different organelles such as endosomes, lysosomes or the PM. Sorting
70 signals identified at the cytosolic regions of transmembrane receptors lead to the
71 specific recruitment of adaptor proteins (APs) or small Rab-GTPases, needed for the
72 incorporation of the cargo inside vesicle carriers. After budding off the TGN
73 membranes, proteins are delivered to their final destination through vesicular
74 transport. It was long being thought that transmembrane receptors use a direct route
75 from the TGN to the PM. Observations of differential trafficking routes suggested
76 otherwise. Indeed, several studies noticed the presence of cargoes inside endocytic
77 compartments before their delivery to the PM (6–8). The nature and fate of these
78 intermediate compartments in protein secretion is still unclear.

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79 To mechanistically understand temporally and spatially the secretory pathway, a few
80 systems were developed. One of the earliest methods developed to study protein
81 secretion was the thermo-sensitive vesicular stomatitis virus glycoprotein (ts045VSV-
82 G) (9). It involves incubation of cells at a restrictive temperature to block ts045VSV-G
83 transport at the ER followed by a shift at a lower permissive temperature to induce
84 the release of the protein to its normal trafficking pathway (10). This method provided
85 valuable analytical information on the dynamics and kinetics of transport of
86 ts045VSV-G from the TGN to the PM.

87 To avoid non-physiological conditions of temperature and monitor different cargo
88 proteins, the RUSH (retention using selective hooks) system was elaborated (11). It
89 allows the retention of a protein of interest in the ER, then its on-demand release
90 following the addition of biotin in the cell media. This method proved to be very
91 powerful (2,12–15), but it requires the transient overexpression of the protein of
92 interest which is a limitation in case of regulated secretion. In addition, the co-
93 existence of the overexpressed tagged and the non-tagged endogenous cargos
94 could confer some limitations for quantitative temporal detection of a receptor at the
95 PM.

96 The vesicular carriers involved in the secretory pathway are difficult to study because
97 of their low abundance at steady state compared to endocytic/recycling vesicles. This
98 is particularly true for the Transferrin Receptor 1 (TfR), which is widely used for
99 recycling studies (for review see (16)). TfR is a ubiquitous transmembrane
100 glycoprotein that mediates iron uptake from circulating transferrin (Tf) at the PM. After
101 formation of the TfR-Tf complex at the cell surface, the receptor is internalized by
102 clathrin-mediated endocytosis and delivered to endosomes. Inside these organelles,
103 TfR dissociates from its ligand and is recycled back to the cell surface. Studies

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104 indicated that an alteration of the expression level of TfR could trigger carcinoma
105 progression (17,18). Indeed, cancer cells expressed a high amount of TfR at their cell
106 surface which makes it a significant anti-cancer target (19,20).

107 Neosynthesized TfR arriving at the PM represents a minor fraction of the total TfR
108 pool expressed at the cell surface at steady state, and thus the pathway of newly
109 synthesized TfR is particularly difficult to investigate. In this study, we developed an
110 approach that combines the RUSH system with CRISPR/Cas9 gene editing that we
111 called “edited-RUSH” or “eRUSH”. We employed eRUSH to investigate the molecular
112 mechanisms involved in the vesicular transport of neosynthesized TfR to the PM. The
113 TfR-eRUSH allowed the spatiotemporal monitoring of the trafficking of the
114 neosynthesized endogenous TfR as well as the identification of the molecular
115 partners involved in this process. In particular, we highlighted that Rab7A, a small
116 Rab GTPase usually described as an endolysosomal marker, is required for efficient
117 arrival of neosynthesized TfR at the PM and was recruited to a subset of post-TGN
118 TfR-containing vesicles, suggesting that Rab7 may play a role in the anterograde
119 trafficking pathway of secretory vesicles.

120 **Results**

121 **Generation and characterization of the TfR-eRUSH system.**

122 The CRISPR/Cas9 strategy that we previously described (21) was used to engineer
123 the breast cancer-derived SUM159 cells to express endogenous TfR fused to the
124 streptavidin-binding peptide (SBP) and EGFP. Lentiviral transduction of a chimera
125 streptavidin-KDEL protein was performed to establish a stable cell line that retains
126 SBP-containing proteins in the ER (see (11) for original description of the RUSH
127 system) and the resulting TfR-eRUSH cells were subsequently characterized.

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128 As depicted in Figure 1A, SBP fused to EGFP was introduced in the genomic
129 sequence of TfR before its stop codon sequence. At the genomic DNA level, both
130 alleles were carrying an extra piece of DNA corresponding to the SBP-EGFP tag
131 (Figure 1B). At the protein level, almost no endogenous TfR was detected (at \approx 84
132 kDa), while an upper band at \approx 117 kDa appeared, corresponding to the expected
133 size of TfR-SBP-EGFP protein (Figure S1A). Of note, the molecular weights were
134 difficult to precisely assess as ladders from different brands were providing dissimilar
135 sizes for a given band. Using an anti-EGFP antibody, we could confirm that TfR-SBP-
136 EGFP was indeed running at an apparent size of 117 kDa (Figure S1B). Depending
137 of the ladder used along our study, the TfR-eRUSH would appear as a band of either
138 \approx 98 kDa or 117 kDa, although both would correspond to the TfR-eRUSH.

139 From the immunoblot, it seemed that less TfR-SBP-EGFP proteins were expressed
140 in the edited cells than the endogenous TfR from WT cells. However, quantification of
141 the amount of proteins from bands of different sizes is not reliable due to different
142 protein transfer efficiency. Thus, an anti-TfR antibody staining on WT and TfR-
143 eRUSH cells was performed and the mean fluorescence intensity of the TfR staining
144 was measured by flow cytometry. We found that TfR-eRUSH cells express less
145 endogenous TfR than their parental cell line (Figure 1C).

146 Next, we carried out 3D confocal live cell imaging on TfR-eRUSH cells to determine
147 whether TfR-eRUSH could be efficiently retained in the ER. We observed that in
148 absence of biotin (0 min), TfR-eRUSH was retained in the ER (Figure 1D, upper
149 panels and corresponding Movie S1). Two to six minutes after biotin addition,
150 vesicles were released from the ER to reach the Golgi apparatus. This trend was
151 successfully quantified by measuring the Pearson's correlation coefficient between
152 TfR and either Calnexin (ER) marker), GM130 (cis-Golgi) or TGN46 (trans-Golgi) at 0

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153 min, 5 min and 15 min post-biotin addition (Figure S1C-D). While the ER released
154 most of its vesicles, a short lag was observed at \approx 12 minutes before observing
155 numerous vesicles exiting from the Golgi apparatus. At 20 min, most of TfR-eRUSH
156 was localized at the Golgi and vesicles were massively released from this location. In
157 parallel, PM gained higher TfR-eRUSH fluorescence intensity (Figure 1D, blue
158 arrowheads and Movie S1), indicating that the first detectable amounts of TfR-
159 eRUSH proteins arrived at the PM at 20 min post-biotin addition.

160 To quantitatively measure the kinetics of TfR-eRUSH arrival at the PM, a flow
161 cytometry assay was optimized (Figure 1E). At different times post-biotin addition,
162 cells were incubated at 4°C to block membrane trafficking and the PM-exposed TfR
163 was labeled using recombinant transferrin coupled to an Alexa Fluor 647 (Tf-A647)
164 (Figure 1E). We noticed that a small fraction of TfR-eRUSH was already found at the
165 PM even in absence of biotin (0 min), suggesting that either some aspecific Tf
166 binding occurred or that a small amount of TfR-eRUSH was not retained by the hook.
167 While the fluorescence signal of Tf-A647 was lowly increasing over the 20 first min
168 after biotin addition, a three-fold increase was observed at 30 min post-biotin
169 addition. This kinetics were confirmed by microscopy (Figure 1F) and are in
170 agreement with our live cell imaging (Figure 1D) in which the first TfR proteins could
171 be readily detected at the PM at \approx 23 min post-biotin addition, then rising over time.

172 In conclusion, our TfR-eRUSH system represents a valid approach to study the
173 molecular mechanism of the TfR secretory pathway in an endogenous synchronized
174 model.

175 **Molecular signature of the TfR-associated membranes using TfR-eRUSH cells**

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176 To identify the molecular partners enriched in the TfR-containing membranes over
177 time, anti-TfR affinity-purification mass spectrometry (AP-LC-MS/MS) experiments
178 using TfR-eRUSH lysates obtained from mechanical cell disruption was performed at
179 different time points post-biotin addition. AP-LC-MS/MS was run in quadruplicate and
180 > 2000 proteins were identified in each sample. Differential temporal analysis
181 identified 557 proteins enriched at T15 compared to T0 (T0-T15), while no significant
182 protein enrichment could be measured at T30 compared to T15 (T15-T30). This
183 absence of protein enrichment between T15 and T30 could be attributed to the lack
184 of temporal resolution and/or the fact that multiple trafficking pathways are
185 overlapping at these times, blurring the final picture. Parallel analyses using STRING
186 (22) (Figure 2A), and the molecular signature database MSigDB (23) (Figure 2B and
187 Table S1) were run on the enriched protein lists from the T0-15 differential analysis.
188 These methods were employed to highlight protein clusters and biological processes
189 associated to neosynthesized TfR trafficking.

190 The pathways “intracellular transport”, “Cellular macromolecular localization”,
191 “intracellular protein transport” and “secretion” were highly enriched compared to T0
192 as shown by the low false-discovery rates (FDR) values, an expected result due to
193 the nature of the assay (Figure 2B). Moreover, the pathways associated to
194 “exocytosis” (FDR = $4.75 \cdot 10^{-23}$) and “Golgi vesicle transport” (FDR = $2 \cdot 10^{-15}$) were
195 also significantly enriched to a lower extend. As a proof of concept, we confirmed that
196 TMED10, a protein identified as enriched in our proteomics analyses was indeed
197 recruited to TfR secretory vesicles (Table S1, Figure S2A). TMED10 is involved in the
198 COPII vesicle-mediated anterograde transport (24) and incorporated in a subset of
199 extracellular vesicles (25), and thus we could confirm the relevance of our differential
200 proteomics approach.

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201 The pathways “oxidation reduction process”, “cellular respiration” and “mitochondrion
202 organization” also scored significant low FDR values. ER and mitochondrial
203 membranes are well-known to tightly interact (26) and recent work proposed that
204 endosome-mitochondria interactions are important for the release of iron (27). Here,
205 the mitochondria-associated proteins identified may be the result of association of
206 distinct membranes during the immunoprecipitation rather than actual presence of
207 TfR within mitochondria. In fact, proximity was observed between TfR-eRUSH and
208 mitotracker-labeled mitochondria (Figure S2B). By live cell imaging, we visualized
209 some rare events of mitochondria “associated” with vesicles containing TfR-eRUSH
210 that seemed to bud off the ER, but the resolution achieved with our spinning disk
211 confocal microscope does not allow us to draw significant conclusion (Figure S2C
212 and Movie S2).

213 Proteins regulating intracellular trafficking may be differentially recruited on vesicular
214 membranes to activate a specific trafficking route. Therefore, we chose to further
215 investigate the role of Rab proteins as they are well-known small GTPase regulators
216 of intracellular membrane traffic. In our AP-LC-MS/MS dataset, we detected a total of
217 20 Rab proteins (Table S2). No Rab proteins were enriched in TfR-containing
218 membranes at T15-T30, but 10 Rab proteins were significantly enriched at T0-T15
219 with a fold change above 1.5 times (Figure 2C). Rab1A, Rab1B and Rab18 were
220 significantly enriched at T15 compared to T0, an expected result as these Rabs
221 regulates vesicle trafficking between the ER and the cis-Golgi (28,29), Rab18 being
222 also found on a subset of extracellular vesicles (25). Rab10, Rab14 and Rab6A were
223 also enriched at T15 compared to T0, although Rab6 did not reach significance
224 (Table S2). These Rabs have previously been involved in post-Golgi trafficking
225 (13,30,31), further indicating that our approach is relevant to identify molecular

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226 partners involved in the secretory pathway. The Rab12 and Rab34 proteins were also
227 identified, but their function has not been extensively studied. Yet they both may play
228 a role in protein degradation (32,33). Finally, Rab7A, a protein usually recruited at the
229 limiting membrane of late endosomes that can serve as degradation signal (34), was
230 significantly enriched at T15 compared to T0. Rab7A showed one of the highest fold
231 enrichment score and the greatest number of unique peptides identified by LC-
232 MS/MS (Figure 2C and Table S2), an intriguing result that we aimed to explore
233 thereafter.

234 To further investigate the relevance of the Rabs identified in our proteomics analyses,
235 the distribution of Rab5, Rab6, Rab7, Ruby3-Rab10 and Rab18 was imaged in TfR-
236 eRUSH cells treated for 12 min with biotin (Figure 2D). While no colocalization was
237 observed between TfR-eRUSH and Rab5 nor Rab18, association with Rab6, Rab7,
238 Rab10 was seen.

239 **Rab7 is significantly enriched onto post-Golgi TfR-eRUSH vesicles**

240 To further characterize the recruitment of Rab7A on TfR-containing secretory
241 vesicles, we performed live cell imaging by spinning disk confocal microscopy on
242 TfR-eRUSH cells transfected with a Ruby3-Rab7A construct under the control of the
243 weak promoter L30 (to minimize overexpression). Starting from 7 min post-biotin
244 addition, we noticed the presence of post-TGN TfR-eRUSH signal associated to
245 Rab7A positive vesicles (Figure 3A and Movie S3). To better appreciate whether TfR-
246 eRUSH and Rab7A were found on the same vesicles (as opposed to two distinct
247 vesicles in close proximity), we artificially swollen these compartments using
248 Apilimod, a PIKfyve inhibitor (35), and indeed, we could identify that TfR-eRUSH-
249 positive vesicles were decorated with Rab7A at their limiting membrane (Figure 3B).
250 These data were reminiscent of a recent work nicely demonstrating that post-Golgi

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251 vesicles were positive for Rab6 (13) and indeed in our model, TfR-eRUSH was also
252 trafficking through Rab6 (Figure S3A).

253 Quantification of TfR-eRUSH association with indicated Rabs was then performed
254 using antibody staining on TfR-eRUSH cells fixed at 15 min post-biotin addition. As
255 expected, the percentage of non-Golgi TfR-eRUSH signal associated to Rab5 was
256 very low (9.3% +/- 1.3), while association with Rab6 and Rab7 was relatively high
257 (31.7% +/- 2.5 and 42.3% +/- 3.3, respectively; Figure 3C-D). Interestingly however,
258 TfR-eRUSH vesicles would harbor either Rab7A or Rab6, but no post-Golgi TfR-
259 eRUSH-Rab6-Rab7A triple colocalization was seen (Figure S3B).

260 Together, our proteomics analysis revealed that several Rabs are enriched onto
261 secretory TfR-containing vesicles and that Rab7A represent an unexpected protein
262 recruited in the neosynthetic secretory pathway.

263 **Neosynthesized TfR associates with non-degradative Rab7 vesicles**

264 Rab7 is known to direct late endosomal compartments toward degradative Lamp1-
265 positive compartments (36). By immunostaining, we observed that a subset of TfR-
266 eRUSH was Rab7-positive and Lamp1-negative (Figure 4A, yellow arrow), but we
267 could also see triple colocalization of TfR-eRUSH, Rab7, and Lamp1 (Figure 4A,
268 white arrowheads). However, mapping the association with Lamp1 is not sufficient to
269 define lysosomal compartments since a recent study demonstrated that TfR is co-
270 sorted with Lamp1 into post-TGN secretory vesicles *en route* to the PM (37).
271 Moreover, Lamp1 was also identified in our proteomics analysis (Table S1).

272 Therefore, to better address whether the TfR-Rab7 vesicles correspond to
273 degradative compartments, pH acidity and proteolytic activity was measured (Figure
274 4B-C). TfR-eRUSH cells were transfected with Ruby3-Rab7A and were visualized by

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275 live imaging at 10 min post-biotin addition. LysoTracker was used as a readout for
276 relative pH acidity (a brighter signal corresponding to a lower pH). Our data shows
277 that TfR-eRUSH vesicles harboring Rab7A had little-to-no LysoTracker signal (Figure
278 4B, white arrow), indicating that these vesicles do not exhibit features of classical
279 proteolytic compartments. To assess for actual degradative properties of these
280 vesicles, we pre-incubated the cells with DQ-BSA, a bovine serum albumin (BSA)
281 protein that contains self-quenched fluorescent dyes that fluoresce only when the
282 BSA is cleaved, and stained the cells with an anti-Rab7 antibody (Figure 4C).
283 Quantification of the percentage of TfR-eRUSH colocalizing with Rab7 or DQ-BSA
284 demonstrated that the TfR were mainly found in Rab7 vesicles devoid of degraded
285 DQ-BSA (Figure 4D). Finally, because the TfR was engineered to incorporate SBP
286 and EGFP, we checked whether a significant subset of protein was sent for
287 degradation. However, cells treated with Bafilomycin A1 (to prevent protein
288 degradation) did not induce an accumulation of TfR-eRUSH, while it induced LC3-II
289 accumulation as expected (Figure 4E). To make sure that the absence of visible
290 degradation was not due to neosynthesized TfR-eRUSH replenishment, cells were
291 co-treated with Bafilomycin A1 and cycloheximide, a translation inhibitor. In this
292 context, we could not observe any accumulation of TfR (Figure 4E) and we were not
293 able to detect degradation products using either anti-TfR or anti-EGFP antibodies
294 (Figure S4), suggesting that TfR-eRUSH is not significantly sent for degradation. Of
295 note, this experiment also indicates that the induction of the eRUSH by the addition
296 of biotin is not accompanied by the induction of autophagy as LC3-II is not
297 upregulated (Figure 4E). To further confirm this, LC3 staining was performed and we
298 could not observe any LC3 staining colocalizing with Rab7-positive TfR-eRUSH
299 vesicles (Figure 4F), indicating that they do not correspond to autophagosomes.

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300 **Rab7A-vesicles are intermediate compartments mediating the transport of a**
301 **subset of neo-synthesized TfR-eRUSH to the PM.**

302 To correlate the enrichment over time of Rab proteins to a biological function, we
303 next carried out a siRNA-based screen targeting 12 members of the Rab protein
304 family. Silencing of 12 Rabs and a non-relevant target was performed using a pool of
305 4 siRNA per target in two independent experiments (Figure S5A). The amount of TfR-
306 eRUSH at the PM was measured by flow cytometry as in Figure 1E, and fold
307 enrichment of T15 over T0 (Figure 5A) was determined. As Rabs may affect other
308 cellular processes, the amount of TfR-eRUSH at steady-state was measured by flow
309 cytometry (Figure S5).

310 At T0-T15, silencing of Rab27A or Rab6A showed significant decrease of PM-
311 associated TfR (Figure 5A) compared to the non-relevant siRNA control. These
312 findings were in agreement with the role of these Rabs in protein secretion
313 (13,38,39), validating our approach. In contrast, Rab10 silencing had no detectable
314 effect on TfR trafficking to the PM, while we found it enriched in our proteomics
315 analysis (Figure 2C). Interestingly however, silencing of Rab7A showed a significant
316 inhibition of TfR-eRUSH arrival at the PM at T0-15. Although Rab7A is known for its
317 role in endocytic retrograde trafficking to late endosomes and lysosomes (34), this is
318 consistent with our AP-LC-MS/MS data (Figure 2C), further indicating that Rab7
319 could participate in the transport of post-TGN TfR vesicles.

320 To directly determine the fate of the Rab-harboring post-Golgi TfR-eRUSH vesicles,
321 total internal reflection fluorescence (TIRF) microscopy was performed on cells
322 transfected with Ruby3-Rab7A (Figure 5B and Movie S4) or Ruby3-Rab6A (Figure
323 5C and Movie S5). After 12 min post-biotin addition, the arrival of TfR-eRUSH was
324 observed in the evanescent TIRF field. We monitored events during which Rab7-

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325 positive vesicles became positive for TfR-eRUSH for several seconds (Figure 5B;
326 from 704 s to 736 s) before the two signals segregated again, followed by a TfR-
327 eRUSH signal burst, indicative of PM fusion (734 s). In sharp contrast, Ruby3-Rab6A
328 remained associated to TfR-eRUSH vesicles until fusion occurred (Figure 5C; 848-
329 868 s).

330 These observations indicated that Rab7A vesicles are used as intermediate
331 compartments in TfR trafficking after its release from the TGN. Unlike Rab6A, Rab7A
332 vesicles do not accompany neosynthesized TfR all the way to the PM and thus, other
333 partners are likely involved downstream of the Rab7-TfR vesicle trafficking.

334

335 **Discussion**

336 Description of the different pathways mediating transport of neo-synthesized
337 receptors to the PM has been studied for decades. Being able to specifically observe
338 the anterograde pathway has always been a challenge as its visualization overlaps
339 with other trafficking routes, including the overrepresented endocytosis and recycling
340 pathways. To visualize protein transport under physiological conditions, we combined
341 the RUSH system to the CRISPR/Cas9 technology. Using TfR as a model, we
342 generated a stable cell line expressing endogenous levels of the receptor fused to
343 EGFP and the SBP tag required for the RUSH system. TfR function and trafficking
344 are well described but the partners involved in neosynthesized TfR trafficking to the
345 PM are not well characterized. The eRUSH (edited-RUSH) approach was coupled to
346 quantitative proteomics experiments and cytometry-based screening to identify the
347 molecular partners involved in the neosynthetic pathway of the TfR. Unexpectedly,

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348 we observed that a significant subset of TfR transits through Rab7-positive vesicles
349 during its trafficking to the PM.

350 The trafficking kinetics of neosynthesized TfR-eRUSH was similar to the
351 overexpressed TfR in the RUSH system which was previously described to reach the
352 PM \approx 30 min post-biotin addition (37). The advantage of our eRUSH is that no or
353 minimal amount of “ghost” untagged proteins are expressed in TfR-eRUSH, allowing
354 quantitative single molecule counting as well as whole TfR functional analysis.
355 Moreover, the eRUSH also represents a powerful knock-away system, similar to
356 other methods (40), but without the problem of competition with the wild type version
357 of the protein co-expressed in the cell. In contrast to classic cDNA transfection,
358 CRISPR/Cas9-based gene editing of TfR allows the conservation of the regulatory
359 genomic environment of the gene. This parameter is particularly important for
360 proteins such as TfR as its transcriptional/translational regulation is a finely regulated
361 process (41,42).

362 Using AP-LC-MS/MS, we could track the local TfR environment at different times
363 post-biotin addition and identify proteins co-distributing with TfR-extracted
364 membranes. Whereas previous siRNA-based screens studying the secretory
365 pathway allowed the identification of important novel partners (43), our eRUSH-
366 based proteomics is based on a non-interfering approach, and thus, it provides new
367 complementary information to previous studies. Pathway analysis revealed relevant
368 enriched biological processes as well as less expected ones. Indeed, an enriched
369 proportion of mitochondrial proteins at 15 min post-biotin addition was observed. We
370 propose that this result is due to ER-mitochondria membrane contacts sites and may
371 not be relevant to the biosynthetic pathway of TfR.

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372 Some Rabs can act together in the exocytosis process, such as Rab6 and Rab8 (44)
373 or Rab3 and Rab27 (45). We detected > 35% of TfR-eRUSH-containing vesicles
374 harboring a Rab6-positive staining (Figure 3D), but our data suggest however that
375 Rab6 and Rab7 do not intervene at the same stage of the secretory pathway and/or
376 in the same type of vesicular transport, as shown by the absence of overlap between
377 the Rab6 and Rab7 staining (Figure S3B). Moreover, by TIRF microscopy (Figure
378 5B-C), we noticed two different processes of TfR transport using Rab6A or Rab7A,
379 further indicating that these two Rabs likely correspond to two distinct secretory
380 routes.

381 Combining results from the AP-MS/MS and siRNA screen, only one Rab was
382 significantly standing out: Rab7. Rab7 is mostly known to mediate cargo trafficking
383 between late endosomes and lysosomes (34) and it was unexpected to find it
384 involved in the neosynthetic pathway. By electron microscopy, a group observed the
385 presence of neosynthesized TfR inside endosome-like structures (6). Moreover, it
386 was demonstrated that Rab7 was not involved in recycling of TfR at the PM as
387 depletion of Rab7A had no effect on TfR re-localization to the PM (34) and thus, it is
388 unlikely that our observations would be the result of marginal PM-associated TfR
389 endocytosis at early times post-biotin addition.

390 A legitimate thought is to believe that the post-golgi Rab7-decorated TfR-eRUSH
391 vesicles correspond to a degradative pathway. However, extensive analyses of these
392 vesicles clearly show that they stain mostly negative/dim to lysotracker, they are not
393 proteolytically active (DQ-BSA marker) nor autophagosomes, while autophagy is not
394 induced by the biotin treatment (Figure 4). Moreover, the full membranes of the
395 western blot analysis show no degradative product, further demonstrating that TfR-
396 eRUSH vesicles harboring Rab7 are not degradative and actually, direct evidence

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397 support PM-targeting of these vesicles (Figure 5B). Yet, the function of these vesicles
398 as compared to the Rab6-positive vesicles, remain to be determined.

399 A recent study by Chen and colleagues indicated that neosynthesized TfR was
400 sorted out with the Lamp1 protein in vesicles exiting the TGN (37). These vesicles
401 were devoid of the mannose-6P receptor (M6PR), which was used as a marker for
402 Golgi-to-endosome route (46). In our hands, we found that M6PR was absent of the
403 TfR-eRUSH vesicles harboring Rab7 (not shown). They concluded that the TfR⁺
404 Lamp1⁺ vesicles were *bona fide* secretory vesicles *en route* to the PM. In our study, a
405 subset of vesicles containing TfR-eRUSH and Lamp1 were also decorated by Rab7
406 at time points corresponding to TGN exit. These vesicles may correlate with the ones
407 described by Chen *et al.* but their comprehensive composition and function in the
408 secretory pathway remains to be fully determined.

409 We suggest that Rab7 could act as an intermediate compartment for neosynthesized
410 TfR transport. Although the role of Rab7 on these secretory vesicles remains to be
411 determined, one could hypothesize that Rab7 regulates the trafficking of cargos with
412 specific post-translationally modifications. Alternatively, this pathway could transport
413 cargos dedicated to specific PM domains. Recently, Rab7 has been mapped not only
414 to late endosomes and lysosomes but also at the ER, TGN and mitochondrial
415 membranes, a localization maintained by the retromer complex (47), and thus, it is
416 likely that Rab7 exerts pleiotropic roles.

417

418 **Materials and Method**

419 ***Cell culture.***

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420 SUM159 cells were cultured in DMEM/F-12 GlutaMAX (GIBCO), supplemented with 5%
421 fetal bovine serum (FBS; Dominique Dutscher), 500 µg/ml penicillin-streptomycin (GIBCO), 1
422 µg/ml hydrocortisone (Sigma-Aldrich), 5 µg/ml insulin (Sigma-Aldrich), and 10 mM HEPES
423 (GIBCO) (complete medium). Cells were maintained at 37°C and 5% CO₂.

424 ***Generation of the TfR-eRUSH CRISPR/Cas9 edited cell line***

425 Gene editing of the SUM159 cells to fuse the GGSGGSGGS spacer, the SBP and
426 EGFP sequences to the C-ter of TfR a CRISPR/Cas9 strategy was used as
427 previously described (21,48). Briefly, three genetic tools were co-transfected using
428 the transfection reagent TransfeX (ATCC): 1) a plasmid coding for CRISPR-
429 associated protein 9 (Cas9), a template plasmid; 2) a linear PCR product used to
430 transcribe the tracrRNA and guide RNA (gRNA) targeting
431 ATAGCTTCCATGAGAACAGC (corresponding to a region near the genomic TfR
432 stop codon) under the control of the human U6 promoter; 3) a donor DNA construct
433 (serving as template during homologous recombination) corresponding to the spacer,
434 SBP and EGFP sequences flanked by ≈ 800 bp upstream and 800 bp downstream of
435 the TfR stop codon. Single cell sorting of EGFP-positive cells was performed and
436 homo/heterozygotic monoclonal cell lines expressing endogenous TfR-eRUSH were
437 screened by PCR using the forward primer 5' CTCACACGCTGCCAGCTTTA 3' and
438 reverse primer 5' TTCAGCAGAGACCAGCCCTT 3'.

439 A clone that was edited on both alleles was further transduced with a lentiviral vector
440 coding the puromycine resistance gene and for the “hook” consisting of the
441 streptavidin protein linked to the KDEL motif (11). Upon puromycin selection, the
442 SUM159 TfR-eRUSH cells were expanded and stocks for the original tube were
443 maintained in liquid nitrogen.

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444 ***Plasmids***

445 The Ruby3-Rab7A, Flag-Apex-Rab7A, Ruby3-Rab6A and Ruby3-Rab10 cDNA
446 constructs cloned into pBS vectors under the control of the weak promoter L30, were
447 generated by the Montpellier Genomics Collections (MGC).

448 ***Antibodies and reagents***

449 For immunofluorescence, primary antibodies used were mouse anti-GM130 (1/1000;
450 BD bioscience), sheep anti-TGN46 (1/1000, Bio-Rad), rabbit anti-Calnexin (1/1000,
451 Elabscience), mouse anti-Lamp1 (1/100, BD bioscience), rabbit anti-Rab7 (1/250,
452 Cell Signaling Technology), rabbit anti-Rab5 (1/1000, Cell Signaling Technology),
453 rabbit anti-Rab6 (1/1000, Cell Signaling Technology), rabbit anti-Rab18 (1/200,
454 Sigma-Aldrich), rabbit anti-TMED10 (1/500, Sigma-Aldrich), mouse anti-LC3 (1/1000,
455 Sigma-Aldrich) and mouse anti-TfR (1/250, Miltenyi Biotec). Secondary antibodies
456 used were Alexa fluor 568 donkey anti-sheep (1/1000, life technologies), Alexa fluor
457 568 donkey anti-rabbit (1/1000, Thermo Fisher Scientific), Alexa fluor 647 donkey
458 anti-mouse (1/1000, Thermo Fisher Scientific). Antibodies used for immunoblotting
459 were rabbit anti-TfR (1/1000, Aviva Systems Biology), mouse anti-beta actin (abcam),
460 mouse anti-GFP (1/1000, Sigma-Aldrich) and rabbit anti-LC3 (1/1000, Sigma-
461 Aldrich). Secondary antibodies used for immunoblotting were Goat anti-mouse IgG
462 HRP antibody (1/10000, Jackson ImmunoResearch), Goat anti-rabbit IgG HRP
463 antibody (1/10000, Jackson ImmunoResearch). Probes used for
464 immunofluorescence were membrane-permeable MitoTracker Orange CM-H₂TMRos
465 (Molecular probes) used at 100 nM to label mitochondria, LysoTracker red (Life
466 Technologies) for acidic compartments used 30 min at 50 nM, DQ-Red BSA (Life
467 Technologies) used at 10 µg/ml in complete medium, and DAPI (1/1000, Sigma-
468 Aldrich) used to stain the nucleus. For flow cytometry Transferrin coupled to Alexa

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469 fluor 647 (molecular probes) was used at 10 µg/ml, anti-mouse TfR (5 µg/ml, Miltenyi
470 Biotec), and IgG mouse used as an isotype control. For deglycosylation,
471 endoglycosidase H was used (NEB).

472 ***Cytometry-based RUSH assay***

473 To detect PM-localized TfR, 40 000 SUM159 cells were plated in 48 well plates and
474 incubated in complete medium containing 0,28 µg/ml avidin (Sigma-Aldrich) for 48
475 hours. To initiate TfR release, cells were incubated in a fresh complete medium
476 containing 40 µM biotin (Sigma) for the indicated amount of time at 37°C and 5%
477 CO₂. Then, cells were placed on ice, the media was replaced with ice-cold PBS and
478 cells were maintained at 4°C for 15 min. Cells were incubated for 20 min with 10
479 µg/ml of Tf coupled to an Alexa Fluor 647 (Tf-A647; Molecular probes) diluted in PBS
480 pH 7.0 at 4°C. Unbound Tf-A647 was washed two times with cold PBS and cells
481 were detached with 5 mM EDTA. Cells were collected and centrifuged at 400 g for 15
482 min at 4°C. Cell fixation was carried out with 4% paraformaldehyde (PFA) for 20 min
483 at room temperature and after three washes, they were resuspended in a flow
484 cytometry buffer (PBS pH7.0, 0.5% BSA, 0.5 mM EDTA). Samples were run on a
485 Cytoflex flow cytometer (Beckman Coulter) equipped with 488 and 640 nm lasers and
486 4 filter set.

487 ***SiRNA screen***

488 A pool of four different siRNAs for each of the 12 selected Rab proteins and a non-
489 targeting siRNA control was purchased as a custom-made siGenome Smart pool
490 cherry-pick library (Dharmacon, Horizon Discovery; see details in Table S3). Forty
491 thousand SUM159 cells were seeded in 48-well plates and on the next day, 3 pmol of
492 siRNA were transfected using lipofectamine 2000 (ThermoFisher Scientific)

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493 according to the manufacturer's instructions. Cells were further incubated 48 h in
494 complete medium in presence of 0.28 µg/ml of avidin. The day of the experiment,
495 cells were incubated at different time points with 40 µM of biotin. The cytometry-
496 based assay for PM-localized TfR described above was used for sample analysis.

497

498 ***Immunoprecipitation of TfR-eRUSH***

499 For immunoprecipitation of the TfR-eRUSH proteins, SUM159 cells were plated in 20
500 mm sterile culture-treated petri dishes (Corning) for 48 h in complete medium with
501 0.28 µg /ml of avidin. Upon TfR-eRUSH release by addition of 40 µM biotin, the cells
502 were incubated on ice, washed with ice-cold PBS and scraped into 1 ml of ice-cold
503 isolation buffer (PBS devoid of Ca²⁺ and Mg²⁺, 0.1% BSA, 2 mM EDTA, pH7.4). Cells
504 were lysed at 4°C by mechanical lysis using a 22G needle and the resulting lysate
505 was centrifuged at 2 000 g for 15 min at 4°C. Supernatants were incubated for 2 h at
506 4°C with 2 µg of anti-TfR antibody previously coupled to Dynabeads (Thermo Fisher
507 Scientific). The immunoprecipitated TfR-eRUSH-containing membrane fractions were
508 washed five times with ice-cold PBS at 4°C before elution.

509 ***Mass spectrometry-based quantitative proteomics***

510 *Sample preparation*

511 The immunoprecipitated samples were resuspended in Laemmli buffer and the
512 antibody-conjugated magnetic beads were removed. Protein concentration was
513 determined using the RC-DC protein assay (Bio-Rad) according to the
514 manufacturer's instructions and a standard curve was established using BSA. For
515 each sample, 8 µg of protein lysate was concentrated on a stacking gel by
516 electrophoresis. The gel bands were cut, washed with ammonium hydrogen

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517 carbonate and acetonitrile, reduced and alkylated before trypsin digestion (Promega).
518 The generated peptides were extracted with 60% acetonitrile in 0.1% formic acid
519 followed by a second extraction with 100% acetonitrile. Acetonitrile was evaporated
520 under vacuum and the peptides were resuspended in 16 μ L of H₂O and 0.1% formic
521 acid before nanoLC-MS/MS analysis.

522 *NanoLC-MS/MS analysis*

523 NanoLC-MS/MS analyses were performed on a nanoACQUITY Ultra-Performance
524 LC-system (Waters, Milford, MA) coupled to a Q-Exactive Plus Orbitrap mass
525 spectrometer (ThermoFisher Scientific) equipped with a nanoelectrospray ion source.
526 Samples were loaded into a Symmetry C18 precolumn (0.18 x 20 mm, 5 μ m particle
527 size; Waters) over 3 min in 1% solvent B (0.1% FA in acetonitrile) at a flow rate of 5
528 μ L/min followed by reverse-phase separation (ACQUITY UPLC BEH130 C18, 200
529 mm x 75 μ m id, 1.7 μ m particle size; Waters) using a binary gradient ranging from
530 1% and 35% of solvent A (0.1 % FA in H₂O) and solvent B at a flow rate of 450
531 nL/min. The mass spectrometer was operated in data-dependent acquisition mode by
532 automatically switching between full MS and consecutive MS/MS acquisitions.
533 Survey full scan MS spectra (mass range 300-1800) were acquired in the Orbitrap at
534 a resolution of 70K at 200 m/z with an automatic gain control (AGC) fixed at $3 \cdot 10^6$
535 ions and a maximal injection time set to 50 ms. The ten most intense peptide ions in
536 each survey scan with a charge state ≥ 2 were selected for MS/MS. MS/MS spectra
537 were acquired at a resolution of 17,5K at 200 m/z, with a fixed first mass at 100 m/z,
538 AGC was set to $1 \cdot 10^5$, and the maximal injection time was set to 100 ms. Peptides
539 were fragmented in the HCD cell by higher-energy collisional dissociation with a
540 normalized collision energy set to 27. Peaks selected for fragmentation were
541 automatically included in a dynamic exclusion list for 60 s. All samples were injected

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542 using a randomized and blocked injection sequence (one biological replicate of each
543 group plus pool in each block). To minimize carry-over, a solvent blank injection was
544 performed after each sample. A sample pool comprising equal amounts of all protein
545 extracts was constituted and regularly injected 4 times during the course of the
546 experiment, as an additional quality control (QC). Protein identification rates and
547 coefficients of variation (CV) monitoring of this QC sample revealed very good
548 stability of the system: 2207 of the 2271 identified proteins, namely 97%, showed a
549 CV value lower than 20% considering all 4 injections.

550 *Data interpretation*

551 Raw MS data processing was performed using MaxQuant software (v 1.5.8.3 (49)).
552 Peak lists were searched against a composite database including all *Homo sapiens*
553 protein sequences extracted from UniprotKB-SwissProt (version April 2019;
554 taxonomy ID: 9606) using the MSDA software suite (50). MaxQuant parameters were
555 set as follows: MS tolerance set to 20 ppm for the first search and 5 ppm for the main
556 search, MS/MS tolerance set to 40 ppm, maximum number of missed cleavages set
557 to 1, Carbamidomethyl (C) set as fixed modification, Acetyl (Protein N-term) and
558 Oxidation (M) set as variable modifications. False discovery rates (FDR) were
559 estimated based on the number of hits after searching a reverse database and was
560 set to 5% for both peptide spectrum matches (minimum length of seven amino acids)
561 and proteins. Data normalization and protein quantification was performed using the
562 LFQ (label free quantification) option implemented in MaxQuant (49) using a “minimal
563 ratio count” of two. The “Match between runs” option was enabled using a 2 min time
564 window after retention time alignment. All other MaxQuant parameters were set as
565 default.

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566 To be validated, proteins must be identified in all four replicates of one condition at
567 least. The imputation of the missing values and differential data analysis were
568 performed using the open-source ProStaR software (51). Two runs of imputation
569 were applied, the “SLSA” mode was applied for the POV (partially observed values)
570 and the “del quantile” for the MEC (missing in the entire condition). Pairwise
571 comparisons were performed using a Limma t-test on protein intensities. P-values
572 calibration was performed using the pounds calibration method and the FDR
573 threshold was set at 5%. The complete proteomics dataset is available via
574 ProteomeXchange (52,53) with identifier PXD010576.

575 ***Gene ontology analysis***

576 Gene set enrichment analysis (GSEA) was run on the protein lists found to be
577 significantly enriched at least 1.5 times in T0-T15, T0-T30 and T15-T30 using the
578 online molecular signature database (MSigDB (23)) v6.2. Significantly enriched gene
579 ontology (GO) pathways related to relevant “biological process” were extracted with
580 their false-discovery rate (FDR). The Table S1 summarizes the relevant GO
581 pathways associated to the T0-T15 time points. No significant enrichment was found
582 at T0-T30 and T15-T30.

583 ***Fluorescence microscopy***

584 50 000 cells were plated on 24 well plates containing 12 mm cover glasses (Electron
585 Microscopy Sciences) and incubated 48 h in complete medium containing 1 µg /ml of
586 avidin. For the different eRUSH assays, cells were incubated at 5 min, 7 min, 12 min,
587 15 min and 30 min in complete medium containing 40 µM of biotin. Cells were fixed
588 with 4% PFA for 20 min at room temperature and were permeabilized for 15 min with
589 PBS containing 0.1% TritonX100 (Sigma-Aldrich), 0.5% bovine serum albumin

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590 (Euromedex). Cells were subsequently incubated one hour at room temperature with
591 different primary antibodies (antibodies section), then one hour with secondary
592 antibodies and DAPI staining. Cells were mounted with mowiol 4-88 (Sigma Aldrich).
593 For LC3 staining, cells were fixed with formalin (Sigma-Aldrich) for 15 min at RT then
594 with cold methanol for 5 min at -20°C, prior antibody staining in PBS containing 0.1%
595 saponin and 1% FBS.

596 Images were taken with an AxioObserver Z1 inverted microscope (Zeiss) mounted
597 with a CSU-X1 spinning disc head (Yokogawa), a back-illuminated EMCCD camera
598 (Evolve, Photometrics) and a X63 (1.45 NA) or X100 (1.45 NA) oil objectives (Zeiss).

599 ***Live imaging***

600 About 250,000 cells seeded on 35 mm #1.5 glass bottom dishes (Ibidi) or on 25 mm
601 cover glasses (Electron Microscopy Sciences) were transfected using JetPrime
602 (Polyplus Transfection) according to manufacturer's instructions. The dish was
603 placed on the microscope stage, maintained in a dark atmosphere-controlled
604 chamber at 37°C and 5% CO₂. Live cell imaging was performed using an
605 AxioObserver Z1 inverted microscope (Zeiss) mounted with a CSU-X1 spinning disc
606 head (Yokogawa), a back-illuminated EMCCD camera (Evolve, Photometrics) and a
607 X100, 1.45 NA oil objective (Zeiss) controlled by VisiView v.3.3.0 software (Visitron
608 Systems). For TIRF microscopy, live imaging was performed with a TIRF PALM
609 STORM microscope from Nikon using a back-illuminated EMCCD camera (Evolve
610 512, Photometrics) and a X100 APO, 1.49NA oil objective controlled by Metamorph,
611 and an iLas² FRAP/TIRF module (BioVision Technologies). The TIRF angle was
612 chosen to obtain a calculated evanescent field depth < 100 nm.

613 ***Preparation of protein extracts***

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614 Cells were seeded at 1.5×10^6 cells per 10 cm dish per conditions in complete medium
615 containing 1 $\mu\text{g/ml}$ of avidin for 48 h. After incubation with biotin for 0 min, 30 min or
616 24 h, cells were washed 3 times with ice cold PBS and lysed with ice cold RIPA
617 buffer (150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1%
618 sodium dodecyl sulfate (SDS), 50 mM Tris, pH 8.0, protease inhibitor (Promega).
619 Cells were placed on ice for 10 min and spun at 10,000 g for 20 min at 4°C. The
620 supernatant was collected and subjected to the Pierce BCA assay kit (ThermoFisher
621 Scientific).

622 **Western blot analysis**

623 A total of 40 μg of protein lysates were run on Bolt 4-12% Bis-Tris plus gels
624 (ThermoFisher Scientific) and proteins were transferred to nitrocellulose membranes.
625 Nitrocellulose membranes were blocked with 5% (w/v) milk in PBS-T (PBS pH 7.4,
626 0.05% Tween 20) for 15 min. Primary antibodies (refer to antibody section) were
627 incubated 1 h at RT or overnight at 4°C in PBS-T containing 5% milk. Secondary
628 antibodies were incubated 1 h at room temperature. After washing with PBS-T,
629 nitrocellulose membranes were incubated with Clarity Max western ECL substrate
630 (Bio-Rad). The specific proteins were visualized with the ChemiDoc imaging system
631 (Bio-Rad).

632 **Software analysis**

633 Image processing was performed using either the FIJI upgrade of ImageJ (54) or the
634 Imaris software v9.2 (Bitplane, Oxford Instruments). Quantifications for colocalization
635 measurements were performed using Imaris software v9.2 (Bitplane, Oxford
636 Instruments). Statistical analyses were performed with Microsoft Excel 2016 and
637 Prism v7.04 (GraphPad). Flow cytometry analysis was done using the FlowJo
638 software v10.4.2 (FlowJo, LLC). Raw mass spectrometry data were first analyzed

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639 using MaxQuant v 1.6.0.16. Differential proteomics data analysis was performed
640 using DAPAR v1.10.3 and ProStaR v 1.10.4.

641

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654

655 **Author contribution**

656 MD and RG conceived the experiments. MD, IC, CD, VL and RG generated and
657 characterized the TfR-eRUSH cell line. IC and RG performed flow cytometry. IC
658 conducted the siRNA-based assays. MD and RG performed the microscopy
659 analyses. FD and AH conducted mass spectrometry an FD, SC, CC and RG
660 analyzed the proteomics data. ES and TX generated constructs for imaging and
661 APEX labeling. GB and FP provided technical and conceptual support. RG and MD
662 wrote the manuscript. RG, MD, CD, FD and CC edited and commented on the
663 manuscript.

664

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665 **Declaration of Interests**

666 The authors declare no competing interests.

667

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- 805
- 806

807 **Fig legends**

808 **Fig 1. Generation and characterization of TfR-eRUSH gene edited cells.**

809 (A) Scheme illustrating the insertion of the linker-SBP-EGFP coding sequence in the
810 chromosomal region containing the stop codon (red) of the *TFRC* gene (Transferrin
811 receptor type 1, referred to as TfR). (B) PCR amplification from genomic DNA using
812 primers flanking the TfR stop codon region confirmed the insertion of the SBP-EGFP
813 sequence on both alleles. (C) Flow cytometry analysis indicates the total amount of
814 TfR expressed in wild type (WT) and TfR-eRUSH cells, 24 h post-biotin treatment.
815 Mouse anti-TfR antibody revealed with anti-mouse Alexa Fluor 647 antibody were
816 used to measure total TfR protein levels. The bar graph indicates the mean
817 fluorescence intensity +/- SD of cell populations expressing TfR. At least 10,000 cells
818 per condition were acquired from n = 6 independent experiments. (D) Live cell
819 imaging of TfR-eRUSH cells started immediately after biotin addition highlights the
820 rapid and dramatic redistribution of TfR over time. Sequential trafficking steps include
821 ER (0 to 6 min) to Golgi (from 4 min) to PM (from 23 min; see blue arrowheads)
822 transport. (E) Flow cytometry analysis representing the mean fluorescence intensity
823 of Tf-A647 bound at the surface of TfR-eRUSH cells. Cells were treated with biotin
824 during indicated times and cells were subsequently switched to 4°C for Tf-A647
825 binding. Background fluorescence was measured by adding an acid wash step,
826 which stripped out all surface bound Tf-A647 (grey bars). The bar graph shows the
827 mean +/- SD of duplicates in which at least 5,000 cells per condition were acquired
828 and is representative of 3 individual experiments. (F) Representative
829 immunofluorescence images detecting the arrival of TfR-eRUSH at the PM. Images
830 were acquired with spinning disk confocal microscope at indicated time points post-
831 biotin addition. TfR-eRUSH at the PM was monitored with Transferrin coupled to

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832 Alexa Fluor 647 (Tf-A647; upper rows) or mouse anti-TfR antibody revealed with a
833 donkey anti-mouse Alexa 647 antibody (TfR Ab; lower rows). The protein was
834 detected at the plasma membrane starting from 20 min post-biotin addition. Scale bar
835 = 10 μ m.

836 **Fig 2. Proteomics analysis of neosynthesized TfR-containing membranes.**

837 (A-C) TfR-eRUSH cells were untreated (T0) or treated with biotin during 15 min (T15)
838 or 30 min (T30). Mechanical cell lysis was performed at 4°C and membrane-
839 containing TfR-eRUSH were isolated by immunoprecipitation using an anti-TfR
840 antibody. LC-MS/MS proteomics analysis was run, and temporal protein enrichment
841 was assessed (see material and methods for details). (A) STRING analysis shows
842 the interaction map of the proteins that were enriched at T15 compared to T0. Color-
843 codes highlight clusters of proteins of related functions. (B) Gene ontology of the
844 proteins enriched at least 1.5 times with a significant p value (< 0.05) at T15
845 compared to T0 (T0-T15) were investigated using the online GSEA online software.
846 Relevant GO pathways and their corresponding FDR (False-discovery rate) values
847 are reported for each differential analysis. (C) Among the 20 Rab proteins identified
848 by LC-MS/MS (see Table S2), the fold enrichment and p values of the ones that were
849 significantly identified at T15 compared to T0 (T0-T15) are reported. No Rab protein
850 were significantly enriched at other differential time points. (D) Representative
851 confocal images from a single z-stack indicate the distribution of TfR-eRUSH treated
852 for 12 min with biotin relative to the endogenous Rab5, Rab6, Rab7, Rab18 proteins
853 and the exogenously expressed Ruby3-Rab10. TfR-eRUSH co-distributed with Rab7
854 and Ruby3-Rab10 (zoomed panel, white arrow). Scale bar = 10 μ m. Zoomed regions
855 from white squares were represented with a scale bar = 1 μ m.

856 **Fig 3. Identification of Rab7 as an intermediate compartment of neosynthesized**
857 **TfR trafficking.**

858 (A) Live cell imaging using a spinning disk confocal microscope shows localization of
859 TfR-eRUSH in Ruby3-Rab7A transfected cells. Representative images were
860 extracted from a single-plane and TfR-eRUSH (green) was visualized within Ruby3-
861 Rab7A (magenta) positive vesicles (white arrows) at 7 min, 9 min and 12 min post-
862 biotin addition, scale bar = 5 μm . (B) TfR-eRUSH cells expressing Ruby3-Rab7A
863 were imaged as in A after treatment with 40 nM Apillimod for 30 min to increase the
864 size of Ruby3-Rab7A vesicles. Biotin was added to release TfR-eRUSH and
865 representative images performed with the spinning disk microscope were extracted
866 as a single-plane at 42- and 44-min post-biotin addition. TfR-eRUSH (green)
867 localizes at the limiting membrane of Ruby3-Rab7A vesicles (magenta) (white
868 square, scale bar = 10 μm). The zoomed regions from the white square highlight
869 single and merge staining of the Ruby3-Rab7A containing TfR-eRUSH (scale bar = 1
870 μm). The white arrows indicate the repartition of TfR-eRUSH at the limiting
871 membrane of the Ruby3-Rab7A vesicle. Of note, trafficking kinetics were much
872 longer following Apilimod treatment. (C-D) TfR-eRUSH cell were treated 15 min with
873 biotin, fixed and stained for endogenous Rab5, Rab6 or Rab7 using specific rabbit
874 antibodies revealed by a donkey anti rabbit Alexa Fluor 647 antibody. (C)
875 Representative images from a single z-stack indicate the localization of TfR-eRUSH
876 relative to the endogenous Rab5, Rab6, Rab7 using spinning disk microscope. TfR-
877 eRUSH colocalization with Rab6, Rab7 is represented with yellow arrows. Scale bar
878 = 5 μm . (D) The graph represents the quantification of the volume of TfR-eRUSH (+/-
879 SEM) colocalizing with Rab5, Rab6 or Rab7. Data represents n = 30 cells (Rab5), n =
880 31 cells (Rab6), n = 27 cells (Rab7) per condition from 3 independent experiments

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881 and student t-test was run to determine significance (* p value < 0.05 and *** p value
882 < 0.001).

883 **Fig 4. TfR-eRUSH transiting through Rab7 vesicles, localizes at the plasma**
884 **membrane.**

885 (A) Representative images from a single z-stack indicate TfR-eRUSH co-distributed
886 with Lamp1 and Rab7 positive vesicles. TfR-eRUSH cells were treated with biotin for
887 7 min, fixed and stained using a rabbit anti-Rab7 antibody and a mouse anti-Lamp1
888 antibody revealed by a donkey anti-rabbit coupled to Alexa Fluor 568 and a donkey
889 anti-mouse coupled to Alexa Fluor 647. The images are single-plane crops from the
890 white square of the upper left image. The snapshots show dual- and triple staining
891 and the merge (lower right panel). TfR-eRUSH (green) co-distributed with Rab7
892 (magenta) and Lamp1 (cyan) in perinuclear localized structures (white arrowheads).
893 Some Rab7 positive vesicles contain TfR-eRUSH but no Lamp1 (yellow arrows). (B)
894 Live cell imaging indicates that Rab7A positive vesicles containing TfR-eRUSH are
895 not labeled by lysotracker. Cells were transfected with Ruby3-Rab7A for 24h. Before
896 imaging, cells were incubated for 30 min with lysotracker (50 nM) then biotin was
897 added to induce the release of TfR-eRUSH. Images extracted at 10 min post-biotin
898 addition, were acquired with spinning disk confocal microscopy and represented as a
899 single plane (scale bar = 10 μ m). White squares showed the zoomed panels with
900 single staining and the merge. The white arrow indicates that Ruby3-Rab7A
901 (magenta) co-distribute with TfR-eRUSH (green) while the yellow arrow shows that
902 lysotracker (cyan) co-distribute with Ruby3-Rab7A (magenta). Scale bar= 2 μ m. (C)
903 Immunofluorescence images indicate that Rab7A vesicles containing TfR-eRUSH
904 does not contain DQ-BSA. TfR-eRUSH cells were incubated with 10 μ g/ml of DQ-
905 BSA for 6 h in the presence of 1 μ g/ml avidin. Then, TfR-eRUSH cells were treated

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906 with biotin for 16 min, fixed and stained with rabbit anti-Rab7 antibody. Secondary
907 antibody donkey anti rabbit Alexa Fluor 647 was used. Images were acquired with
908 spinning disk confocal microscopy and are represented as a single plane (scale bar =
909 10 μ m). White squares indicated zoomed regions with single staining and the merge.
910 TfR-eRUSH (green) co-distributed with Rab7 (cyan) (square 1) and DQ-BSA
911 (magenta) co-distributed with Rab7 (cyan) (square 2). Scale bar = 2 μ m. (D) The bar
912 graph represents the quantification of the TfR-eRUSH vesicles colocalizing with
913 Rab7A or DQ-BSA. Data represents n = 32 cells (DQ-BSA) and n = 28 cells (Rab7)
914 from 3 independent experiments (+/- SEM). Student t-test was run to determine
915 significance (***) p value < 0.001). (E) Western blot analysis indicate that TfR-eRUSH
916 is not degraded following biotin addition. TfR-eRUSH cells were incubated for 4 h in
917 the presence or absence of 50 μ g/ml cycloheximide and 100 nM bafilomycin A1 as
918 indicated. Biotin was added for 0 or 30 min and cells were lysed for western blot
919 analysis. Actin was used as a loading control. The presence of LC3-II over LC3-I
920 confirmed the inhibitory effect of bafilomycin A1 on protein degradation. Low
921 molecular weight protein marker (LMW) was used for molecular weight estimation.
922 (F) Immunofluorescence images indicates that LC3 does not colocalize with Rab7
923 and TfR-eRUSH. Cells were treated with biotin for 15 min, fixed and stained with
924 rabbit anti-Rab7 antibody, and mouse anti-LC3 antibody. Secondary antibody donkey
925 anti rabbit Alexa Fluor 647 and donkey anti-mouse Alexa 561 were used. Images
926 were acquired with spinning disk confocal microscopy and are represented as a
927 single plane (scale bar = 10 μ m). White squares indicated zoomed regions with
928 single staining and the merge. TfR-eRUSH (green) co-distributed with Rab7
929 (magenta) but not with LC3 (cyan) Scale bar = 2 μ m.

930

931 **Figure 5. Rab7A is involved in transport of TfR-eRUSH at the plasma**
932 **membrane.**

933 (A) TfR-eRUSH cells were treated with siRNA sequences targeting 12 different Rab
934 mRNAs and a non-targeting siRNA control. After 48 h post-transfection, cells were
935 untreated (T0) or treated with biotin for 15 min (T15). Measure of the amount of TfR-
936 eRUSH at the PM was performed by flow cytometry as in Fig 1E. The bar graph
937 represents the mean fold change +/- SD corresponding to the ratio between the Tf-
938 A647 MFI measured at 15 min and 0 min from two individual experiments performed
939 in duplicates in which at least 2,000 cells were analyzed. Anova and Student t-test
940 were run to assess for significance (red bar graph: p value < 0.05 for siRab2A,
941 siRab6A and siRab7A and p value < 0.001 for siRab27A). (B) TfR-eRUSH cells
942 transfected with Ruby3-Rab7A were imaged by TIRF microscopy 24 h post-
943 transfection. Cells were imaged from 5- to 25-min post-biotin addition. A
944 representative image extracted from the Movie S4 was shown at 726 s (scale bar =
945 10 μ m). The white square indicates the cropped region represented in the right
946 panels. In these panels, a Ruby3-Rab7A (magenta) vesicle carrying TfR-eRUSH
947 (green) was tracked from 704 s to 736 s. Scale bar = 1 μ m. (C) TfR-eRUSH cells
948 transfected with Ruby3-Rab6A were imaged by TIRF microscopy 24 h post-
949 transfection. Cells were imaged from 5- to 25-min post-biotin addition. A
950 representative image extracted from movie S5 was shown at 860 s (scale bar = 10
951 μ m). The white square indicates the cropped region represented in the right panels.
952 In the right panels a Ruby3-Rab6A (magenta) vesicle carrying TfR-eRUSH (green)
953 was tracked from 848 s to 868 s. It indicates a Rab6A vesicle releasing a TfR-eRUSH
954 directly to the PM. Scale bar = 1 μ m.

956 **Supporting information**

957 **Fig. S1. Characterization and distribution of TfR-eRUSH over time post-biotin**
958 **addition.**

959 (A-B) The western blot represents TfR expression in edited cells (TfR-eRUSH)
960 compared to non-edited cells (wild type). Two molecular weight markers were used
961 LMW (low molecular weight) and HMW (high molecular weight) to confirm TfR
962 molecular size. Actin was used as a loading control. Anti-TfR was used in (A) and
963 Anti-GFP in (B). (C) TfR-eRUSH cells were incubated for indicated times with biotin,
964 fixed and stained with anti-calnexin antibodies (ER marker), anti-GM130 (cis-Golgi
965 marker), anti-TGN46 (TGN marker) and revealed with appropriate secondary
966 antibodies. The snapshots of cropped merged images from a single plane show TfR-
967 eRUSH (green), Dapi staining (blue) and the indicated organelle marker (magenta).
968 (D) Quantification of TfR-eRUSH colocalization with calnexin, GM130 or TGN46 at
969 indicated times post-biotin addition. The Pearson's correlation coefficient in the total
970 volume of the cell was measured using the Imaris software. The graph shows mean
971 Pearson's correlation coefficient +/- SD and n = 17-20 cells from 2 independent
972 experiments. Statistics were measured using unpaired t-test. * p value < 0.05, ** p
973 value < 0.01, *** p value < 0.001. ns = non-significant.

974 **Fig. S2. TfR-eRUSH co-distributes with TMED10 but does not transit through**
975 **mitochondria.**

976 (A) Representative images from a single z-stack indicate the localization of TfR-
977 eRUSH relative to the endogenous TMED10 protein. TfR-eRUSH cells were treated
978 for 12 min with biotin and images were acquired with a spinning disk confocal
979 microscope. Representative images from a single plane show TfR-eRUSH (green),

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980 TMED10 (magenta) and nucleus (blue). Scale bar = 10 μ m. Zoomed regions from
981 white squares indicate a vesicle with TfR-eRUSH co-distributing with TMED10. Scale
982 bar = 1 μ m. (B) TfR-eRUSH cells were incubated for indicated times with biotin and
983 mitochondria were visualized with 100 nM Mitotracker. Representative images from a
984 single plane show TfR-eRUSH (green), mitochondria (magenta) and nucleus (blue).
985 White dashed squares represent zoomed regions (lower panel). (C) Live imaging of
986 TfR-eRUSH cells incubated with MitoTracker and biotin was performed at 30 s per
987 frame using a spinning disk confocal microscope. An isolated event of TfR-
988 mitochondria colocalization was observed 6 min post-biotin addition, although our
989 spatial resolution limits the extend of this observation. The red square represented
990 zoomed regions (left panel).

991 **Fig. S3. Rab7A vesicles transporting TfR-eRUSH does not contain Rab6A.**

992 (A) TfR-eRUSH cells transfected with Ruby3-Rab6A were imaged by TIRF
993 microscopy 24 h post-transfection. Representative images were extracted at 10, 12
994 and 13 min post-biotin addition (scale bar = 10 μ m). The white arrows show the
995 Ruby3-Rab6A vesicles co-distributing with TfR-eRUSH. The yellow arrows indicate
996 the zoomed regions of a Ruby3-Rab6A vesicle (magenta) co-distributing with TfR-
997 eRUSH (green). Scale bar = 1 μ m. (B) TfR-eRUSH cells were transfected with
998 Ruby3-Rab7A for 24 h. Biotin was added for 15 min and images were taken with the
999 spinning disk confocal microscope. Two cells were represented as images from a
1000 single z-stack. The staining indicates TfR-eRUSH (green), Ruby3-Rab7A (magenta),
1001 endogenous Rab6 (cyan), and the merge. The white arrows show Rab7A vesicles
1002 containing TfR-eRUSH but no Rab6. Scale bars = 5 μ m.

1003 **Fig. S4. TfR-eRUSH is not degraded following biotin addition.**

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1004 (A-B) Western blots representing the absence of degradation of TfR-eRUSH in
1005 presence of cycloheximide. Wild type and TfR-eRUSH cells were treated for 4 h with
1006 or without 50 µg/ml of cycloheximide and subsequently treated for 30 min with biotin.
1007 Actin was used as a loading control. Anti-TfR antibodies were used for visualization
1008 and HMW protein marker was used for molecular weight estimation in (A). Anti-GFP
1009 antibodies were used for visualization and LMW protein marker was used for
1010 molecular weight estimation in (B).

1011 **Fig. S5. siRNA screen carried out on TfR-eRUSH cells**

1012 Effect of Rab silencing on TfR-eRUSH expression. TfR-eRUSH cells were treated
1013 with siRNA sequences targeting 12 different Rab mRNAs and a non-targeting siRNA
1014 control. After 48 h post-transfection, the amount of total TfR-eRUSH at time 0 min
1015 was monitored by flow cytometry using EGFP fluorescence intensity. Anova test was
1016 run to assess for significance (* p value < 0.05, ** p value < 0.01, *** p value <
1017 0.001).

1018 **Table S1. Pathway enrichment analysis of proteins binding to TfR-eRUSH**
1019 **membranes at T0-T15.**

1020 LC-MS/MS identification of the proteins associated to TfR-eRUSH membranes
1021 enriched > 1.5-fold at T15 compared to T0 and their associated ontology pathways.

1022 **Table S2. Analysis of the Rab family members identified by mass spectrometry.**

1023 LC-MS/MS identification of the proteins from the Rab GTPase family associated to
1024 TfR-eRUSH membranes. Fold change is shown for all detected Rabs. The red Rabs
1025 indicate that they are significantly enriched at T15 compared to T0.

1026 **Table S3. List of the target sequences used in the siRNA screen.**

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1027 Oligonucleotides contained in the custom-made siGenome Smart pool cherry-pick
1028 library and used for the siRNA screen.

1029 **Movie S1. Transport of neosynthesized TfR toward the PM.**

1030 TfR-eRUSH cells were incubated with biotin and imaged using 3D spinning disk
1031 confocal microscopy. The images correspond to a z-stack spaced by 0.6 μm
1032 acquired every 30 s for 40 min. Three-dimensional reconstruction was performed
1033 using Imaris.

1034 **Movie S2. TfR-eRUSH trafficking is mostly independent of the mitochondrial
1035 distribution.**

1036 TfR-eRUSH cells were incubated with biotin and MitoTracker then imaged using 3D
1037 spinning disk confocal microscopy. The movie corresponds to a single plane from a
1038 z-stack spaced by 0.5 μm acquired every 10 s for 10 min. TfR-eRUSH is shown in
1039 green and MitoTracker in magenta. Scale bar = 2 μm .

1040 **Movie S3. A subset of neosynthesized TfR-eRUSH traffics through Rab7-
1041 positive vesicles.**

1042 TfR-eRUSH cells were transfected with Ruby3-Rab7A for 24 h and imaged in the
1043 presence of biotin using 3D spinning disk confocal microscopy. The movie
1044 corresponds to a single plane from a z-stack spaced by 0.3 μm acquired every 10 s
1045 from 7 min to 24 min. TfR-eRUSH is shown in green and Ruby3-Rab7A in magenta.
1046 Scale bar = 2 μm .

1047 **Movie S4. TfR-eRUSH vesicles transiently interact with Rab7A.**

1048 TfR-eRUSH cells were transfected with Ruby3-Rab7A for 24 h and imaged in the
1049 presence of biotin using TIRF microscopy. The movie represents a cropped vesicle

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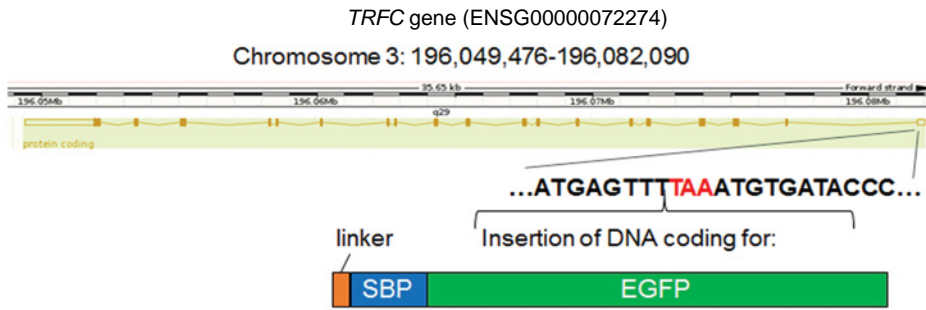
1050 starting from 706 s to 748 s post-biotin addition. A single evanescent field is acquired
1051 in TIRF mode every 2 s. TfR-eRUSH is shown in green and Ruby3-Rab7A in
1052 magenta. Scale bar = 2 μ m.

1053 **Movie S5. TfR-eRUSH vesicles interact with Rab6A.**

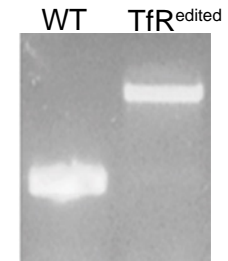
1054 TfR-eRUSH cells were transfected with Ruby3-Rab6A for 24 h and imaged in the
1055 presence of biotin using TIRF microscopy. The movie represents a cropped vesicle
1056 starting from 838 s to 878 s post-biotin addition. A single evanescent field is acquired
1057 in TIRF mode every 2 s. TfR-eRUSH is shown in green and Ruby3-Rab6A in
1058 magenta. Scale bar = 2 μ m.

1059

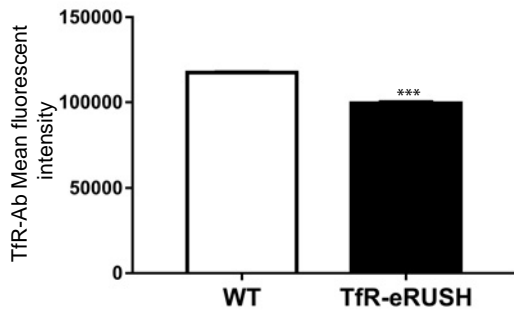
A



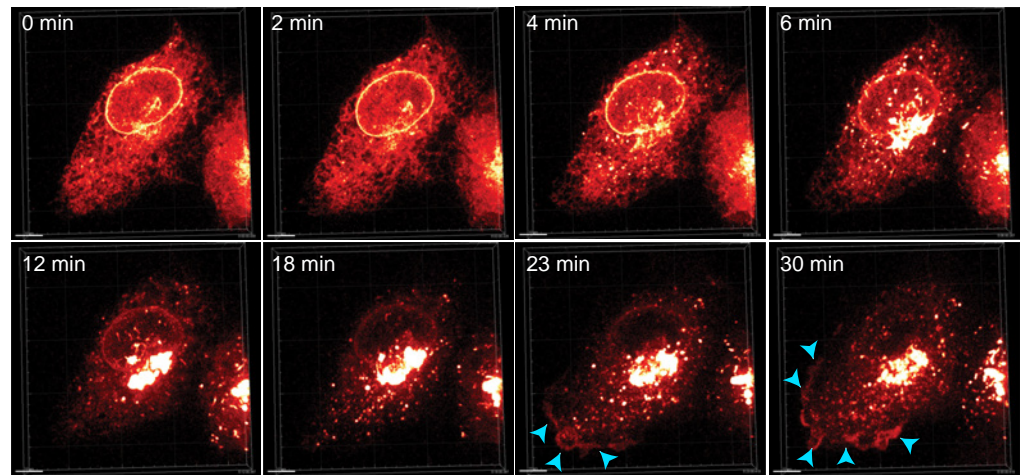
B



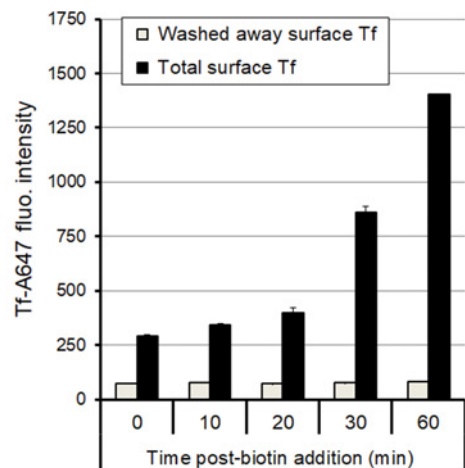
C



D



E



F

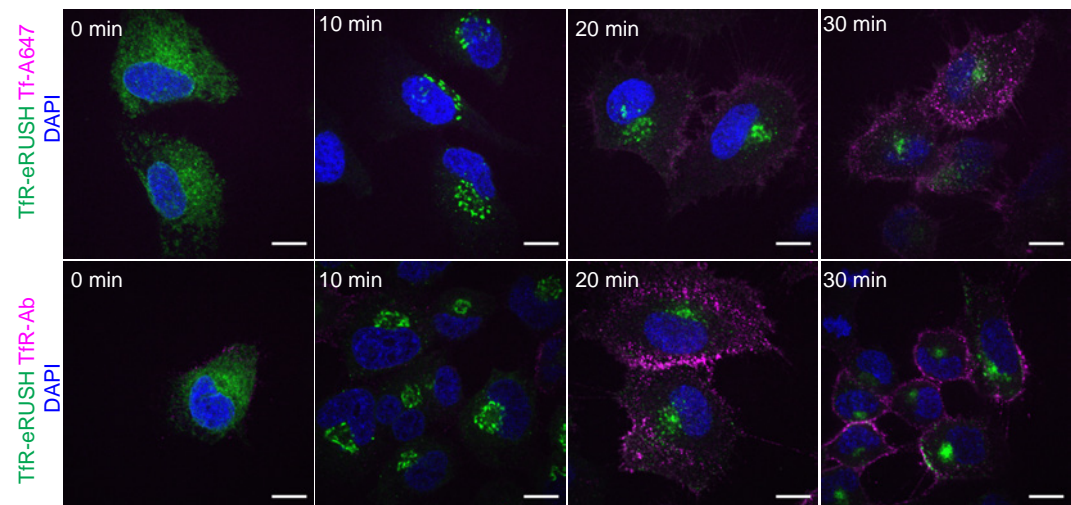
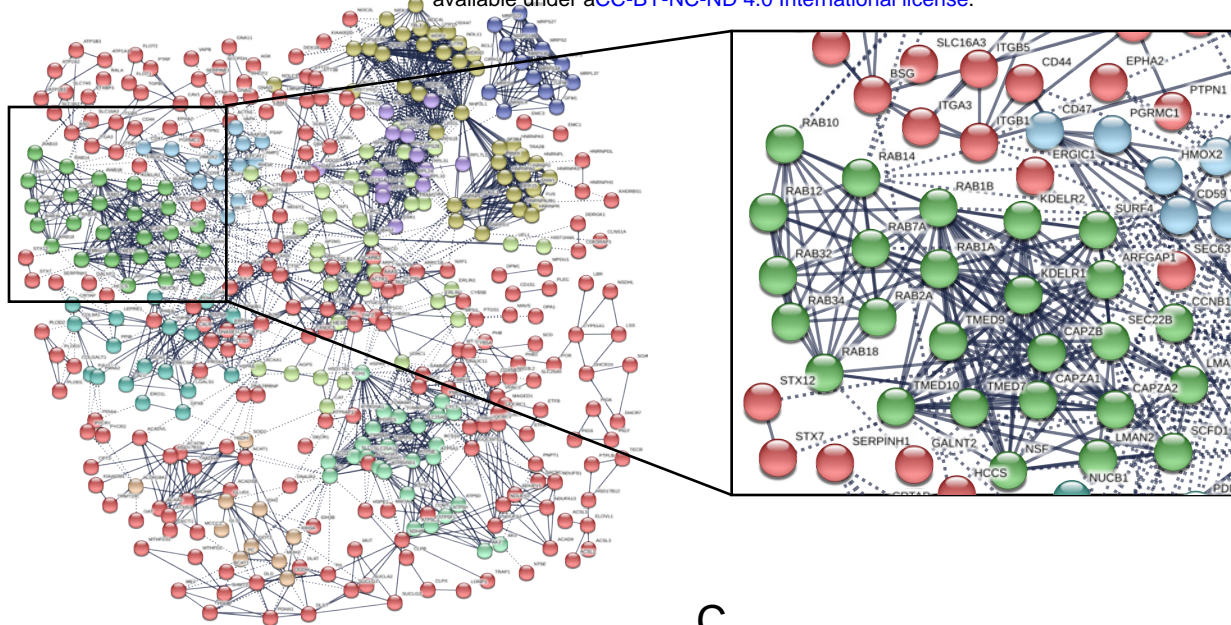


Fig 1. Deffieu et al.

A



B

Pathway (biological process)	FDR q-value
Oxidation reduction process	$5.61 e^{-71}$
Intracellular transport	$8.01 e^{-51}$
Organic acid metabolic process	$2.70 e^{-45}$
Cellular macromolecule localization	$4.77 e^{-37}$
Cellular respiration	$1.79 e^{-36}$
Intracellular protein transport	$3.67 e^{-34}$
Response to endoplasmic reticulum stress	$1.08 e^{-29}$
lipid metabolic process	$3.61 e^{-29}$
Mitochondrion organization	$1.36 e^{-27}$
Secretion	$3.69 e^{-26}$

C

Protein	Fold enrichment	p value
RAB18	1.89	0.0021
RAB32	1.82	0.0026
RAB7A	1.73	0.0080
RAB10	1.70	0.0071
RAB2A;RAB2B	1.70	0.0102
RAB1B	1.64	0.0233
RAB14	1.61	0.0093
RAB1A	1.60	0.0053
RAB34	1.54	0.0183
RAB12	1.52	0.0113

D

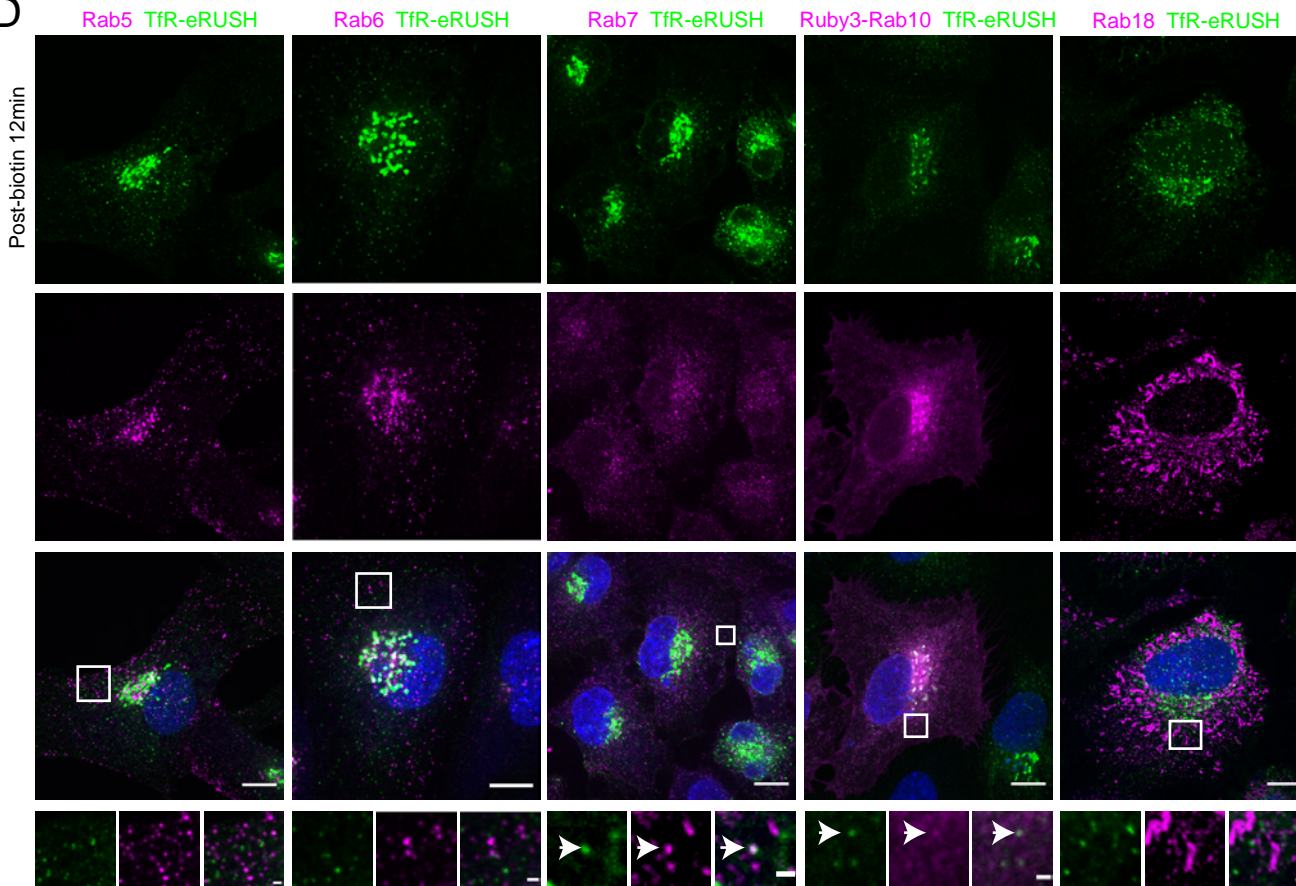
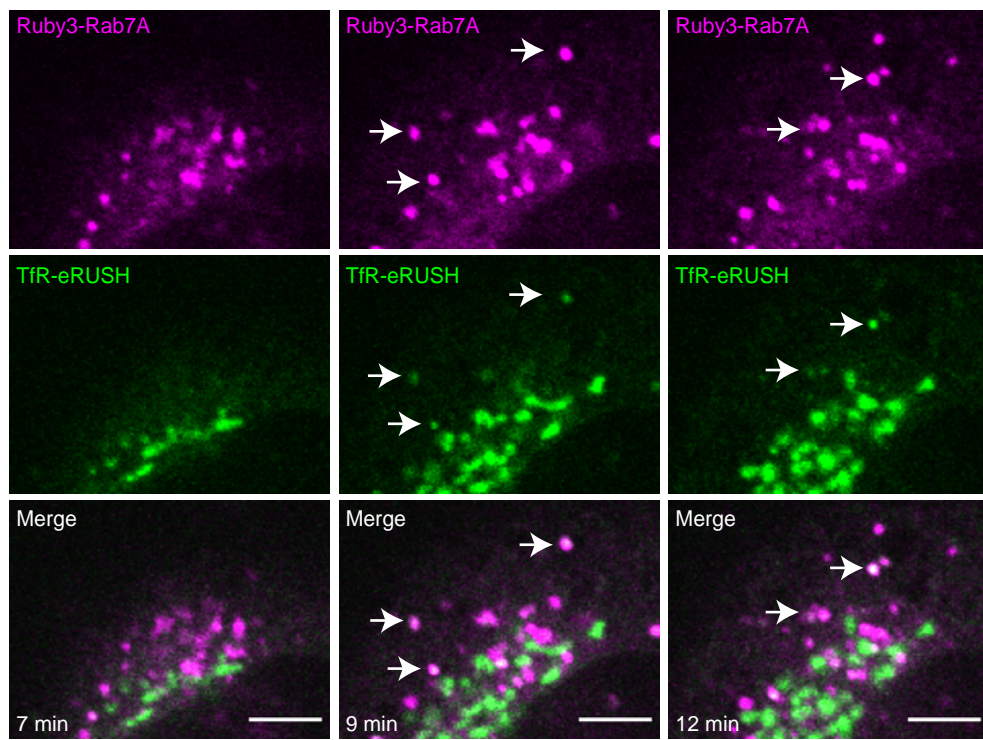
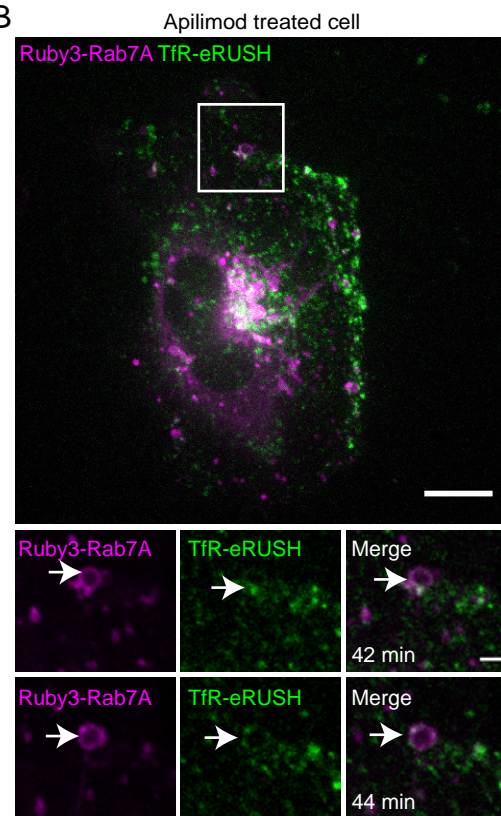


Fig 2. Deffieu et al.

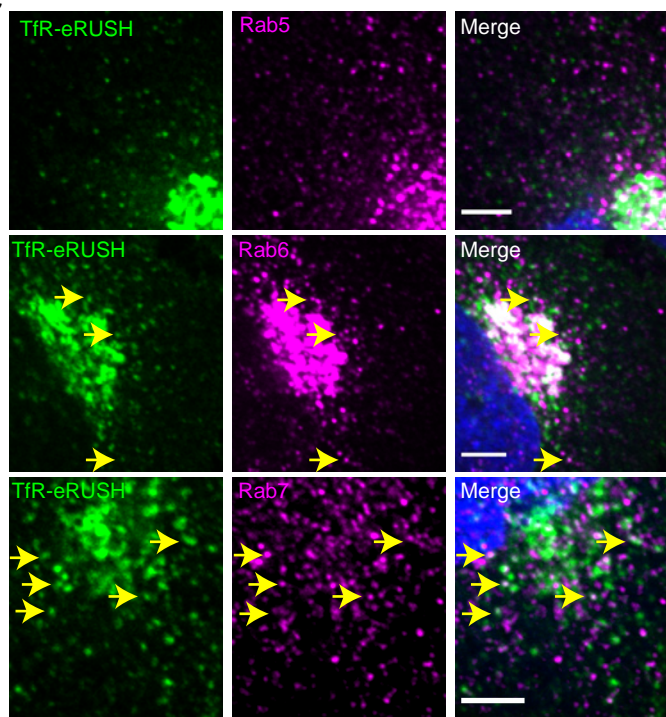
A



B



C



D

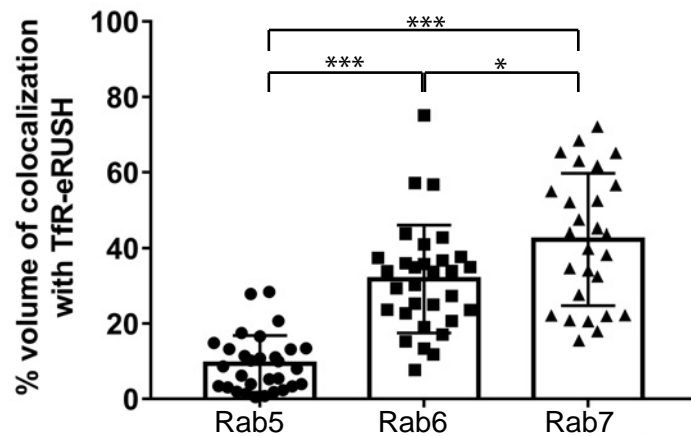
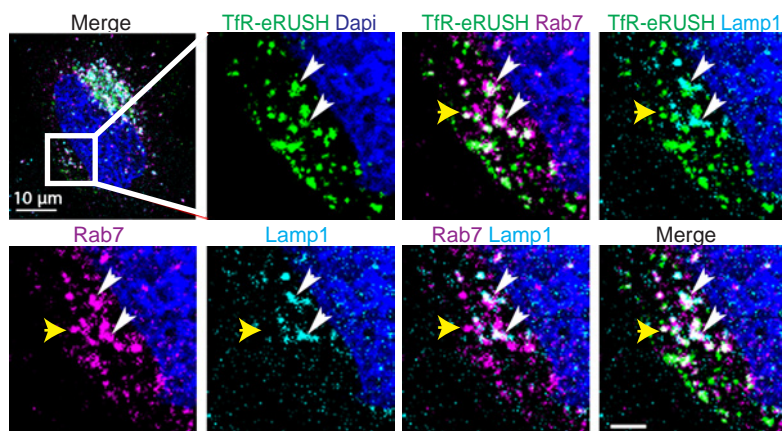
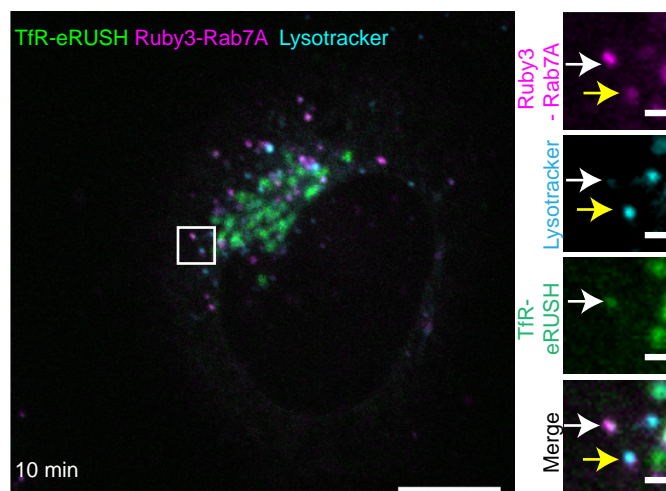


Fig 3. Deffieu et al.

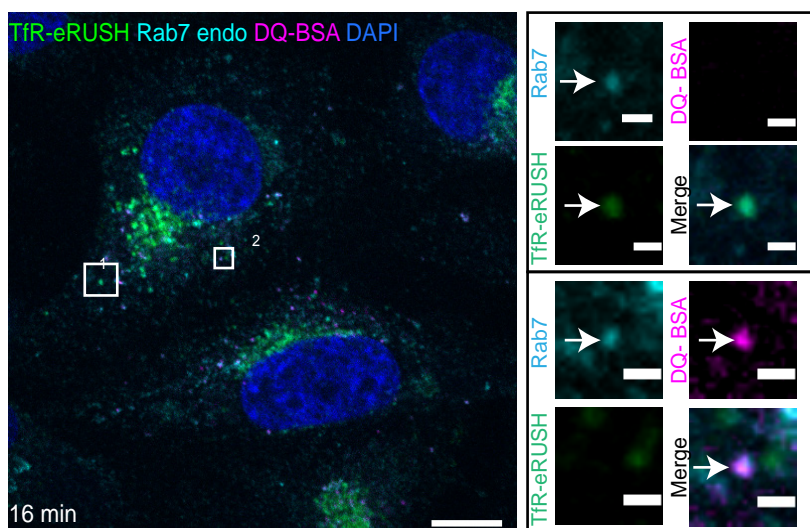
A



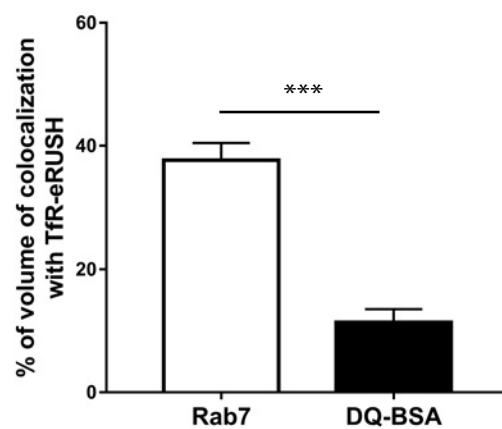
B



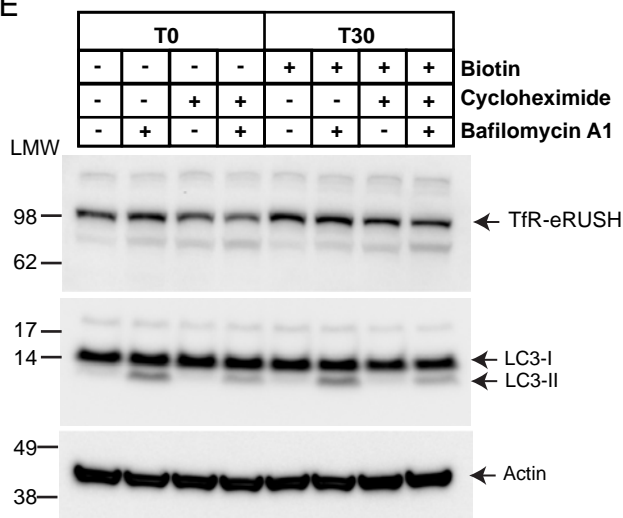
C



D



E



F

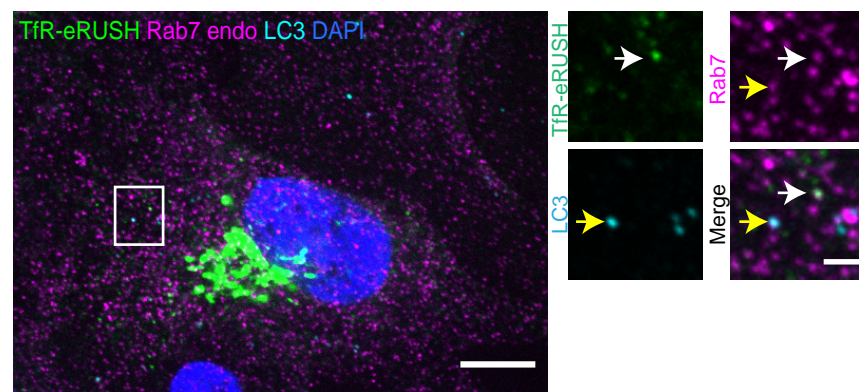
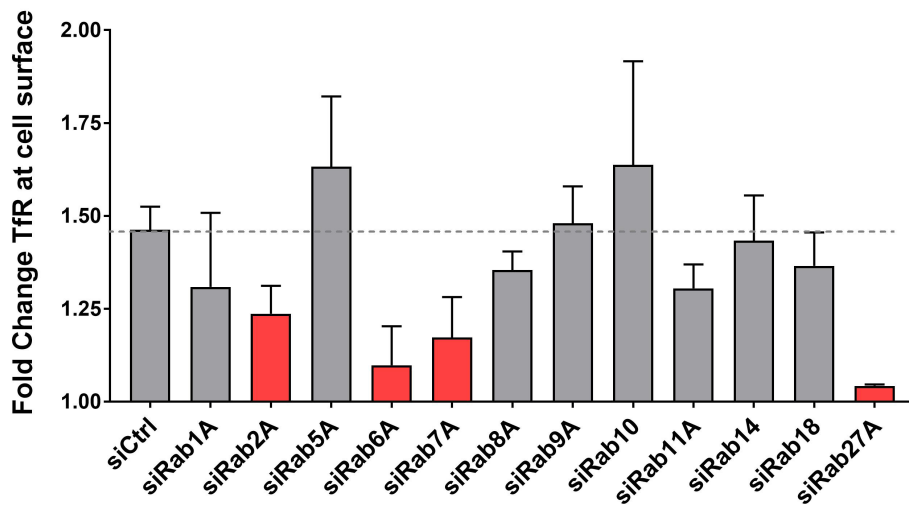
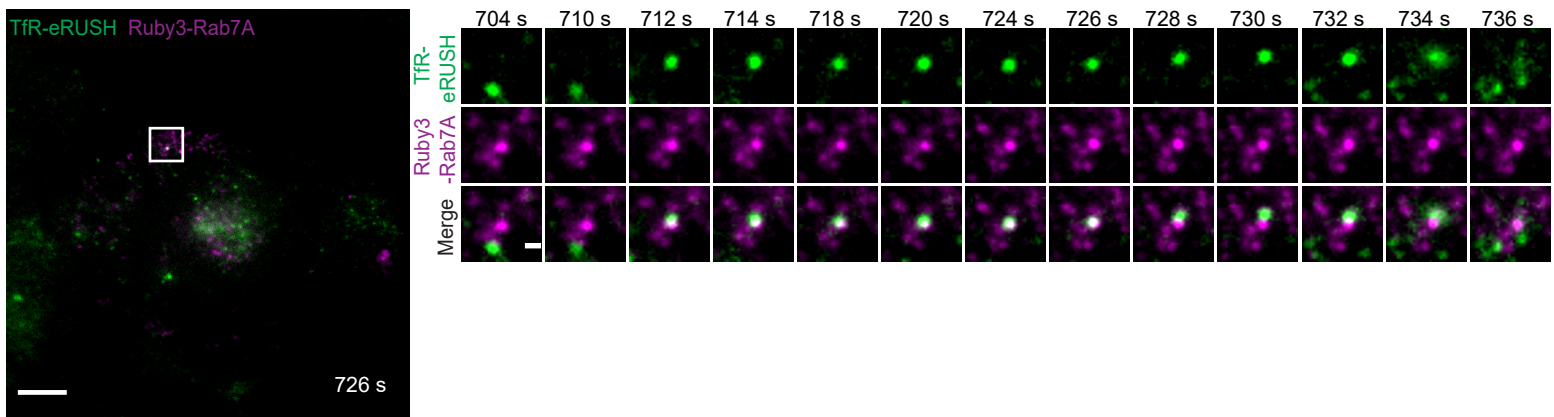


Fig 4. Deffieu et al.

A



B



C

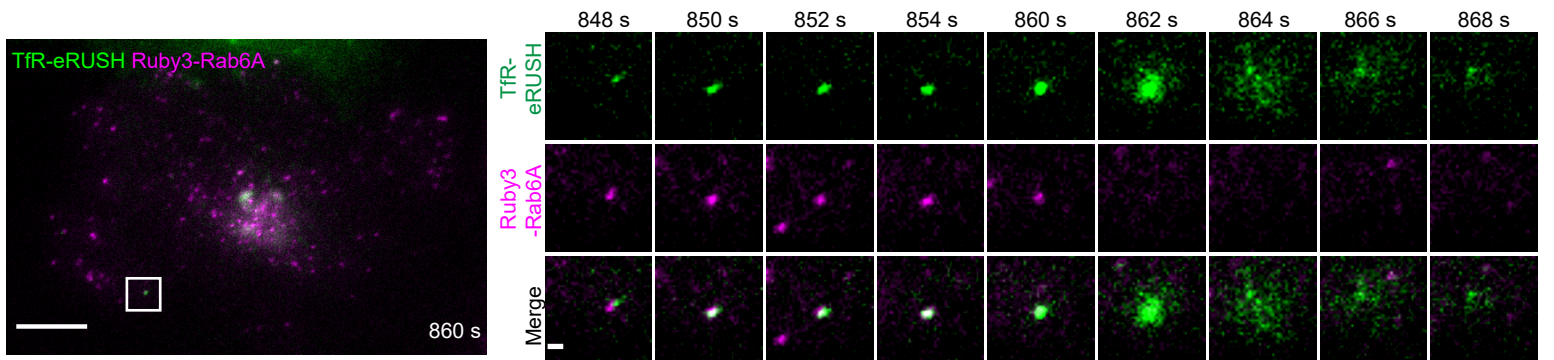


Fig 5. Deffieu et al.