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► To cite this version:

José Ignacio Valenzuela, Franck Perez. Localized intercellular transfer of ephrin-As by trans-endocytosis enables long-term signaling. *Developmental Cell*, 2020, 52 (1), pp.104-117.e5. <10.1016/j.devcel.2019.11.013>. <hal-03090958>

HAL Id: hal-03090958

<https://hal.science/hal-03090958v1>

Submitted on 30 Dec 2020

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1 **Localized intercellular transfer of ephrin-As** 2 **by trans-endocytosis enables long-term** 3 **signaling**

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10 **Summary**

11 Ephrins can elicit either contact-mediated cell-cell adhesion or repulsion, depending on the
12 efficiency of removal of their ligand/receptor complexes from the cell surface, thus
13 controlling tissue morphogenesis and oncogenic development. However, the dynamic of
14 turnover of the newly assembled ephrin/Eph complexes during cell-cell interactions
15 remains mostly unexplored. Here we show that ephrin-A1/EphA2 complexes are locally
16 formed at the tip of filopodia, at cell-to-cell contacts. Clusters of ephrin-A1 from donor
17 cells surf on filopodia associated to EphA2-bearing sub-domains of acceptor cells. Full-
18 length ephrin-A1 is transferred to acceptor cells by trans-endocytosis through a proteolysis-
19 independent mechanism. Trans-endocytosed ephrin-A1 bound to its receptor enables
20 signaling to be emitted from endo-lysosomes of acceptor cells. Localized trans-endocytosis
21 of ephrin-A1 sustains contact-mediated repulsion on cancer cells. Our results uncover the
22 essential role played by local concentration at the tip of filopodia and the trans-endocytosis
23 of full-length ephrin to maintain long-lasting ephrin signaling.

24 **Introduction**

25 Cell contact-mediated repulsion is a basic mechanism of cell and axon navigation that
26 underlies tissue boundary formation and patterning (Batlle and Wilkinson, 2012). The
27 fundamental principle relies on the high-affinity interaction between a surface-bound
28 repellent and its receptor on opposing cell. The repellent-receptor complexes form a
29 physical tether that must be removed after cell-cell contact to allow cell detachment and
30 repulsion. This can be achieved by two conceptually different mechanisms: i) regulated
31 cleavage of the ligand-receptor complex (Hattori et al., 2000; Janes et al., 2005), ii) trans-
32 endocytosis of the complete ligand-receptor cluster (Gaitanos et al., 2016; Marston et al.,
33 2003; Zimmer et al., 2003). Eph receptors (EphRs) are the largest subfamily of tyrosine
34 kinases receptors and together with ephrins, their membrane-bound ligands, constitute
35 important cell guiding cues during development and oncogenesis (Batlle and Wilkinson,
36 2012; Boyd et al., 2014; Kania and Klein, 2016). EphRs activate signaling pathways that
37 directly impact cytoskeleton regulating cell-cell interactions, guiding cell migration,
38 neuronal pathfinding and tissue patterning (Kania and Klein, 2016). EphRs are classified
39 into two subfamilies; EphA and EphB, depending on the membrane anchoring nature of the
40 ligand they preferentially bind, either glycosylphosphatidylinositol (GPI)-anchored ephrins
41 (ephrin-As type) or transmembrane ephrins (ephrin-Bs type) respectively (Eph
42 Nomenclature Committee, 1997). Ephrin's linkage to membranes facilitates its clustering,
43 which is essential for the efficient receptor activation (Davis et al., 1994; Stein et al., 1998).
44 The membrane localization of the ligands provides to the ephrin-Eph pathway the unique
45 property of transducing reverse signals into the cells that express the ligand, in addition to
46 the forward signals in the cells that contain the EphRs (Klein, 2009; Pasquale, 2008).

47 Under the current paradigm of ephrin-mediated repulsion, ephrin-B/EphB cell tethers are
48 removed from the cell surface by trans-endocytosis of the whole complex including
49 portions of the surrounding membranes (Marston et al., 2003), by a mechanism that is
50 bidirectional (Gaitanos et al., 2016; Zimmer et al., 2003) and depends on the intracellular
51 domains of both proteins and the Rac pathway (Gaitanos et al., 2016; Marston et al., 2003;

52 Zimmer et al., 2003). In contrast, ephrin-A/EphA cell tethers are broken by the controlled
53 proteolysis of the ephrin ectodomain followed by endocytosis of the cleaved ligand-
54 receptor complex (Greene et al., 2014; Hattori et al., 2000; Janes et al., 2005, 2009; Yoo et
55 al., 2011). The main metalloprotease involved in the ephrin-A cleavage is ADAM10 (A
56 disintegrin and metalloprotease 10). Thereby, ephrin-A2 and ephrin-A5 are cleaved by
57 ADAM10 which may act both in *cis* or *trans* (Hattori et al., 2000, Janes et al., 2005, 2009).
58 ADAM10 is recruited to sites of ephrin-A1/EphA2 interaction where ephrin-A1 removal
59 from membranes is sensitive to ADAM10/17 inhibition (Greene et al., 2014). Ephrin-A1
60 might also be processed by ADAM12 (Ieguchi et al., 2014). While proteolytic cleavage of
61 ephrin-B/EphB have also been observed (Georgakopoulos et al., 2006; Litterst et al., 2007;
62 Tomita et al., 2006), only the shedding mechanism has been reported for the ephrin-
63 A/EphA-dependent cell-to-cell detachment.

64 The spatial organization of the EphRs at the plasma membrane (PM) is essential for their
65 response to ephrins and subsequent endocytosis (Davis et al., 1994; Greene et al., 2014;
66 Schaupp et al., 2014; Seiradake et al., 2013; Shaw et al., 2014; Stein et al., 1998).
67 Nevertheless, very little is known about the arrival to the PM of ephrins and EphRs and
68 how it defines the sites of assembly and removal of the cell tether during cell-cell
69 interactions. Here, we have synchronized the secretory trafficking of ephrin-As and its
70 receptor in order to control their membrane delivery and spatiotemporally characterize the
71 subcellular dynamics and fate of their signaling complexes under a native membrane
72 context. We focused on the trafficking of ephrin-A1 and its receptor EphA2. EphA2 is
73 frequently overexpressed and associated with poor prognosis in many different types of
74 cancer (Boyd et al., 2014; White and Getsios, 2014; Wykosky and Debinski, 2008). Here
75 we report that ephrin-A1 locally forms signaling clusters with EphA2Rs on opposing cells
76 at sites of filopodia-filopodia and filopodia-cell interaction. Receptor-expressing cells
77 internalize full-length ephrin-A1. Importantly, we show that trans-endocytosis of
78 ephrin-A1 is independent of its cleavage. This adds a new layer of complexity to the
79 accepted model of how ephrin-As regulate cell-to-cell contact and reveals the unexpected

80 role of the trans-endocytosis of ephrin to sustain long-lasting signaling from
81 endomembranes.

82 **Results**

83 **Synchronization of the secretion of EphA2 and ephrin-A1**

84 To finely tune the delivery of EphA and ephrin-As to the PM we used the RUSH (Retention
85 Using Selective Hooks) system (Boncompain et al., 2012). RUSH allows the
86 synchronization of the secretory trafficking of a chosen protein using biotin-sensitive cargo
87 retention in the ER (Fig. 1A). We focused on the trafficking of ephrin-A1 and its receptor
88 EphA2. We used HeLa cells because their morphology allows to easily identify various
89 cellular compartment and they recapitulate *in vitro* the cell signaling through filopodia that
90 have been observed *in vivo* (Lidke et al., 2005; Moti et al., 2019). We performed
91 simultaneous live-cell imaging of SBP-Cherry-ephrin-A1 and EphA2-EGFP-SBP. Both
92 cargoes were efficiently retained in the ER and rapidly exported from it after biotin
93 addition, reaching the Golgi apparatus (GA) in ~5 min (Fig. 1 C, D). We measured the
94 fluorescence intensity of the cargoes at the GA over the time as an indication of their
95 trafficking kinetic. We observed that both cargoes peak at ~15 min (Fig. 1C, D) before
96 experiencing an intra-GA segregation. A difference in GA exit kinetic is also observed,
97 with ephrin-A1 residing longer than EphA2 in the GA (Fig. 1C to E and video S1). This
98 coincides with the sorting of EphA2 and ephrin-A1, which are partially segregated in post-
99 Golgi vesicles in route to the PM (Fig. 1C, E). As expected, and as a control, SBP-EGFP-
100 ephrin-A1 and SBP-Cherry-ephrin-A1 fully co-localized during their secretory transport
101 (Fig. 1E and video S1).

102 We observed that ephrin-A1, but not EphA2 was concentrated in filopodia-associated
103 subdomains of the PM upon arriving at the cell surface (Fig. 1F). It then seemed to be
104 exported into neighboring cells at later time points. We imaged the trafficking of SBP-
105 EGFP-ephrin-A1 in live-cells co-expressing a mCherry-tagged PM marker (PM-Cherry).
106 We observed that ephrin-A1 clusters were formed at the base or all along filopodia ~20 min

107 after biotin addition, where they often localize at the tip before being exported from ligand
108 producing cells later on (Fig. 1G-I and video S2).

109 **Ephrin-As are sorted into signaling filopodia**

110 This phenomenon was also observed for another GPI-anchored ephrin-A. ephrin-A5 (SBP-
111 EGFP-ephrin-A5) was also targeted to, and released from, the tip of filopodia upon arriving
112 at the cell surface (Fig. 2A, and video S3). This suggests that the localized secretion on
113 filopodia may be a general behavior for ephrin-As. We tested if this was a common behavior
114 of GPI-anchored proteins (GPI-APs). We used the RUSH system to co-express ephrin-A1
115 together with different GPI-APs: a fully synthetic one (SBP-Cherry-GPI) or the naturally
116 occurring GPI-APs (SBP-Cherry-CD59 and SBP-Cherry-ALPP). While all the GPI-APs
117 we analyzed were co-transported with ephrin-A1 at early stages, they were excluded from
118 the ephrin-A1-specific clusters at the PM and were not exported from transfected cells (Fig.
119 2B and video S4).

120 Because ephrin-A1 clusters were secreted from actin-rich filopodia, as visualized with the
121 actin-binding peptide F-tractin-EGFP (Belin et al., 2014) (Fig. 2C), we questioned the role
122 of actin dynamics in this process. We used cytochalasin-D to depolymerize actin in cells
123 co-transfected with SBP-EGFP-ephrin-A1 and PM-Cherry. Live-cell imaging in control
124 cells confirmed that, as observed above for SBP-EGFP- ephrin-A5, SBP-EGFP-ephrin-A1
125 clusters were only formed at the cell-cell interfaces (Fig. 2A and D, video S3 and S5).
126 Interestingly, single filopodia can support many rounds of ephrin-A1 export, with the
127 clusters being subsequently detected into neighboring cells. Surprisingly, despite the
128 decrease in number and length of filopodia observed after actin disorganization (Fig. 2F-
129 G), ephrin-A1 clusters were still produced with timing comparable to control cells.
130 However they were rarely found at the tips of cellular extension and their transfer to
131 neighboring cells was strongly impaired (Fig. 2D-E and H, video S5). End-point
132 quantifications of fixed cells showed a significant decrease in the number of transferred
133 ephrin-A1 clusters to the surrounding cells after cytochalasin-D or latrunculin-A treatments

134 (Fig. 2H). These data suggest that newly synthesized ephrin-As might be targeted to
135 specialized signaling filopodia and that transport to surrounding cells is actin-dependent.

136 **Cleavage-independent trans-endocytosis of ephrin-A1**

137 Our experiments suggested that ephrin-A1 was transferred from ligand-producing cells
138 (donor) towards acceptor cells, but it was still possible that these externalized clusters
139 existed outside from neighboring cells. We thus evaluated whether externalized ephrin-A1
140 clusters were localized inside acceptor cells. We induced the delivery of SBP-EGFP-
141 ephrin-A1 to the PM using the RUSH system, stopped the transport on ice and
142 immunolabeled extracellular SBP-EGFP-ephrin-A1 in non-permeabilizing conditions.
143 Then we permeabilized the cells and stained the total SBP-EGFP-ephrin-A1. Clusters
144 immunostained only after permeabilization thus corresponded to an intracellular pool of
145 ephrin-A1. Such clusters were clearly detected inside acceptor cells, implicating that
146 ephrin-A1 expressed on a donor filopodia underwent endocytosis into acceptor cells (Fig.
147 3A).

148 To explain how a membrane-attached ephrin-A1 could be transferred to neighboring cells
149 we examined whether it was cleaved at the tip of filopodia by ADAMs. We synchronized
150 the trafficking of SBP-Cherry-ephrin-A1 after inhibition of ADAMs with the broad
151 spectrum inhibitor TAPI-1 (Janes et al., 2005). Surprisingly, the intercellular transfer of
152 ephrin-A1 from filopodia remained unaffected under ADAM inhibition (Fig. 3B, C),
153 suggesting that it can proceed independently of proteolytic shedding, in contrast to
154 proposed models (Greene et al., 2014; Hattori et al., 2000; Janes et al., 2005, 2009). It was
155 however still possible that inhibition was incomplete or that an unrelated protease was
156 responsible for the cleavage. Thus, we developed a fluorescent assay to unambiguously
157 determine whether full-length ephrin-As can undergo trogocytosis as reported for ephrin-
158 Bs (Gaitanos et al., 2016; Gong et al., 2019; Marston et al., 2003; Zimmer et al., 2003). We
159 engineered a fusion protein, which consists of ephrin-A1 tagged at its N-terminal extremity
160 with SBP-mCherry and at its C-terminal end, just before the GPI-anchor sequence, by
161 EGFP (SBP-Cherry-ephrin-A1-EGFP-GPI) (Fig. 3D-F). Depending on the mode of

162 transfer of ephrin-A1, two possible output were expected: i) if ephrin-A1 was transferred
163 to the acceptor cells as a product of its proteolytic cleavage, the clusters in the acceptor cells
164 should be mCherry-positive and EGFP-negative, conversely ii) if ephrin-As were
165 transferred without being cleaved, the clusters in the acceptor cells should be both mCherry-
166 positive and EGFP-positive. Imaging synchronized transport of SBP-Cherry-ephrin-A1-
167 EGFP-GPI to the cell surface, we consistently observed that clusters of ephrin-A1 positive
168 for both mCherry and EGFP were trans-endocytosed from donor filopodia by the acceptor
169 cells (Fig. 3D-F and video S6A). As a control, we inserted two consensus ADAM cleavage
170 sites (2xADAMtide) (Caescu et al., 2009) between SBP-mCherry and the N-terminal of
171 ephrin-A1-EGFP-GPI (SBP-Cherry-2xADAMtide-ephrin-A1-EGFP-GPI) (Fig. S1A).
172 When SBP-Cherry-2xADAMtide-ephrin-A1-EGFP-GPI arrived to the PM, ADAMs
173 induced the shedding of the SBP-Cherry portion and green, but not red, clusters were
174 transferred to the acceptor cells (Fig. S1B-D). Importantly, the cleavage of SBP-Cherry-
175 2xADAMtide-ephrin-A1-EGFP-GPI was efficiently reverted after ADAM inhibition with
176 TAPI-1 or GI254023X (Fig. S1E-J).

177 Our data indicated that ephrin-A1 is trans-endocytosed in cleavage-independent manner.
178 Two possible mechanisms were considered: the trans-endocytosis of ephrin-A1 may
179 depend on the removal of the GPI-anchors (i.e. by phospholipase C), or full-length ephrin-
180 A1 may be trans-endocytosed as a GPI-AP. To distinguish between these possibilities we
181 designed a chimera in which we replaced the GPI-anchor sequence of SBP-Cherry-ephrin-
182 A1 by the transmembrane domain of ephrin-B1 (EB1) followed by an EGFP in the
183 cytoplasmic domain (SBP-Cherry-ephrin-A1-TMD-EB1-EGFP). If trans-endocytosis of
184 ephrin-A1 depends on GPI-anchor cleavage, the ephrin chimera should stay bound to the
185 PM of the donor cell and should not be transferred to the acceptor cells. On the contrary, if
186 the whole ephrin-A undergoes trans-endocytosis, transfer may occur and the trans-
187 endocytosed clusters should be mCherry- and EGFP-positive. Live-cell imaging revealed
188 that clusters of SBP-Cherry-ephrin-A1-TMD-EB1-EGFP were pinched-off from the donor
189 filopodia and trans-endocytosed as mCherry- and EGFP-positive clusters (Fig. 2G-I and

190 video S6B). Our results thus demonstrate that ephrin-As can be transferred to neighboring
191 cells as full-length proteins probably by trogocytosis.

192 **Dynamin-dependent trans-endocytosis of ephrin-A1 from cytonemes**

193 There are two main structures reported to connect donor and target cells through filopodia-
194 based membrane bridges, namely cytonemes and tunneling nanotubes (TNTs) (Sherer and
195 Mothes, 2008). Cytonemes are specialized filopodia that connect distant cells through
196 synaptic-like contacts, transporting signaling molecules, participating, for example, to
197 establish a gradient of membrane bound morphogens *in vivo* (Kornberg and Roy, 2014).
198 Unlike cytonemes, TNTs are cell extensions that directly connect the cytoplasm of donor
199 and acceptor cells, enabling the intercellular transfer of cytosolic content such as protein
200 aggregates, endosomes and lysosomes (Abounit et al., 2016; Burtey et al., 2015; Gousset
201 et al., 2009; Sherer and Mothes, 2008). In contrast to cytonemes, intercellular transfer of
202 cargoes through TNTs is insensitive to endocytosis block (e.g. dynamin inhibition)
203 (Abounit et al., 2016; Burtey et al., 2015). Thus, to differentiate between both mechanisms,
204 we first inhibited dynamin-dependent endocytosis by pre-incubating SBP-EGFP-ephrin-A1
205 transfected cells and acceptor cells with the dynamin inhibitor Dyngo4A. Dyngo4A
206 treatment efficiently inhibited endocytosis (as seen by transferrin uptake) as well as the
207 intercellular transfer of SBP-EGFP-ephrin-A1 (Fig. 4A-C). We further blocked dynamin-
208 dependent endocytosis specifically in acceptor cells by co-culturing SBP-EGFP-ephrin-A1
209 expressing cells with cells overexpressing either wild-type dynamin-1-CFP (Dynamin-1-
210 WT) or the GTPase-defective/dominant-negative dynamin-1 (Dynamin-1-K44A). As
211 expected, ephrin-A1 clusters were efficiently transferred through SBP-EGFP-ephrin-
212 A1/Dynamin-1-WT interfaces (Fig. 4D and video S7). However, the internalization of
213 ephrin-A1 clusters was strongly inhibited in Dynamin-1-K44A expressing cells, tending to
214 accumulate at the donor/acceptor interface (Fig. 4D and video S7). These data indicate that
215 ephrin-A1 is trans-endocytosed from cytoneme-like filopodia rather than transferred
216 through TNTs.

217 We then analyzed the endocytic pathway followed by ephrin-A1 upon transfer into
218 receiving cells. SBP-EGFP-ephrin-A1 expressing cells were mixed with cells expressing
219 either wild-type or GTP-locked Rab5 mutant (mRFP-Rab5-WT or mCherry-Rab5-Q79L,
220 respectively). Rab5 localizes at early endosomes and its GTP-bound form causes early
221 endosome fusion. Importantly, mCherry-Rab5-Q79L expression in acceptor cells blocked
222 ephrin-A1 inside Rab5-enlarged endosomes (Fig. 4E). Early endosomes-bearing trans-
223 endocytosed ephrin-A1 mature to late endosomes, as visualized by the co-culture of SBP-
224 Cherry-ephrin-A1 expressing cells with cells expressing Rab7 (EGFP-Rab7-WT) or the
225 active GTPase-deficient mutant of Rab7 (EGFP-Rab7-Q76L) (Fig 4F). In contrast, trans-
226 endocytosed ephrin-A1 is sorted apart from recycling endosomes, as indicated by the same
227 type of experiments done using cells overexpressing Rab11 (EGFP-Rab11-WT) or the
228 dominant negative GDP-bound form of Rab11 (DsRed-Rab11-S25N) (Fig. S2).

229 **Localized trans-endocytosis of ephrin-A1 in complex with EphA2**

230 We wondered whether the high local concentration of ephrin-A1 at filopodia tip might
231 confine the ephrin signaling. We performed immunostaining after SBP-EGFP-ephrin-A1
232 secretion using an anti-phosphotyrosine antibody or an antibody that recognizes the active
233 form of the EphARs (pEphA). We observed a strong co-localization between ephrin-A1
234 clusters and either phosphotyrosine signal or pEphA at the PM and inside the acceptor cells
235 (Fig. 5A). Signaling started at the PM because when trans-endocytosis was blocked,
236 activation of EphARs was still observed (Fig S3A). Signaling at the cell surface was
237 actually linked to trans-endocytosis. Pharmacological inhibition of EphA2 signaling with
238 Dasatinib (Chang et al., 2008) efficiently impaired trans-endocytosis of ephrin-A1 (Fig. 5B
239 and Fig. S3B). In these conditions, steady clusters of ephrin-A1 were observed at the
240 interface between donor filopodia and acceptor cells (Fig. 5B-C).

241 To evaluate the dynamics of ephrin-A1/EphA2 signaling complexes, we co-cultured SBP-
242 EGFP-ephrin-A1 expressing cells with EphA2-Cherry-SBP expressing cells and performed
243 live-cell imaging upon trafficking synchronization. Under these conditions, EphA2-bearing
244 cells extend filopodia towards the ephrin-A1-filopodia. We detected the local formation of

245 high-order ligand/receptor clusters in subdomains of trans-interaction between donor and
246 acceptor filopodia (Fig. 5D). Ephrin-A1/EphA2 complexes were preferentially transported
247 through EphA2-containing filopodia towards the cell body of the acceptor cells where they
248 underwent trans-endocytosis (Fig. 5D and video S17) although we sometimes observed
249 clusters of EphA2Rs being pulled towards the cell body of ephrin-A1-producing cells (Fig.
250 5D and video S8). We questioned the trans-endocytosis of EphA2 by synchronizing the
251 trafficking of extracellular-tagged SBP-EGFP-EphA2 co-cultured with cells transfected
252 with SBP-Cherry-ephrin-A1. (Fig. 5E-F). We observed that a minor population of
253 EphA2Rs might also undergo intercellular transfer.

254 **Long-lasting signaling of trans-endocytosed ephrin-A1/EphA2 complexes in cancer** 255 **cells**

256 RUSH-based synchronization of transport was essential to reveal filopodia-dependent
257 trans-endocytosis, however the intercellular transfer of ephrin-A1 to EphA2-expressing
258 cells was massive at steady state (no hook expression) (video S9). This ruled-out possible
259 artifacts due to trafficking synchronization. We then monitored transfer in cells known to
260 use the ephrin-A1/EphA2 feedback loop. We used the highly aggressive breast cancer cell
261 MDA-MB231, because they connect to the endothelia through filopodia promoting the
262 formation of a metastatic niche in mice (Connor et al., 2015). We co-cultured MDA-MB231
263 cells stably expressing SBP-EGFP-EphA2 (MDA-EphA2) with MDA-MB231 cells stably
264 expressing SBP-Cherry-ephrin-A1 (MDA-ephrin-A1). Live imaging revealed that ephrin-
265 A1 undergoes massive trans-endocytosis in a complex with the EphA2R. Intracellularly;
266 ephrin-A1/EphA2 clusters accumulate into large and round endomembrane compartments
267 of MDA-EphA2 cells (Fig. 6A). Ephrin-A1 was still detected inside those compartments
268 more than 7 h after release from the ER. We analyzed whether MDA-MB231 cells use
269 lysosomes for the long-term storage of ephrin-A1. We co-cultured donor SBP-Cherry-
270 ephrin-A1 expressing cells with acceptor MDA-EphA2 cells and we labeled the lysosomes
271 using the SiR-lysosome dye, which binds to the lysosomal proteinase cathepsin-D. We
272 observed that ephrin-A1/EphA2 complexes are co-internalized and transported to large

273 endo-lysosomal compartments (Fig. S4). To analyze whether sustained signaling
274 accompanied this internalization, we co-cultured donor MDA-ephrin-A1 cells with wild-
275 type MDA-MB231 cells used as acceptors. We labeled the lysosomal-associated membrane
276 protein 1 (LAMP1) and active pEphARs. This experiment showed that trans-endocytosed
277 ephrin-A1 was stored in lysosomes as signaling complexes. To better characterize the
278 signaling compartment, we assessed for the acidity and degradative properties of the ephrin-
279 A1-lysosomes by live-cell imaging using the pH-sensitive dye lysotracker and the
280 fluorogenic protease substrate DQ-BSA respectively. We observed that trans-endocytosed
281 ephrin-A1 is trafficked to acidic lysosomes, but just a minor population was found in
282 degradative lysosome (Fig. S4). Interestingly, ephrin-A1-positive endo-lysosomes were
283 slightly larger and concentrated a significantly higher amount of active EphARs than their
284 ephrin-A1-negative counterpart (Fig. 6B-D).

285 In addition to protease-dependent cleavage, trans-endocytosis may thus be an alternative
286 mechanism to allow cell separation and repulsion upon ephrin signaling. To measure
287 contact-mediated cell repulsion we used a cell-confrontation assay (Porazinski et al., 2016).
288 It consists in a two-compartment cell culture insert separated by a fixed gap. After removal
289 of the insert, cells migrate to fill the gap and opposing cells meet and establish cell-to-cell
290 contacts. As a control, when two monolayers of MDA-ephrin-A1 cells were confronted,
291 both populations stopped migrating upon contact (Fig. 7A, B), suggesting that no repulsion
292 signal was emitted. In contrast, when acceptor MDA-EphA2 cells were confronted to donor
293 MDA-ephrin-A1 cells, the acceptor MDA-EphA2 cells were repelled backward;
294 meanwhile the MDA-ephrin-A1 cells persisted migrating forward (Fig. 7A, B). MDA-
295 EphA2 repulsion correlated with the transfer of ephrin-A1 to acceptor cells, suggesting that
296 trans-endocytosis of ephrin-A1 was used to remove the cell-cell tether formed by ephrin-
297 A1 and EphA2.

298 Remarkably, when a MDA-EphA2 acceptor cell undergoes cell division short after ephrin-
299 A1 trans-endocytosis, ephrin-A1 was inherited to the daughter cells inside an
300 endomembrane compartment (Fig. 7C and video S10). To determine if activated EphARs

301 can be inherited during cell division, MDA-MB231 cells labeled with a cell dye were
302 seeded on gridded cell dishes and synchronized at the G2 phase of the cell cycle with the
303 CDK1 inhibitor RO3306. Then MDA-ephrin-A1 cells were seeded on top of the acceptor
304 cells in presence of biotin. Once the acceptor endosomes were filled with ephrin-A1, biotin
305 and RO3306 were washed out and we imaged live the acceptor cells mitosis (Fig. 7D). We
306 corroborated that trans-endocytosed ephrin-A1 is transmitted to daughter cells. When
307 acceptor cells completed mitosis, we fixed them and co-stained with antibodies against
308 LAMP-1 and pEphA. Importantly, we observed that around 60% of the inherited ephrin-
309 A1 was localized into lysosomes and about 40% of inherited ephrin-A1-clusters contained
310 pEphA (Fig. 7D-E), suggesting that a memory of ephrin signaling might be transmitted
311 over generations.

312 **Discussion**

313 Membrane-bound ephrins and EphRs are ubiquitous signaling molecules present in most
314 of cell types and regulating a myriad of biological processes. The localization and clustering
315 of ephrins at the PM is required for the efficient receptor activation (Davis et al., 1994;
316 Stein et al., 1998), restricting the ephrin/Eph communication to the cell vicinity at sites of
317 cell-to-cell contact. However, there are some exceptions to this juxtacrine ephrin signaling.
318 Ephrin-A1 can be released in soluble forms by matrix metalloproteases activating an
319 EphA2-dependent signaling at paracrine level (Beauchamp et al., 2012; Wykosky et al.,
320 2008). Ectodomains of EphAs, EphBs and ephrin-Bs can also be released by proteolytic
321 shedding, leading to signaling inhibition (Barquilla and Pasquale, 2015). Additionally, cells
322 can display EphAs, EphBs and ephrin-Bs on extracellular vesicles (EVs); which would
323 expand the signaling to the endocrine level (Gong et al., 2016). Ephrin-As and -Bs may
324 thus differ on their mechanisms of long-range communication.

325 Here we have uncovered a mechanism supporting long-range action for ephrin-As, namely
326 cytonemes. Contacts produced by cytonemes are considered as a subtype of synaptic
327 signaling (Kornberg and Roy, 2014). Transport of ephrin-A on cytonemes brings several
328 advantages: i) it allows ephrin-A to be concentrated at the tip of filopodia for efficient

329 signaling; ii) the length of these filopodia enables the cells to sustain long-distance
330 communication with EphARs displayed on acceptor PM; iii) concentration of the ligand at
331 the tip of a very fine structure allows its efficient trans-endocytosis. This phenomenon,
332 while massive at steady state, may have been overlooked so far. Synchronization of
333 transport allowed generating a pulse that revealed this phenomenon.

334 The RUSH system enables the coordinated delivery of ephrins and EphRs to the PM, which
335 is a powerful approach to the study of ephrin signaling and trans-endocytosis without the
336 need for artificial clustering of soluble and non-physiological ligands. PM association
337 endows ephrins with the particularity of transduce bidirectional signaling (Pasquale, 2008).
338 The fact that a small proportion of EphA2Rs are trans-endocytosed in ephrin-A1 producing
339 cells suggests that bidirectional signaling may take place at cytonemes. Interestingly, in sea
340 urchin embryos a transmembrane ephrin has been observed to localize to cytonemes at the
341 basal surface of ectodermal cells (Krupke et al., 2016). In zebrafish, the ephrin-B1/EphB3b
342 signaling regulates cytonemal contacts between hepatoblast and the adjacent lateral plate
343 mesoderm, coordinating liver morphogenesis and laterality (Cayuso et al., 2016). In
344 mammalian cells, myosin1b regulates the formation of filopodia at the ephrin-B1/EphB2
345 cellular interfaces, contributing to cell repulsion (Prospéri et al., 2015). Considering that
346 ephrin-B/EphBR clusters are removed from the cell surface by trans-endocytosis (Gaitanos
347 et al., 2016; Marston et al., 2003; Zimmer et al., 2003), it is reasonable to propose that
348 filopodia localization and trans-endocytosis are closely linked signal transduction
349 processes.

350 Importantly, we show that removal of ephrin-A/EphA complexes is cleavage-independent.
351 This finding suggests an alternative to the accepted model of cell-tether removal and
352 EphAR downregulation upon ephrin-A stimulation, which relies on the ADAM-dependent
353 cleavage (Hattori et al., 2000; Janes et al., 2005, 2009). While it is still unclear whether
354 trans-endocytosis has a distinct role from simple ephrin-A shedding, our results suggest
355 that ADAMs might play an indirect role on cell-cell repulsion. For example, they may be
356 used to regulate the shedding of E-cadherin, which have been observed on ephrin-B1/EphB

357 boundaries (Solanas et al., 2011) or the shedding of EGF and further down-regulation of E-
358 cadherin, as shown for ephrin-A1/EphA2 cell interfaces (Kaplan et al., 2018). This would
359 explain how the recruitment of ADAM10 to the ephrin-A/EphA interfaces can regulate
360 repulsion independently of ephrin-A cleavage.

361 Ephrin-Bs also undergo bidirectional trogocytosis (Gaitanos et al., 2016; Gong et al., 2019;
362 Marston et al., 2003; Zimmer et al., 2003), suggesting that all sorts of ephrins may be
363 subjected to it. Trans-endocytosis of ephrin-As depends on actin. Similarly, actin and Rac1
364 have been reported to be involved in trans-endocytosis of ephrin-Bs (Gaitanos et al., 2016;
365 Marston et al., 2003). Rac-1 has also been implicated in ephrin-A1-induced EphA2
366 endocytosis (Zhuang et al., 2007) and in ephrin-A5-induced EphA8 endocytosis during the
367 development of the retinocollicular topographic map in mice (Yoo et al., 2010, 2011). In
368 neurons, ephrin-A2 induces Rac-1 activation, stimulating endocytosis in growth cones
369 leading to their collapse (Jurney et al., 2002). We have shown that ephrin-As interact with
370 EphARs at filopodia. In embryonic retinal explants, ephrin-A2 and EphA4 colocalize in
371 axons regulating the density of axonal interstitial filopodia (Fiore et al., 2019). Early after
372 birth, EphA4 receptors are located at the tip of axonal and dendritic filopodia in rat
373 hippocampus (Tremblay et al., 2009). These data support the role of filopodia on ephrin-
374 A-signaling *in vivo*.

375 Trogocytosed clusters of ephrin-A1 often contain small amounts of donor PM. In contrast
376 to the shedding mechanism, trans-endocytosis allows the transfer of a membrane-attached
377 ligand, hence keeping the ephrin in an oligomeric state and enabling efficient EphR
378 activation. This would explain how trans-endocytosed ephrins are able to emit long-lasting
379 signaling from lysosomes. Trans-endocytosed ephrin-A1/EphA2 complexes are transported
380 using Rab5- and Rab7-endosomes (Fig. 7F). Similarly, fibroblast growth factor (FGF)
381 forms a complex with cytoneme-localized FGF receptors and Rab5, which is retrogradely
382 transported to the cell body (Koizumi et al., 2012). EGF (epidermal growth factor)/ErbB1
383 complexes are also endocytosed at the base of filopodia (Lidke et al., 2005), suggesting that
384 different cytoneme-related signaling molecules may follow similar endocytic pathways.

385 Ephrin-A1-dependent EphA2 activation and internalization leads to a transient inactivation
386 of ERK and Akt pathways (Sabet et al., 2015). Ephrin-A1-dependent Akt inhibition
387 captures EGFR in Rab5 endosomes, therefore suppressing the pro-migratory Akt signaling
388 from the PM while preserving proliferative endosomal ERK activity (Stallaert et al., 2018).
389 In this context, cells with reduced cell-cell contact and therefore reduced ephrin signaling
390 (e.g. at tissue boundaries) would increase their exploratory response to EGF, while cells
391 located deeper into the tissue, would retain their proliferative response and fill the empty
392 space despite their massive cell-cell contacts (Stallaert et al., 2018). A consequence of the
393 endosomal sorting process is that it allows the transmission of ephrin-A/EphA complexes
394 to the cellular progeny. This is possible because lysosomes are partitioned during cell
395 division (Bergeland et al., 2001). Ephrin-loaded lysosomes may act then as long-term
396 signaling unit, endowing the cell with a kind of memory. However, it will be essential to
397 demonstrate that, in contrast to cleavage-dependent ephrin-A regulation, trans-endocytosis
398 and complex storage in lysosomes provide a memory of signaling *in vivo*.

399 Long-distance communication through cytonemes, followed by storage in endomembranes
400 may also promote the release of EphARs on EVs, switching to a cell contact-independent
401 signaling modality (Fig. 7G). EphA2 on EVs may then both, activate reverse ephrin-
402 signaling or titer down free ephrin-As on remote tissues. Because TNTs also participate in
403 the intercellular transport of lysosomes (Abounit et al., 2016) they may help to transfer
404 signaling lysosomes between cells. This might be especially important in tumor cells, in
405 which specialized filopodia, were proposed to configure a network that provides resistance
406 to therapies (Osswald et al., 2015). Notably, EphA2 overexpression decreases the
407 sensitivity of breast cancer to tamoxifen and trastuzumab (Lu et al., 2003; Zhuang et al.,
408 2010). It would be important to know if the potential transfer of lysosomes containing active
409 EphA2Rs might spread a resistance to otherwise sensitive cells. A pending challenge is to
410 decipher the role in both normal and pathological development of the pathway described
411 here for ephrins, from transport and concentration on cytoneme to trans-endocytosis and
412 long-term signaling. Such a pathway may also be at work for other signaling routes linking
413 cell-cell adhesion to signaling and may represent a signature of cytoneme-based signaling.

414 **Acknowledgments**

415 The authors wish to thank V. Fraisier and L. Leconte from the PICT-IBiSA Lhomond
416 Imaging facility of Institut Curie and the members of the Recombinant Antibodies Platform
417 of the Institut Curie; Z. Gouveia for her help with lentiviral transductions; S. Divoux for
418 her help with some experiments and G. Boncompain for constructs and advices; J. Ecard
419 and M. Maurin for assistance with the Fiji macros. The work in the laboratory of F.P. was
420 supported by the Institut Curie, the Centre National de la Recherche Scientifique (CNRS),
421 the French Agence Nationale de la Recherche (ANR-12-BSV2-0003-01), Fondation
422 Recherche Médicale (DEQ20120323723). LabEx CelTisPhyBio (ANR-10-LBX-0038 part
423 of the IDEX PSL no. ANR-10-IDEX-0001-02; to FP and J.I.V.). Long-Term EMBO
424 Fellowship (ALTF 607-2015) co-funded by the European Commission FP7 (Marie Curie
425 Actions, LTFCOFUND2013, GA-2013-609409; to J.I.V.).

426 **Author Contributions**

427 J.I.V. conceived the study, conducted the experiments and analyzed the data. J.I.V. and F.P.
428 designed the experiments and wrote the manuscript. F.P. supervised the study and acquired
429 funding.

430 **Declaration of Interests**

431 The authors declare no competing interests.

432 **Figure Legends**

433 **Figure 1 Synchronization of the secretion of EphA2 and ephrin-A1**

434 **(A)** Scheme of the RUSH system.

435 **(B)** Scheme of SBP-Cherry-ephrin-A1 and EphA2-EGFP-SBP reporters.

436 **(C)** Time-lapse imaging of transport of SBP-Cherry-ephrin-A1 (red) and EphA2-EGFP-
437 SBP (green) in a RUSH set-up (t = time after addition of biotin). Bottom panels (high-
438 magnification images).

439 **(D)** Intensity plots of SBP-EGFP-ephrin-A1 or EphA2-EGFP-SBP at the GA (mean \pm
440 SEM).

441 **(E)** Percentage of colocalization (mean \pm SEM) of EphA2-EGFP-SBP and SBP-Cherry-
442 ephrin-A1 or SBP-EGFP-ephrin-A1 and SBP-Cherry-ephrin-A1. (D-E) Light red region is
443 before Golgi exit, light green region is after Golgi exit.

444 **(F)** EphA2-EGFP-SBP (top panel, green) or SBP-EGFP-ephrin-A1 (bottom panel, green)
445 transfected cells 90 min after biotin addition. Nuclei (magenta), GA (red). Right panels
446 (high-magnification images). Arrows indicate filopodia.

447 **(G)** Live-cell imaging of the RUSH construct SBP-EGFP-ephrin-A1 (green) and the PM
448 marker (PM-Cherry, red). Bottom panels (high-magnification images). Arrow indicates an
449 ephrin-A1 cluster exported from filopodia.

450 **(H)** Temporal projection of SBP-EGFP-ephrin-A1 and PM-Cherry imaged live. Bottom
451 panels (high-magnification images).

452 **(I)** Boxplot of the time of appearance of the first ephrin-cluster at the PM and the first
453 ephrin-cluster exported from the PM.

454 (C, F-H) Scale bars, 10 μ m. (***) $t < 0.001$, two-tailed t-test) See also video S1 and S2.

455 **Figure 2 ephin-As are sorted into signaling filopodia**

456 **(A)** Live-cell imaging of the RUSH construct SBP-EGFP-ephrin-A5 (green) and the PM
457 marker (PM-Cherry, red). F-actin (blue). Bottom panels (high-magnification images). (D)
458 Donor cell, (A) Acceptor cell.

459 **(B)** Co-localization after synchronous transport to the cell surface of SBP-EGFP-ephrin-
460 A1 with SBP-Cherry-GPI (left panels), SBP-Cherry-CD59 (middle panels) or SBP-Cherry-
461 ALPP (right panels). ephrin-A1 (middle row), GPI reporters (bottom row). Nuclei (blue).

462 **(C)** Live-cell imaging of the RUSH construct SBP-Cherry-ephrin-A1 (red) and F-tractin-
463 EGFP (green). Bottom panels (high-magnification images).

464 **(D)** Live-cell imaging of SBP-EGFP-ephrin-A1 (green) and PM-Cherry (red) under control
465 conditions (top panels) or cytochalasin-D treatment (bottom panels). F-actin (blue).

466 **(E)** Kymographs analysis of one cluster of ephrin-A1 at cell-cell interface. The dotted line
467 indicates the time of cluster formation.

468 **(F)** Number of filopodia in control (DMSO) and cytochalasin-treated (Cyto-D) cells (mean
469 \pm SD).

470 **(G)** Filopodia length distribution in control (DMSO) and cytochalasin-treated (Cyto-D)
471 cells.

472 **(H)** Boxplot showing the normalized number of transferred ephrin-A1 clusters in control
473 (DMSO) and cytochalasin-D (Cyto-D) or latrunculin A-treated (Lat-A) cells. (A-D) Scale
474 bars, 10 μ m.

475 (F, H) * $t < 0.05$; ** $t < 0.01$; ** $t < 0.01$, two tailed t-test. See also video S3-S5.

476 **Figure 3 Cleavage-independent trans-endocytosis of ephrin-A1**

477 **(A)** RUSH SBP-EGFP-ephrin-A1 (green) after 95 min of biotin addition. Surface ephrin-
478 A1 (blue), total ephrin-A1 (red). Right panels (high-magnification images).

479 **(B)** RUSH SBP-Cherry-ephrin-A1 (red) in the presence (bottom) or absence (top) of
480 TAPI-1. F-actin (green), nuclei (magenta). Right panels (high-magnification images).

481 **(C)** Boxplot of the normalized number of transferred ephrin-A1 clusters in control cells
482 (DMSO) or cells treated with TAPI-1. (n.s., non-significant, two tailed t-test).

483 **(D)** Time-lapse imaging of the SBP-Cherry-ephrin-A1-EGFP-GPI sensor (drawn at the
484 top). EGFP and Cherry signals are shown. Cell-cell boundaries are visualized with F-actin
485 (blue). Bottom panels (high-magnification images).

486 **(E)** Kymograph analysis of one cluster of SBP-Cherry-ephrin-A1-EGFP-GPI into an
487 acceptor cell.

488 **(F)** Line-scan analysis and EGFP/Cherry intensity ratio from (E).

489 **(G-I)** Same as in (D-F), but for the SBP-Cherry-ephrin-A1-TMD-ephrin-B1-EGFP sensor
490 (drawn at the top). Red lines show a linear fit of the data.

491 (A-B, D-E, G-H) Scale bars, 10 μ m. See also Fig. S1 and video S6.

492 **Figure 4 Dynamin-dependent trans-endocytosis of ephrin-A1 from cytonemes**

493 **(A)** Cells expressing SBP-EGFP-ephrin-A1 (green) and PM-Cherry (red) untreated
494 (DMSO) or incubated with Dyngo4A and synchronized with biotin. Tfr-647 (cyan) shown
495 endocytosed transferrin. Arrowheads indicate the clusters of ephrin-A1 inside acceptor
496 cells.

497 **(B)** Line-scan analysis of colocalization between ephrin-A1 clusters and Tfr-647
498 endosomes.

499 **(C)** Boxplot of the normalized number of transferred ephrin-A1 clusters. (** $t < 0.01$, two
500 tailed t-test).

501 **(D)** SBP-EGFP-ephrin-A1-expressing cells (green) co-cultured with Dynamin-I-WT-CFP-
502 expressing (blue, indicated with asterisks, top panels) or Dynamin-I-K44A-CFP-expressing
503 cells (blue, indicated with asterisks, bottom panels). F-actin (red). Right panels (high-
504 magnification images).

505 **(E)** Time-lapse imaging of SBP-EGFP-ephrin-A1-expressing cells (green) co-cultured with
506 cells expressing mRFP-Rab5-WT (red, top panels) or Cherry-Rab5-Q79-L (red, bottom
507 panels). Arrows indicate an example of trans-endocytosed SBP-EGFP-ephrin-A1 cluster in
508 an acceptor cell. Arrowheads indicate an mRFP-Rab5-WT or Cherry-Rab5-Q79-L-positive
509 endosomes. F-actin (blue).

510 **(F)** Same as (E) but for cells expressing SBP-Cherry-ephrin-A1 (red) co-cultured with cells
511 expressing EGFP-Rab7-WT (green, top panels) or EGFP-Rab7-Q76-L (green, bottom
512 panels).

513 (A, D-F) Scale bars, 10 μ m. See also Fig. S2 and video S7.

514 **Figure 5 Localized trans-endocytosis of ephrin-A1 in complex with EphA2**

515 (A) SBP-EGFP-ephrin-A1 or SBP-Cherry-ephrin-A1 (green) expressing cells immuno-
516 stained for phosphotyrosinated proteins (pTyrosine; top panels, red) or pEphARs (pEphA;
517 bottom panels, red). Nuclei (blue). Right panels (high-magnification images).

518 (B) Time-lapse imaging of SBP-Cherry-ephrin-A1 (red) and PM-EGFP (green) under
519 control conditions (upper panels) or after Dasatinib treatment. F-actin (blue). Arrows
520 indicate stable clusters of ephrin-A1.

521 (C) Percentage of donor-acceptor interfaces in which we observed clustering and/or transfer
522 or no clustering of ephrin-A1 (mean \pm SEM).

523 (D) Mix culture of HeLa cells expressing SBP-EGFP-ephrin-A1 (green) or EphA2-Cherry-
524 SBP (red) imaged live. F-actin (blue). The arrow indicates a failed phagocytosis of an
525 EphA2-Cherry-SBP filopodia by a SBP-EGFP-ephrin-A1 expressing cell. High-
526 magnifications images are presented below. SBP-EGFP-ephrin-A1 (top panels), EphA2-
527 Cherry-SBP (bottom panels). Arrowheads indicate two events of filopodia-related co-
528 internalization of SBP-EGFP-ephrin-A1 with EphA2-Cherry-SBP. Percentage of cell-cell
529 interfaces in which EphA2-Cherry-SBP-cells behave as a donor, as an acceptor cell, or
530 when the interfaces is bidirectional (mean \pm SEM).

531 (E) RUSH SBP-Cherry-ephrin-A1 (red) cells co-cultured with SBP-EGFP-EphA2-
532 expressing cells (green). Surface staining of SBP-EGFP-EphA2 (magenta). High-
533 magnifications images (right panels). Arrowheads indicate clusters of SBP-EGFP-EphA2
534 trans-endocytosed into the ephrin-A1 expressing cells.

535 (F) Normalized number of transferred EphA2 clusters (mean \pm SD).

536 (A-B, D-E) Scale bars, 10 μ m. (C, F) * $t < 0.05$; ** $t < 0.01$, two tailed t-test. See also Fig. S3
537 and video S8-S9.

538 **Figure 6 Long-lasting storage and signaling of trans-endocytosed ephrin-A1/EphA2**
539 **complexes in cancer cells**

540 (A) Live-cell imaging of MDA-EGFP-EphA2 cells (green) co-cultured with MDA-Cherry-
541 ephrin-A1 cells (red). High-magnification images: MDA-EGFP-EphA2 (top panels),

542 MDA-Cherry-ephrin-A1 (middle panels), merged images (bottom panels). Arrowheads
543 indicate trans-endocytosed SBP-Cherry-ephrin-A1 stored inside the acceptor cell.

544 **(B)** HeLa cells expressing SBP-Cherry-ephrin-A1 (red) co-cultured with MDA-MB231-
545 WT, co-stained with antibodies against LAMP1 (green) and pEphARs (white). Nuclei
546 (blue). Right panels (high-magnification images) **(B')** High-magnification images of an
547 individual lysosome boxed in B (right panels). Line-scan analysis (right plot) of ephrin-A1
548 (red), LAMP1 (green) and pEphA (gray).

549 **(C)** Plot showing the integrated intensity density (mean \pm SD) of SBP-Cherry-ephrin-A1,
550 LAMP1 and pEphA in acceptor MDA-MB-231-WT cells inside ephrin-A1-positive
551 lysosomes (ephrin-A1+ / LAMP1+), ephrin-A1-negative lysosomes (ephrin-A1- /
552 LAMP1+) or ephrin-A1- and lysosome-free cellular area (ephrin-A1- / LAMP1-).

553 **(D)** Lysosome area (μm^2) of ephrin-A1-positive and ephrin-A1-negative lysosomes (mean
554 \pm SD). (A-B) Scale bars, 10 μm ; (B') scale bar 1 μm . (C-D) * $t < 0.05$; *** $t < 0.001$; n.s. non-
555 significant, two tailed t-test. See also Fig. S4.

556 **Figure 7 Trans-endocytosed ephrin-A1 participates in contact-mediated repulsion**

557 **(A)** Cell confrontation assay of MDA-Cherry-ephrin-A1 labeled with a cell mask (left
558 panels, green) confronted to MDA-Cherry-ephrin-A1 (left panels, red). Nuclei (blue).
559 Trans-illumination images (gray). Right panels show a similar experiment done using
560 MDA-EGFP-EphA2 (green) confronted to MDA-Cherry-ephrin-A1 cells (red). The cyan
561 line depict the point of collision (time; h: min).

562 **(B)** Distance (μm) traveled by the marginal cells 20 h after collision (mean \pm SD).

563 **(C)** Time-lapse of the collision and subsequent cell division of a MDA-Cherry-ephrin-A1
564 cell (red) with a MDA-EGFP-EphA2 cell (green). Trans-illumination images (gray).
565 Capital letters indicates the donor cell (D), acceptor cell (A) and daughter cells (A', A'').

566 **(D)** Live-cell imaging of acceptor MDA-MB-231-WT cells labeled with a cell mask (A,
567 green) co-cultured with donor MDA-Cherry-ephrin-A1 cells (D, red). Trans-illumination

568 images (gray). The timeline at the top summarizes the different steps of the experiment.
569 Time indicates the time after washout of RO3306 (top panels). Correlative immunostaining
570 with antibodies against pEphARs (blue) and LAMP1 (magenta, bottom panels). High-
571 magnification of the boxed region (bottom right panels). Arrows indicate inherited ephrin-
572 A1 clusters.

573 **(E)** Percentage of trans-endocytosed ephrin-A1 clusters co-localizing with LAMP-1,
574 pEphA or both (mean \pm SEM).

575 **(F)** Schematic model of the ephrin-As trans-endocytosis.

576 **(G)** Functional overview. Removal of the Ephrin-A/EphA cell tether by trans-endocytosis
577 permits cell-cell repulsion to occur. Long-distance communication through cytonemes may
578 promote the sorting of EphARs to multivesicular bodies (MVB) and/or to signaling
579 lysosomes. EphARs can be secreted in extracellular vesicles (EVs) interacting with Ephrin-
580 As on distant cells (green cell). Ephrin-A-containing lysosomes may also be transferred to
581 neighboring cells through TNTs.

582 (A, C) Scale bars, 20 μ m; (D) scale bars, 10 μ m. (B, E) * $t < 0.05$; *** $t < 0.001$. two tailed t-
583 test. See also video S10.

584

585 **STAR Methods**

586 **LEAD CONTACT AND MATERIALS AVAILABILITY**

587 Further information and request for reagents may be directed to, and will be fulfilled by the
588 Lead Contact, Dr. Franck Perez (franck.perez@curie.fr). RUSH plasmids are already
589 available through Addgene and we will deposit additional ones upon publication.

590

591 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

592 **Cells and transfection**

593 HeLa and MDA-MB231 wild-type cells were cultured in Dulbecco's modified Eagle
594 medium (DMEM), high glucose, GlutaMAX™ (Life Technologies) supplemented with
595 10% fetal calf serum (FCS) (GE Healthcare), penicillin, streptomycin and 1mM sodium
596 pyruvate (Life Technologies) at 37°C and 5% of CO₂. HeLa and MDA-MB231 stable cell
597 lines expressing the streptavidin-KDEL hook (Boncompain et al., 2012) and the SBP-
598 Cherry-ephrin-A1 or SBP-EGFP-EphA2 reporters (cloned in a pCDH-CMV-MCS-EF1 α -
599 Puro vector) were generated by transduction with lentiviral particles generated in HEK293T
600 cells and cultured as described above, but supplemented with 4 μ g/ml of puromycin
601 (Invitrogen) and 0.5 mg/mL geneticin (Life Technologies). HeLa cells were transfected
602 with calcium phosphate 24-48 h before fixation or imaging as previously described (Jordan
603 et al., 1996).

604

605 **METHOD DETAILS**

606 **Plasmids**

607 RUSH plasmids of cargoes retain by their luminal domain (SBP-EGFP-ephrin-A1, SBP-
608 Cherry-ephrin-A1, SBP-EGFP-ephrin-A5, SBP-EGFP-EphA2) were constructed as
609 FseI/PacI inserts as previously described (Boncompain and Perez, 2012). SBP-Cherry-
610 CD59 and SBP-Cherry-ALPP plasmids were previously published (Fourriere et al., 2019).

611 SBP-Cherry-GPI has been previously described (Boncompain et al., 2012) (Addgene
612 plasmid #65295). Cytoplasmic retained EphA2 was amplified from pDONR223-EPHA2
613 (Johannessen et al., 2010) (Addgene plasmid #23926) and constructed like an AscI/MfeI
614 insert (EphA2-EGFP-SBP and EphA2-Cherry-SBP) (Boncompain and Perez, 2012). SBP-
615 Cherry-ephrin-A1-EGFP-GPI was generated by inserting the EGFP sequence (flanked by
616 BamHI and XhoI sites) between the amino acids H181 and S182 of ephrin-A1. SBP-
617 Cherry-ephrin-A1-TMD-ephrin-B1-EGFP contains the trans-membrane domain of ephrin-
618 B1 (amino acids 229D to 279A) fuse to EGFP after the amino acid H181 of ephrin-A1.
619 SBP-Cherry-2xADAMtide-ephrin-A1-EGFP-GPI was created by replacing the mCherry of
620 the SBP-Cherry-ephrin-A1-EGFP-GPI construct by mCherry fused to 2 consensus ADAM
621 cleavage sites (2XADAMtide: PRAEALKGGPRAEALKGG) (Caescu et al., 2009). The
622 plasmid containing myristoylation and palmitoylation signals (MyrPalm) fused to EYFP
623 (PM-EGFP) was kindly provided by Roger Y. Tsien (UCSD, USA) (Zacharias et al., 2002);
624 PM-Cherry was generated by the insertion of the MyrPalm signals as a NheI/AgeI fragment
625 in pmCherry-C1 (Takara Clontech). F-tractin-EGFP was a gift from Dyche Mullins (Belin
626 et al., 2014) (Addgene plasmid #58473). Dynamin-1-WT-CFP and Dynamin-1-K44A-CFP
627 were created by replacing the EGFP tag of Dynamin-1-WT-EGFP (Addgene plasmid
628 #34680) or Dynamin-1-K44A-EGFP (Addgene plasmid #34681) by CFP (Song et al.,
629 2004). mRFP-Rab5-WT was kindly provided by Marino Zerial (MPI-CBG, Germany)
630 (Rink et al., 2005); mCherry-Rab5-Q79L was a gift from Sergio Grinstein (Bohdanowicz
631 et al., 2012) (Addgene plasmid # 35138); DsRed-Rab11-S25N was a gift from Richard
632 Pagano (Choudhury et al., 2002) (Addgene plasmid # 12680). Rab7 and Rab7-Q76L were
633 cloned in in a pEGFP-C1 (Takara Clontech) by SmaI blunt digestion (EGFP-Rab7-WT and
634 EGFP-Rab7-Q76L, respectively). The plasmids will be made available upon request. If the

635 number of requests is high enough, those plasmids will be added to a public repository (e.g.
636 Addgene).

637 **Immunofluorescence and antibodies**

638 For immunofluorescence, cells were fixed for 15 min at room temperature with 3%
639 paraformaldehyde (Electron Microscopy Sciences), washed in PBS and permeabilized with
640 0.05% saponin in PBS supplemented with 2 mg/mL of BSA. Coverslips were mounted in
641 Mowiol (Calbiochem). Cell surface staining were performed with the primary antibody
642 added in ice-cold PBS on non-fixed cells during 40 min as previously described
643 (Boncompain and Perez, 2012). Cells were then fixed for 10 min with 2%
644 paraformaldehyde. The mouse monoclonal anti-GFP antibody was purchased from Roche
645 (Roche Cat# 11814460001, RRID:AB_390913; 1:300). The rabbit polyclonal Anti-Eph
646 receptor A2+A3+A4 (phospho Y588 + Y596) antibody was purchased from abcam (Abcam
647 Cat# ab62256, RRID:AB_942240; 1:500). The mouse monoclonal anti-LAMP1 antibody
648 was purchased from Santa Cruz (Santa Cruz Biotechnology Cat# sc-20011,
649 RRID:AB_626853; 1:500). The rabbit polyclonal anti-GFP (1:400), the mouse monoclonal
650 Anti-phosphotyrosine (clone 4G10; 1:500) and the human monoclonal anti-giantin (clone
651 TA10; 1:100) antibodies were obtained from the Recombinant Antibodies Platform (Institut
652 Curie). Fluorescent-conjugated secondary antibodies were obtained from Jackson
653 ImmunoResearch. SiR-DNA, SiR-Actin and SiR-Lysosome probes were purchased from
654 Spirochrome, phalloidin-FITC was purchased from Sigma. Fixed cells were imaged with
655 an epifluorescence microscope (Leica) equipped with a Coolsnap camera (Roper
656 Scientific).

657 **Live-cell imaging**

658 Cells were seeded onto glass coverslips of 25 mm of diameter. Before imaging, coverslips
659 were transferred into L-shape tubing Chambridge chamber (Live Cell Instrument). Chambers
660 were filled with Leibovitz's medium (Life Technologies) pre-warmed at 37°C. For
661 trafficking synchronization, the medium was removed and D-biotin (Sigma-Aldrich) at 40
662 μ M in Leibovitz's medium was introduced using the tubing system of the chamber. Time-

663 lapse imaging were obtained in a thermostat-controlled chamber at 37°C with an Eclipse
664 80i microscope (Nikon) equipped with a spinning disk confocal head (Perkin) and an
665 Ultra897 iXon camera (Andor). Image acquisitions were performed using MetaMorph
666 software (Molecular Device) on workstations of the PICT-IBiSA Lhomond Imaging
667 facility of Institut Curie.

668 **Treatments**

669 Cells seeded on coverslips were pre-treated for 1 h at 37°C with either 0.5 µM Cytochalasin
670 D (Sigma-Aldrich), 0.5 µM Latrunculin A (Interchim), 50 µM TAPI-1 (Calbiochem), 20
671 µM GI254023X (Tocris Bioscience), 2.5 µM Dasatinib (Cell Signaling Technology) or
672 DMSO (Sigma-Aldrich) as a control. The trafficking of the reporters was synchronized by
673 addition of biotin (40 µM at 37°C) in the presence of the respective drugs. For the
674 Cytochalasin D and Latrunculin A treatments, we corroborated the disruption of the actin
675 microfilaments by assessing the SiR-Actin staining (0.5 µM, 1 h at 37°C). For TAPI-1 and
676 GI254023X, the inhibition of ADAM metalloprotease was corroborated by using the SBP-
677 Cherry-2XADAMtide-ephrin-A1-EGFP-GPI sensor. Inhibition of EphA2 signaling with
678 Dasatinib was controlled by performing a pEphA immuno-staining.

679 To assure that dynamin-dependent endocytosis was inhibited after Dyngo4A (Abcam)
680 treatment, we checked for transferrin internalization. Briefly, cells seeded on coverslips
681 were washed 3 times with PBS and starved from serum during 5 h at 37°C in DMEM
682 supplemented with 0.1% BSA and penicillin/streptomycin (DMEM-BSA). Cells were then
683 pre-treated with 10 µM Dyngo4A or DMSO (Control) for 1h at 37°C in DMEM-BSA. The
684 following incubation and washing steps were performed with DMEM-BSA containing
685 either DMSO or 10 µM Dyngo4A. The trafficking of SBP-EGFP-ephrin-A1 was
686 synchronized by addition of biotin (40 µM, 90 min at 37°C). Cells were then incubated for
687 10 min on ice with 50 µg/mL Transferrin Alexa Fluor™ 647 conjugate (Tfr-647, Molecular
688 Probes) and washed 3 times with cold DMEM-BSA to remove the unbound Tfr-647.
689 Transferrin internalization was allowed by incubating the cells for 10 min at 37°C. Cells
690 were then washed 3 times with DMEM-BSA and the remnant surface-bound Tfr-647 was

691 stripped out by an acidic wash with Glycine buffer (0.1 M Glycine + 150 mM NaCl, pH
692 2.5, 30-40 sec). Finally, cells were washed in PBS, fixed with 3% PFA and mounted for
693 imaging.

694 Degradative properties of the lysosomes were assessed by incubating the cells with DQTM-
695 Green-BSA (10 µg/mL at 37°C) (ThermoFisher Scientific). Acidic lysosomes were
696 visualized by treating cells with 75 nM LysoTracker Blue DND-22 (Invitrogen) at 37°C
697 and imaging them live for 5 min.

698 Arrest of the cell cycle at G2/M border was performed by treating the cells with 9 µM
699 RO3306 (Sigma-Aldrich) for 20 h at 37°C (Vassilev et al., 2006).

700 **Cell confrontation assay**

701 Cell confrontation assays were performed using cell culture inserts and 35 mm-High plates
702 from Ibidi (Ibidi GmbH) following the instructions of the manufacturer as previously
703 described (Porazinski et al., 2016). Cell dyes were used for better visualization; MDA-SBP-
704 Cherry-ephrin-A1 or MDA-SBP-EGFP-EphA2 cells were labeled in suspension with Cell
705 Proliferation Dye eFluor® 670 or 5-(and 6)-Carboxyfluorescein diacetate succinimidyl
706 ester (CFSE-Cell Proliferation Dye) (Invitrogen), respectively (5 µM, 10 min at 37°C). The
707 labeling was stopped by incubating the cells with ice-cold DMEM medium supplemented
708 with 20% FCS during 5 min, followed of three washes with 20% FCS medium (5 min, 1200
709 x g each). 3-4 x 10⁴ cells were then seeded into each compartment of the insert and
710 incubated in presence of 80 µM of D-biotin at 37°C for 12-20 h before the removal of the
711 insert. Upon reaching confluence, the insert was removed. Non-adhered cells were gently
712 washed out with PBS and the dish was filled with 2 mL of 80 µM D-biotin-containing
713 DMEM. 8-12 h after insert removal the medium was switched to Leibovitz's medium

714 supplemented with 80 μ M of D-biotin and 10% FBS. The cells were imaged-live using a
715 spinning disk confocal microscope with a 20X objective.

716 **Co-culture assay**

717 HeLa cells were first electroporated with the appropriate plasmid and then mixed.
718 Electroporation were performed using the NEPA21 system (Nepa Gene Co.) according to
719 the manufacturer's instructions. Briefly, cells were electroporated in Opti-MEM medium
720 (Invitrogen) supplemented with 7 μ g/mL of avidin (Sigma-Aldrich) to neutralize the traces
721 of biotin present in the medium. 1×10^6 of HeLa cells in suspension were mixed with 10
722 μ g of DNA up to a final volume of 100 μ L. For the electroporation of mRFP-Rab5-WT,
723 mCherry-Rab5-Q79L, EGFP-Rab11-WT, DsRed-Rab11-S25N, EGFP-Rab7-WT and
724 EGFP-Rab7-Q76L, 5 μ g of each plasmid were mixed with 5 μ g of an empty vector. Cells
725 were then transferred to 2 mm gap cuvettes and electroporated using 2 poring pulses of 125
726 V, 7.5 msec length and 50 msec interval, followed by 5 transfer pulses of 20 V, 50 msec
727 length and 50 msec interval. Equal amounts of donor and acceptor cells were then seeded
728 onto 35 mm coverslip with pre-warmed medium and mixed by pipetting until a mono-
729 disperse cell suspension was achieved. Before imaging, cells were gently washed 3 times
730 with PBS to remove dead cells and debris.

731 **Ephrin inheritance assay**

732 Wild-type MDA-MB231 cells (acceptor) were labeled in suspension with CFSE-Cell
733 Proliferation Dye and seeded on gridded 35 mm-High plates from Ibidi (Ibidi GmbH)
734 following the instructions of the manufacturer. After 24h the cell cycle was arrested at
735 G2/M transition using the reversible CDK1 inhibitor RO3306 (9 μ M, 20 h at 37°C)
736 (Vassilev et al., 2006). Then HeLa-SBP-Cherry-ephrin-A1 cells (donor) were seeded on
737 top of the monolayer of acceptor cells and were let adhere in the presence of 40 μ M D-
738 biotin-containing DMEM for 2-4 h before imaging. The imaging field were chosen and the
739 coordinates of the grid were registered. Then the cells were washed 5 times for 1 min with
740 Leibovitz's medium and imaged live using a spinning disk confocal microscopy with a 63X

741 objective as described before. After completion of mitosis, the cells were fixed for 15 min
742 at room temperature with 3% paraformaldehyde and processed for immunofluorescence
743 with antibodies against LAMP1 and pEphA receptors. The cells were located using the
744 coordinates on the gridded plate and imaged using the same microscope and objective as
745 above. Images were then manually aligned using Fiji software.

746

747 **QUANTIFICATION AND STATISTICAL ANALYSIS**

748 **Image quantification**

749 Normalized Golgi intensity was obtained by measuring the integrated intensity over the
750 time in a mask of the Golgi apparatus, whose position was determined from latter time
751 points as previously described (Boncompain et al., 2012). The same mask was then
752 positioned in a cell-free region (background) and the integrated intensity over the time was
753 measured and subtracted to the integrated Golgi intensity. The values were normalized to
754 the maximal value, and the measurements before the addition of biotin were adjusted to
755 zero.

756 Colocalization percentages over the time were determined in background subtracted
757 cropped images containing individual cells. Single channel masks were generated by
758 thresholding of each channel. The colocalization area was measured and normalized to the
759 total area of each channel for every time point. The values were then translated in
760 percentages. Random colocalization was determined by displacing the red channel 5x5
761 pixels in the *xy* plane for all the time points.

762 The start of clustering and secretion were determined by visual inspection of videos of cells
763 co-transfected with the RUSH reporter SBP-EGFP-ephrin-A1 and PM-Cherry to delimit
764 the donor cell. The start of clustering was considered as the time point at which the first
765 bright ephrin-A1 structure appeared at the PM of a given cell before to be exported. The
766 start of the secretion was stated as the time point at which the first ephrin-A1 cluster was
767 distinguishable separated from the PM of its donor cell. The normalized number of

768 transferred clusters was extracted from fixed epifluorescence images acquired with a 63X
769 objective. The total number of exported clusters in each field was determined by manual
770 counting and multiplied by the ratio of donor / acceptor cells.

771 For lysosomes-related quantifications, we measured the integrated intensity and area of
772 hand-drawn region of interests (ROIs) comprising ephrin-A1-containing lysosomes,
773 ephrin-A1-negative lysosomes or cytosolic lysosomes-free ROIs of the same size. For the
774 cell confrontation assays we determined the distance traveled by the marginal cells after
775 collision by measuring the distance between two parallels lines depicting the point of the
776 first cell-to-cell contact and the position of the front of migration 20 h after this contact
777 respectively. All these quantifications were made using Fiji software (Schindelin et al.,
778 2012) and Microsoft® Excel® 14.7.1 for drawing the plots. For boxplots; boxes represent
779 25th and 75th percentiles, the whiskers extend to the most extreme data points and the
780 median is indicated as a band inside the box. The data were compared by using the two-
781 tailed t-test.

782

783 **DATA AND CODE AVAILABILITY**

784 This study did not generate/analyze datasets or codes

785

786 **Additional information**

787 **SUPPLEMENTARY INFORMATION**

788 **Correspondence and request for materials** should be addressed to J.I.V. or F.P.

789

790 **SUPPLEMENTARY VIDEOS**

791 **Supplementary Video 1 (Related to Figure 1) Partial sorting of EphA2 and ephrin-A1**
792 **at the Golgi apparatus**

793 **Supplementary Video 2 (Related to Figure 1) Ephrin-A1 is exported from filopodia of**
794 **ligand-producing cells**

795 **Supplementary Video 3 (Related to Figure 2) Ephrin-A5 is transferred to neighboring**
796 **cells from filopodia**

797 **Supplementary Video 5 (Related to Figure 2) Actin-dependent trans-endocytosis of**
798 **ephrin-A1 from filopodia**

799 **Supplementary Video 6 (Related to Figure 3) Cleavage-independent trans-endocytosis**
800 **of ephrin-A1**

801 **Supplementary Video 7 (Related to Figure 4) Dynamin-dependent trans-endocytosis**
802 **of ephrin-A1**

803 **Supplementary Video 8 (Related to Figure 5) Localized trans-endocytosis of ephrin-**
804 **A1/EphA2 complexes**

805 **Supplementary Video 9 (Related to Figure 5) Trans-endocytosis of ephrin-A1 is**
806 **massive at steady state**

807 **Supplementary Video 10 (Related to Figure 7) Inheritance of trans-endocytosed**
808 **ephrin-A1 in cancer cells**

809

810 **SUPPLEMENTARY FIGURES**

811 **Supplementary Figure 1 Synthetic sensor of ADAM protease-based trans-**
812 **endocytosis of ephrin-A1 (Related to Figure 3)**

813 **(A)** Scheme of the experimental design of the ADAM-cleavage sensor SBP-Cherry-
814 2XADAMtide-ephrin-A1-EGFP-GPI. The blue fragment represents the position of the
815 inserted repetitions (2x) of the consensus ADAM cleavage site (ADAMtide).

816 **(B)** Time-lapse imaging of HeLa cells transfected with the SBP-Cherry-2XADAMtide-
817 ephrin-A1-EGFP-GPI sensor. EGFP and Cherry signals are shown. Cell-cell boundaries
818 are visualized with the actin probe SiR-Actin (blue). Bottom panels (high-magnification
819 images of a donor/acceptor cell interface).

820 **(C)** Kymograph analysis of one cluster of SBP-Cherry-2XADAMtide-ephrin-A1-EGFP-
821 GPI into an acceptor cell.

822 **(D)** Line-scan analysis and EGFP/Cherry intensity ratio obtained from the kymograph in
823 (C).

824 **(E-G)** Same as in (B-D), but for cells treated with the metalloprotease's inhibitor TAPI-1.

825 **(H-J)** Same as in (B-D), but for cells treated with the ADAM-10 inhibitor GI254023X.
826 Scale bars, 10 μ m.

827
828 **Supplementary Figure 2 Trans-endocytosed ephrin-A1 is sorted apart from Rab11-**
829 **recycling endosomes (Related to Figure 4)**

830 **(A)** Time-lapse imaging of HeLa cells expressing SBP-Cherry-ephrin-A1 co-cultured with
831 cells expressing EGFP-Rab11-WT. Single channels are shown in inverted colors for better
832 visualization (ephrin-A1 in the top row and Rab11 in the middle row). Merge images are
833 shown (bottom panels: ephrin-A1, red; Rab11, green). Cell-cell interfaces were visualized
834 by the SiR-Actin labeling (blue). Arrows indicate an example of trans-endocytosed SBP-
835 Cherry-ephrin-A1 cluster in an acceptor cell.

836 **(B)** Same as in (A) but for cells expressing SBP-EGFP-ephrin-A1 (top row) and co-cultured
837 with cells expressing DsRED-Rab11-S25N mutant (middle row). Scale bars, 10 μ m.

838
839 **Supplementary Figure 3 Activation of EphA receptors at the plasma membrane is**
840 **necessary for ephrin-A1 trans-endocytosis (Related to Figure 5)**

841 **(A)** HeLa cells expressing SBP-EGFP-ephrin-A1 (green) were co-cultured with HeLa cells
842 expressing Dynamin-I-WT-CFP (blue, top panels) or Dynamin-I-K44A-CFP (blue, bottom
843 panels). Cells were treated with biotin (90 min), fixed and stained with an antibody against
844 active phosphorylated EphA receptors (pEphA, red). Right panels show high-magnification

845 images of the boxed area. Arrows indicate examples of trans-endocytosed SBP-EGFP-
846 ephrin-A1 clusters in an acceptor cell co-localizing with active EphA receptors.

847 **(B)** HeLa cells co-expressing SBP-Cherry-ephrin-A1 (red) and PM-EGFP (blue), under
848 control conditions (upper panels) or after EphA2 signaling inhibition with Dasatinib
849 (bottom panels). Cells were fixed after 90 min of biotin addition. Immuno-staining with an
850 antibody against active phosphorylated EphA receptors (pEphA, green) are shown. Right
851 panels show high-magnification images of donor/acceptor cell interfaces (boxed area in the
852 left panel). Arrows indicate examples of trans-endocytosed SBP-Cherry-ephrin-A1 clusters
853 in an acceptor cell co-localizing with active EphA receptors. PM-EGFP is show in inverted
854 colors (right panels) to highlight the long donor filopodia inside the acceptor cell after EphA
855 receptor inhibition. Scale bars, 10 μ m.

856
857 **Supplementary Figure 4 Trans-endocytosed ephrin-A1 is co-transported with EphA2**
858 **and stored for a long time in acidic lysosomes in MDA-MB231 cells (Related to Figure**
859 **6)**

860 **(A)** Live-cell imaging of MDA-MB231 cells stably expressing the RUSH construct SBP-
861 Cherry-ephrin-A1 (SBP-Cherry-ephrin-A1, red) co-cultured with MDA-MB231 cells
862 expressing steady-state SBP-EGFP-EphA2 (SBP-EGFP-EphA2 in the absence of hook
863 expression, green). Lysosomes were stained with the SiR-lysosome dye (blue). High-
864 magnification images are presented below in black and white: SBP-Cherry-ephrin-A1 (top
865 row), SBP-EGFP-EphA2 (middle-upper row) and SiR-lysosome (middle-lower row).

866 Merge images are shown (bottom row). Arrows indicate an example of a trans-endocytosed
867 SBP-Cherry-ephrin-A1 cluster co-transported with SBP-EGFP-EphA2 towards a large
868 lysosomal structure in an acceptor cell.

869 **(B)** Single frame from live-cell imaging of MDA-MB231 cells stably expressing the RUSH
870 construct SBP-Cherry-ephrin-A1 (SBP-Cherry-ephrin-A1, red) co-cultured with wild-type
871 MDA-MB231 cells (left pannel). High magnification images of an acceptor cell are shown.
872 Right panels shown the same cell after a short treatment with the acidotropic dye
873 LysoTracker (blue). Arrows indicate trans-endocytosed SBP-Cherry-ephrin-A1 in acidic
874 lysosomes.

875 **(C)** Live-cell imaging of MDA-MB231 cells stably expressing the RUSH construct SBP-
876 Cherry-ephrin-A1 (SBP-Cherry-ephrin-A1, red) co-cultured with wild-type MDA-MB231
877 cells. Lysosomes were stained with the SiR-lysosome dye (blue). Degradative
878 compartements were detected by the fluorogenic protease substrate DQ-BSA (green). High-
879 magnification images are presented below in black and white: SBP-Cherry-ephrin-A1 (top
880 row), DQ-BSA (middle-upper row) and SiR-lysosome (middle-lower row). Merge images
881 are shown (bottom row). Arrows indicate trans-endocytosed SBP-Cherry-ephrin-A1 in
882 degradative lysosomes. Scale bars, 10 μ m.

883

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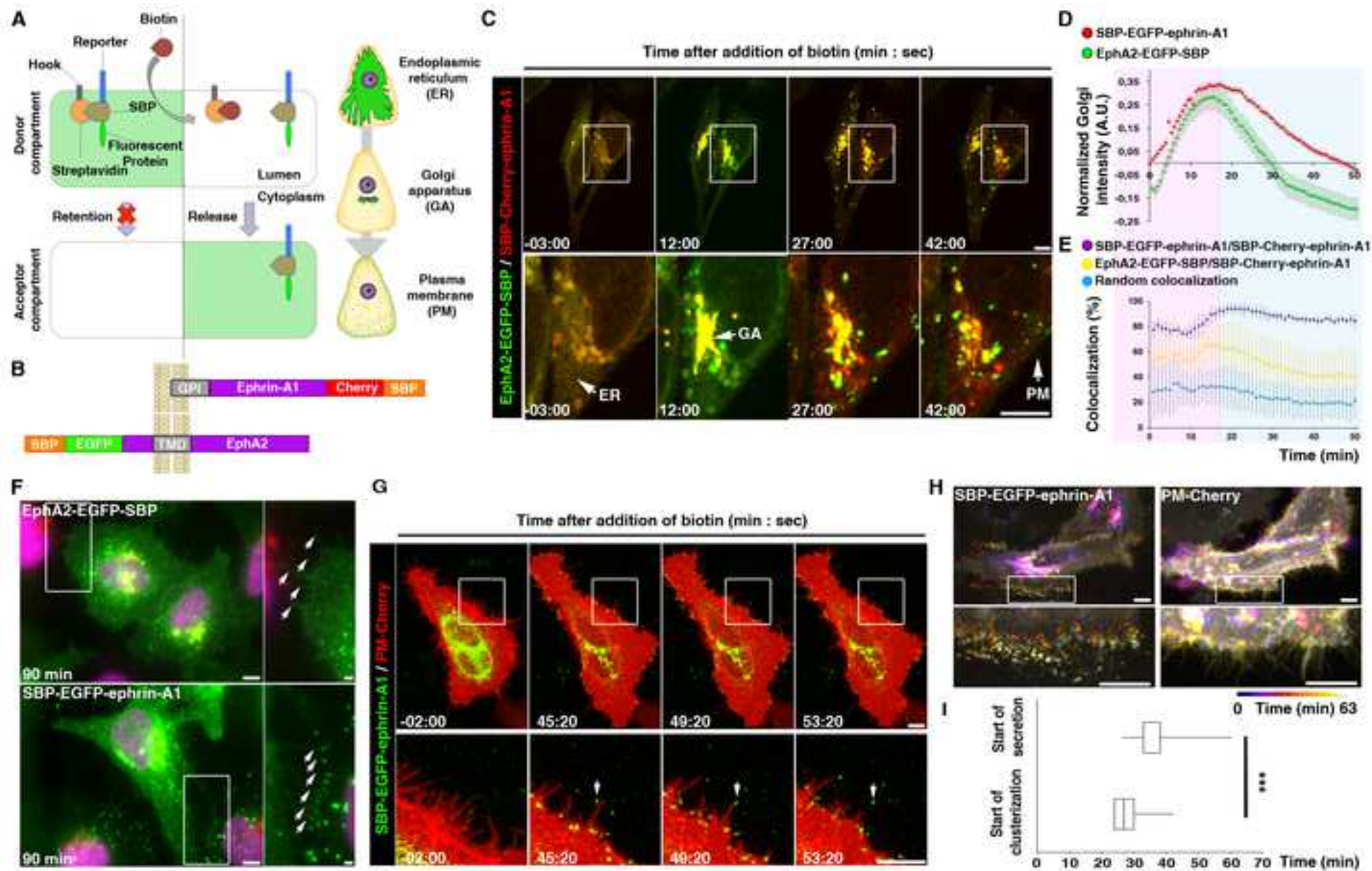
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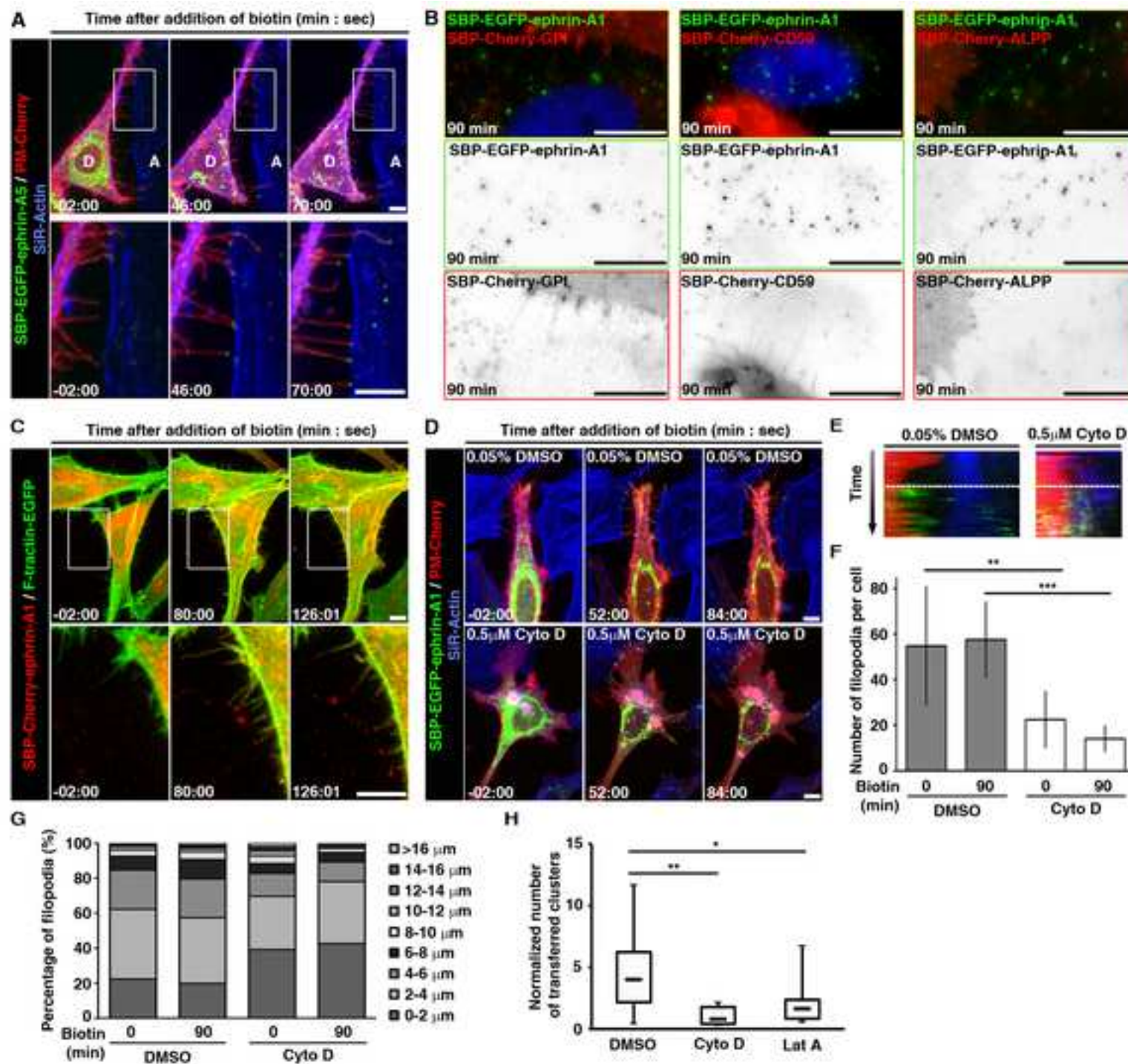
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-GFP	Roche	Cat#11814460001; RRID:AB_390913
Rabbit polyclonal Anti-Eph receptor A2+A3+A4 (phospho Y588 + Y596)	Abcam	Cat#ab62256; RRID:AB_942240
Mouse monoclonal anti-LAMP1	Santa Cruz Biotechnology	Cat#sc-20011; RRID:AB_626853
Rabbit polyclonal anti-GFP	Recombinant Antibodies Platform (Institut Curie)	A-P-R#06
Mouse monoclonal Anti-phosphotyrosine (clone 4G10)	Recombinant Antibodies Platform (Institut Curie)	A-M-M#09
Human monoclonal anti-giantin (clone TA10)	Recombinant Antibodies Platform (Institut Curie)	A-R-H#03
Bacterial and Virus Strains		
Biological Samples		
Chemicals, Peptides, and Recombinant Proteins		
Avidin	Sigma-Aldrich	Cat#A-9275
Cell Proliferation Dye eFluor® 670	Invitrogen	Cat#65-0840-90
5-(and 6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE-Cell Proliferation Dye)	Sigma-Aldrich	Cat#21888
Cytochalasin D	Sigma-Aldrich	Cat#C8273
Dasatinib	Cell Signaling Technology	Cat#9052S
D-biotin	Sigma-Aldrich	Cat#B4501
DMSO	Sigma-Aldrich	Cat#D2438
DQ™-Green-BSA	ThermoFisher Scientific	Cat#D12050
Dyngo4A	Abcam	Cat#Ab120689
GI254023X	Tocris Bioscience	Cat#3995
Latrunculin A	Interchim	Cat#L-12370
LysoTracker Blue DND-22	Invitrogen	Cat#L7525
Phalloidin-FITC	Sigma-Aldrich	Cat#P5282

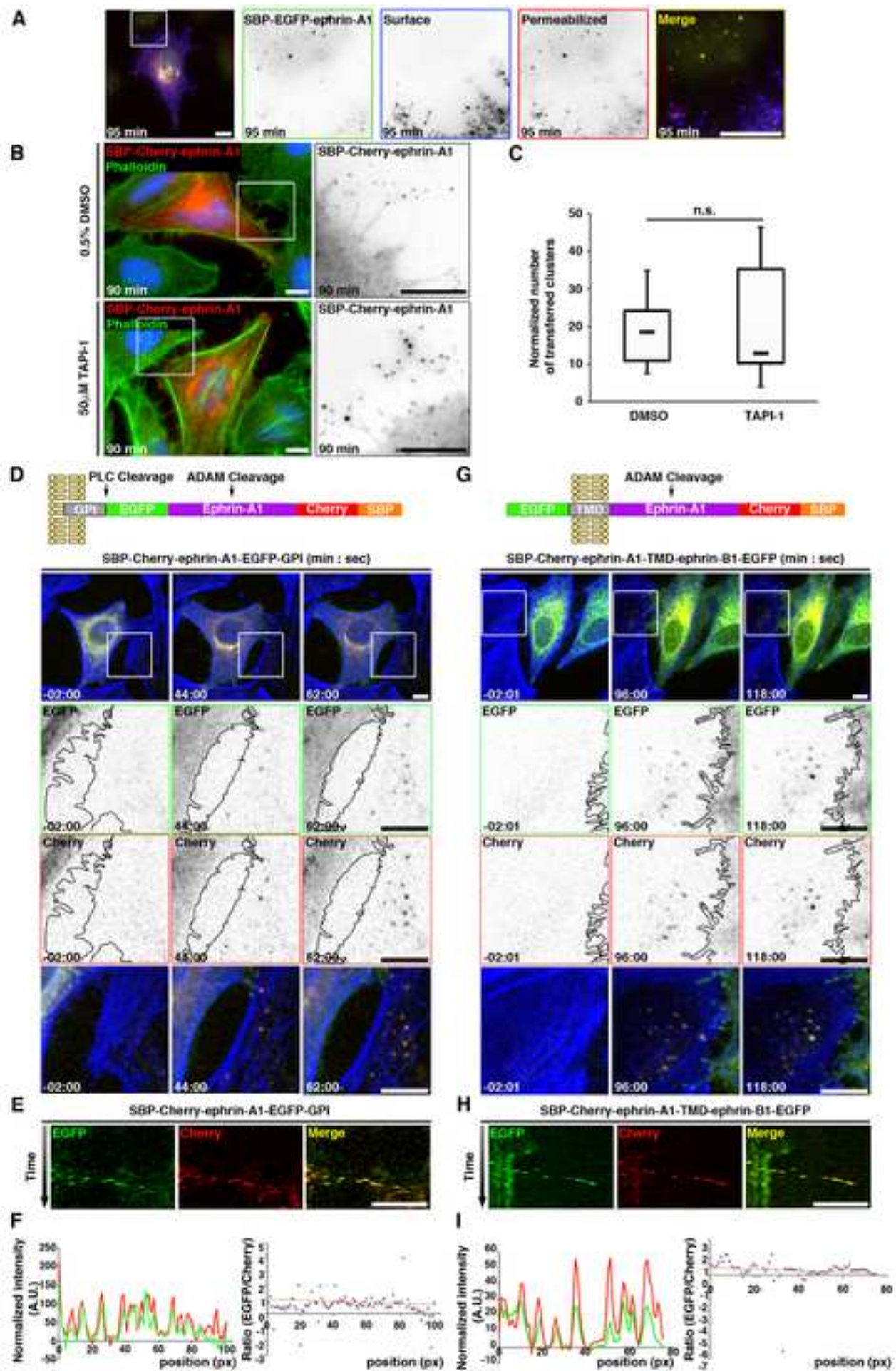
RO3306	Sigma-Aldrich	Cat#SML0569
SiR-Actin	Spirochrome	Cat#SC001
SiR-DNA	Spirochrome	Cat#SC007
SiR-Lysosome	Spirochrome	Cat#SC012
TAPI-1	Tocris Bioscience	Cat#6162
Transferrin Alexa Fluor™ 647	Molecular Probes	Cat#T23366
Critical Commercial Assays		
Culture-Insert 3 Well in μ -Dish 35 mm, high	Ibidi	Cat#80366
Deposited Data		
Experimental Models: Cell Lines		
Human: HeLa	ATCC	ATCC CCL-2
Human: MDA-MB-231	ATCC	ATCC HTB-26
Experimental Models: Organisms/Strains		
Oligonucleotides		
Recombinant DNA		
pmCherry-C1	Clontech	Cat#632524
pEGFP-C1	Clontech	This plasmid has been discontinued
pCDH-CMV-MCS-EF1 α -Puro	System Biosciences	Cat#CD510B-1
Dynamin-1-WT-EGFP	(Song et al., 2004)	Addgene plasmid #34680
Dynamin-1-K44A-EGFP	(Song et al., 2004)	Addgene plasmid #34681
F-tractin-EGFP	(Belin et al., 2014)	Addgene plasmid #58473

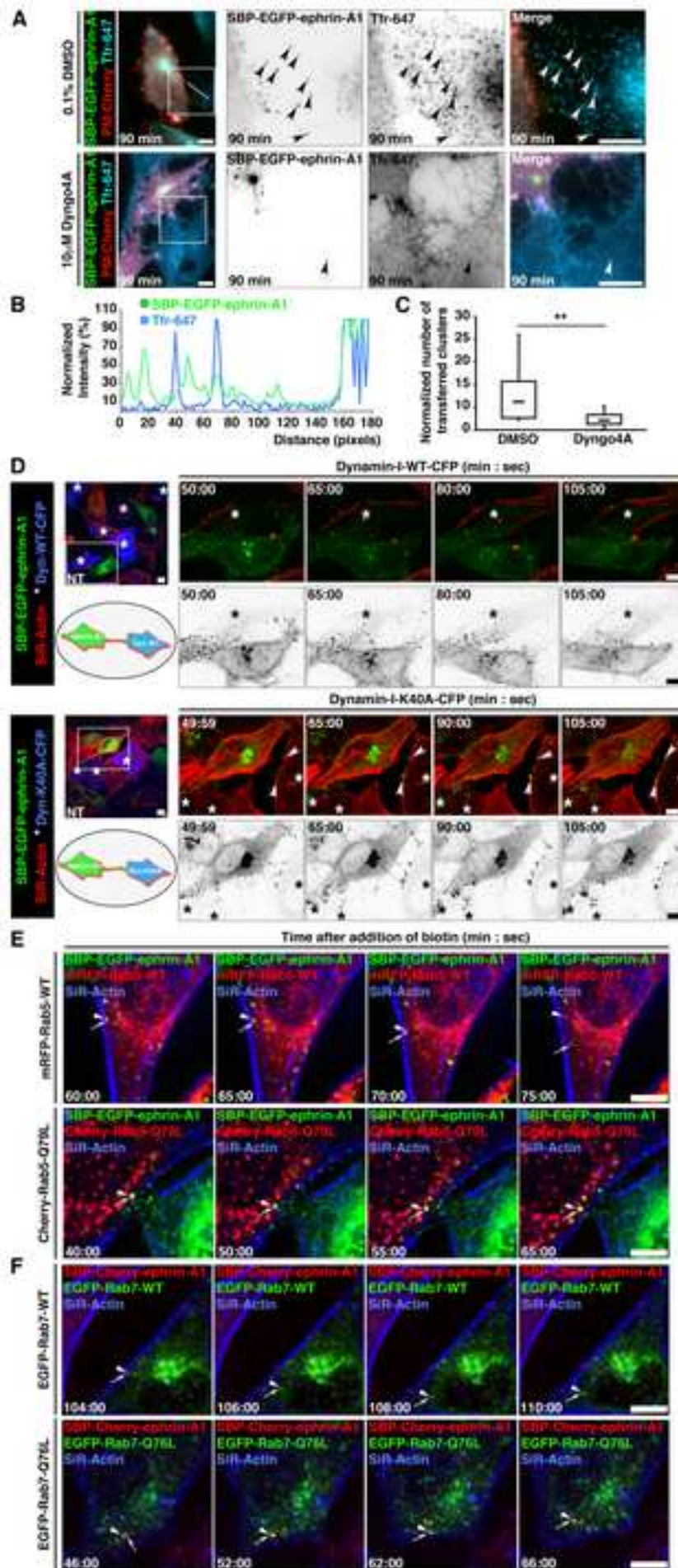
pDONR223-EPHA2	(Johannessen et al., 2010)	Addgene plasmid #23926
DsRed-Rab11-S25N	(Choudhury et al., 2002)	Addgene plasmid #12680
SBP-Cherry-GPI	(Boncompain et al., 2012)	Addgene plasmid #65295
PM-EGFP	(Zacharias et al., 2002)	N/A
mRFP-Rab5-WT	(Rink et al., 2005)	N/A
SBP-Cherry-ALPP	(Fourriere et al., 2019)	N/A
SBP-Cherry-CD59	(Fourriere et al., 2019)	N/A
EGFP-Rab7-WT	This paper	N/A
EGFP-Rab7-Q76L	This paper	N/A
Dynamin-1-WT-CFP	This paper	N/A
Dynamin-1-K44A-CFP	This paper	N/A
PM-Cherry	This paper	N/A
SBP-EGFP-ephrin-A1	This paper	N/A
SBP-Cherry-ephrin-A1	This paper	N/A
SBP-EGFP-ephrin-A5	This paper	N/A
SBP-EGFP-EphA2	This paper	N/A
EphA2-EGFP-SBP	This paper	N/A
EphA2-Cherry-SBP	This paper	N/A
SBP-Cherry-ephrin-A1-EGFP-GPI	This paper	N/A
SBP-Cherry-ephrin-A1-TMD-ephrin-B1-EGFP	This paper	N/A
SBP-Cherry-2xADAMtide-ephrin-A1-EGFP-GPI	This paper	N/A
Software and Algorithms		
Fiji	(Schindelin et al., 2012)	https://fiji.sc/
MetaMorph software	Molecular Device	
Other		

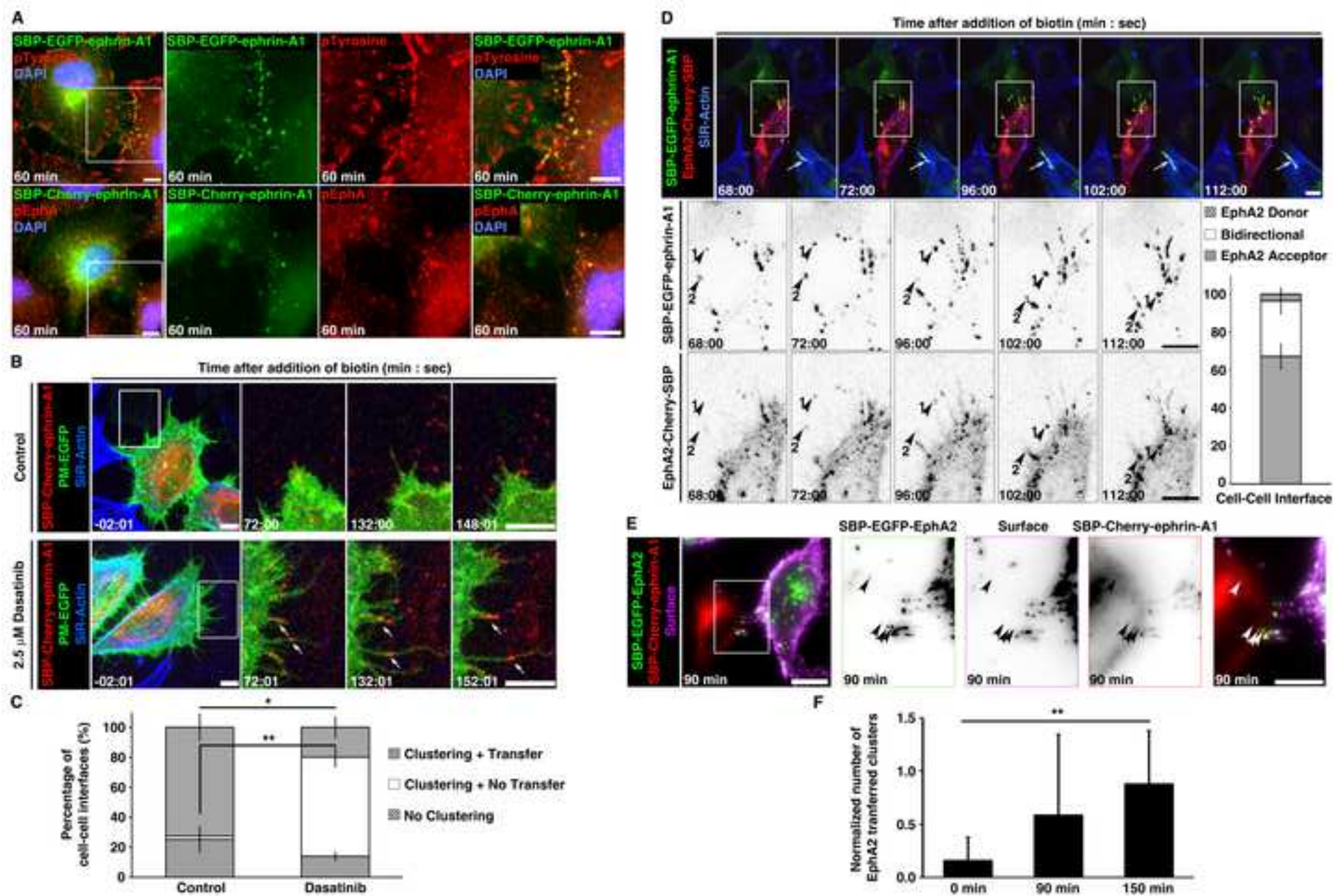
(Perez & Valenzuela - *Localized intercellular transfer of ephrin-As by trans endocytosis provides a memory of signaling*)

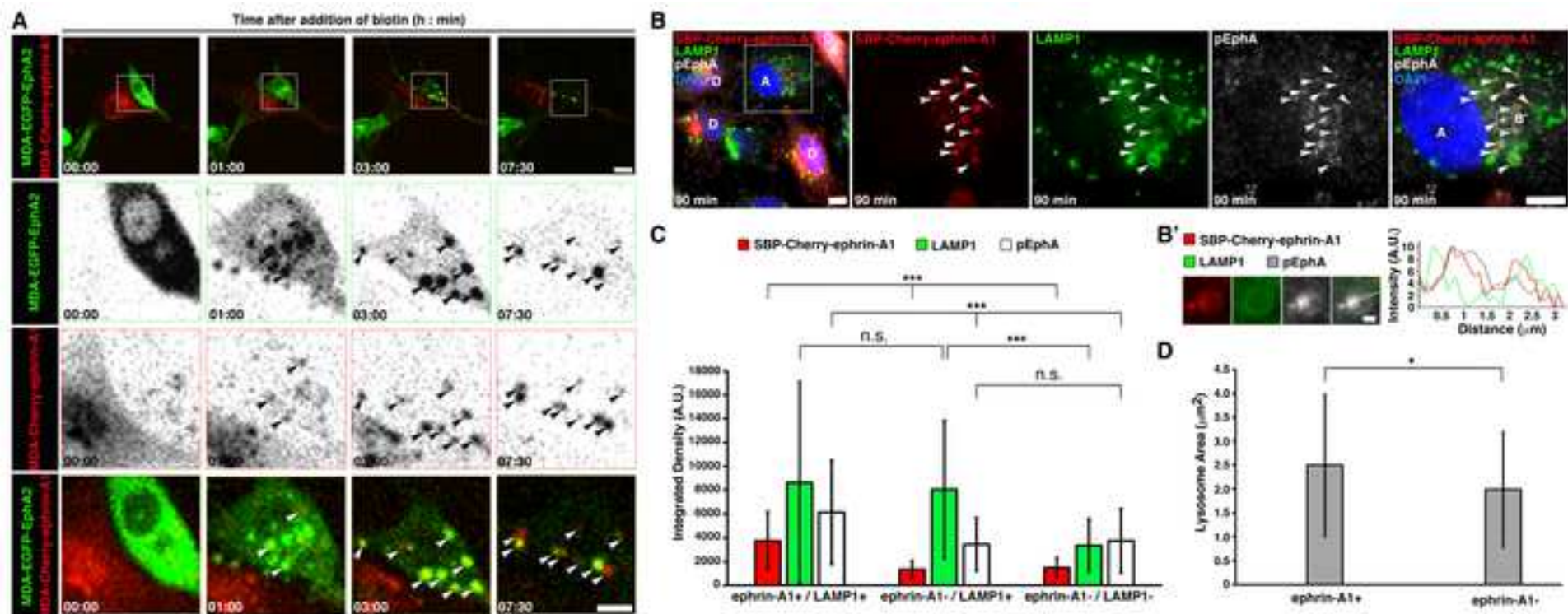


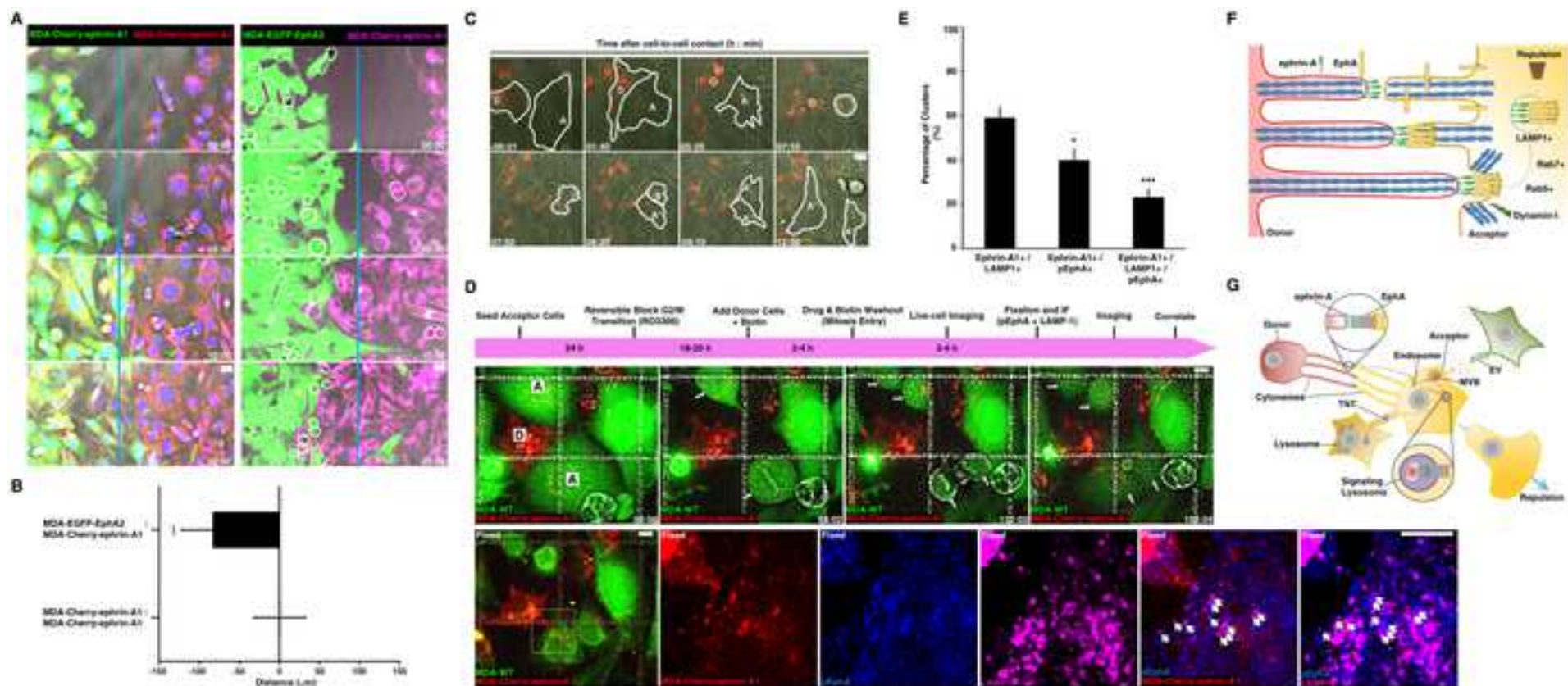




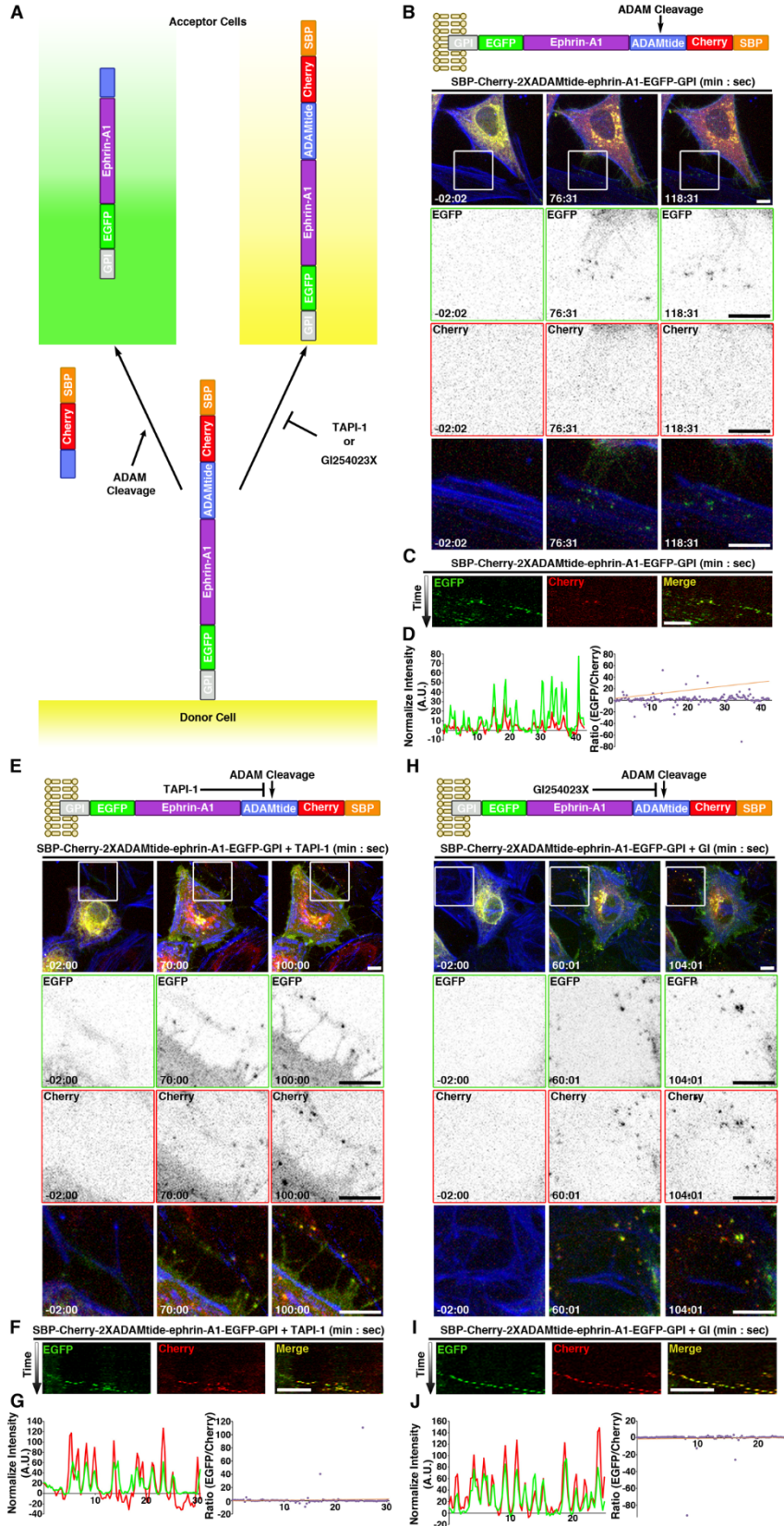








SUPPLEMENTARY FIGURES



Supplementary Figure 1 Synthetic sensor of ADAM protease-based trans-endocytosis of ephrin-A1 (Related to Figure 3)

(A) Scheme of the experimental design of the ADAM-cleavage sensor SBP-Cherry-2XADAMtide-ephrin-A1-EGFP-GPI. The blue fragment represents the position of the inserted repetitions (2x) of the consensus ADAM cleavage site (ADAMtide).

(B) Time-lapse imaging of HeLa cells transfected with the SBP-Cherry-2XADAMtide-ephrin-A1-EGFP-GPI sensor. EGFP and Cherry signals are shown. Cell-cell boundaries are visualized with the actin probe SiR-Actin (blue). Bottom panels (high-magnification images of a donor/acceptor cell interface).

(C) Kymograph analysis of one cluster of SBP-Cherry-2XADAMtide-ephrin-A1-EGFP-GPI into an acceptor cell.

(D) Line-scan analysis and EGFP/Cherry intensity ratio obtained from the kymograph in (C).

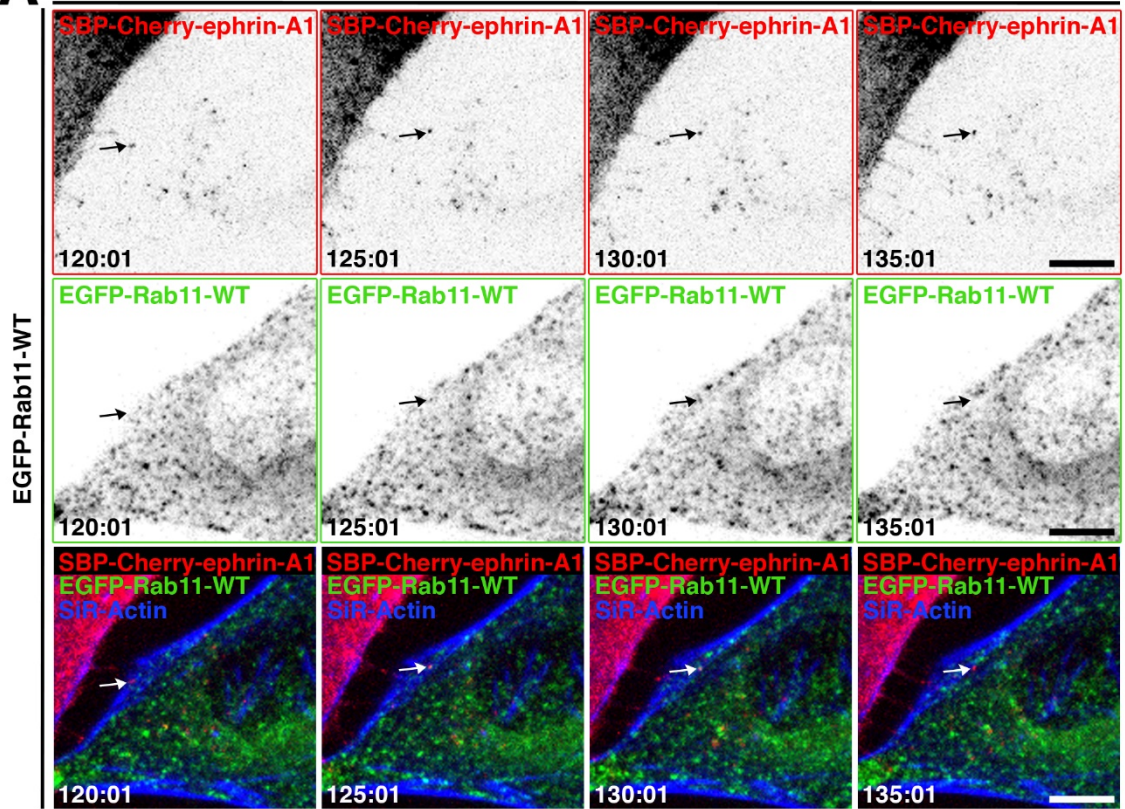
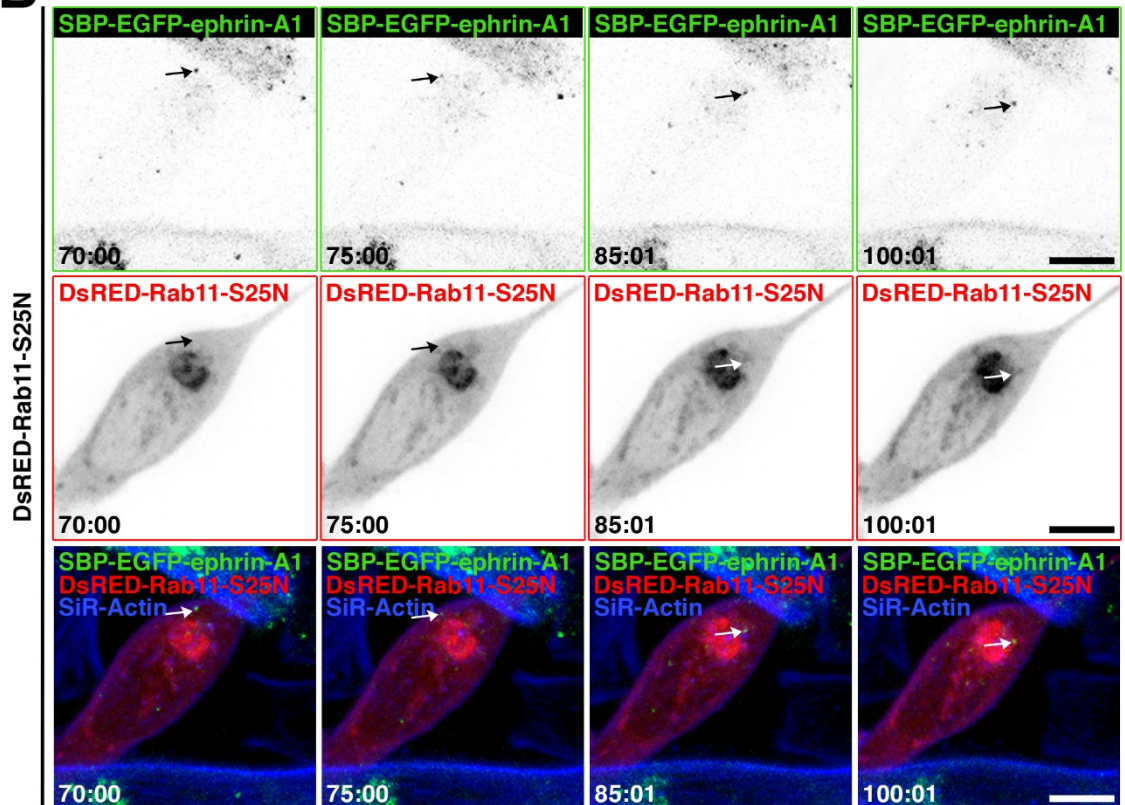
(E-G) Same as in (B-D), but for cells treated with the metalloprotease's inhibitor TAPI-1.

(H-J) Same as in (B-D), but for cells treated with the ADAM-10 inhibitor GI254023X.

(B-C, E-F, H-I) Scale bars, 10 μm .

A

Time after addition of biotin (min : sec)

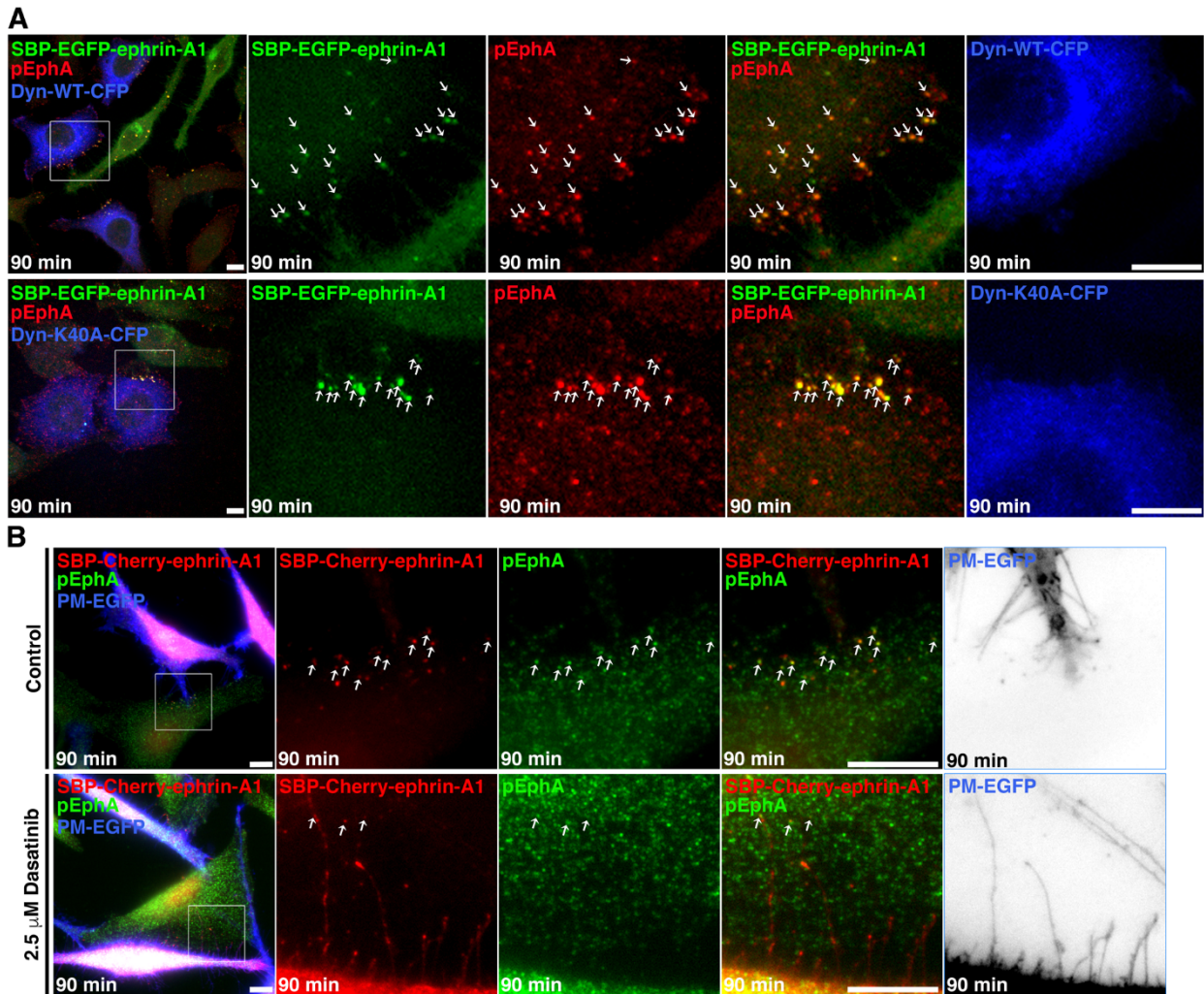
**B**

Supplementary Figure 2 Trans-endocytosed ephrin-A1 is sorted apart from Rab11-recycling endosomes (Related to Figure 4)

(A) Time-lapse imaging of HeLa cells expressing SBP-Cherry-ephrin-A1 co-cultured with cells expressing EGFP-Rab11-WT. Single channels are shown in inverted colors for better visualization (ephrin-A1 in the top row and Rab11 in the middle row). Merge images are shown (bottom panels: ephrin-A1, red; Rab11, green). Cell-cell interfaces were visualized by the SiR-Actin labeling (blue). Arrows indicate an example of trans-endocytosed SBP-Cherry-ephrin-A1 cluster in an acceptor cell.

(B) Same as in (A) but for cells expressing SBP-EGFP-ephrin-A1 (top row) and co-cultured with cells expressing DsRED-Rab11-S25N mutant (middle row).

(A-B) Scale bars, 10 μm .

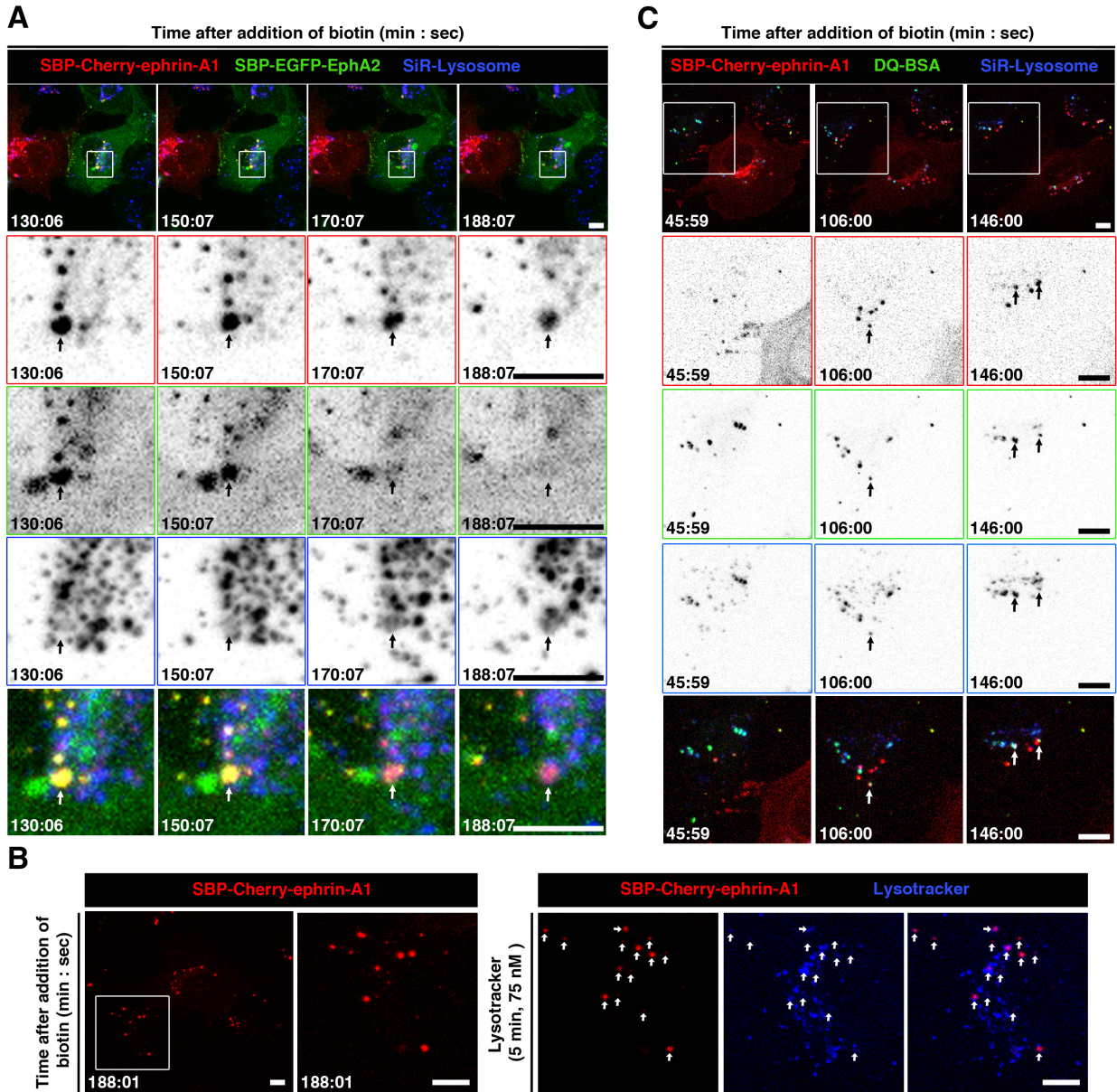


Supplementary Figure 3 Activation of EphA receptors at the plasma membrane is necessary for ephrin-A1 trans-endocytosis (Related to Figure 5)

(A) HeLa cells expressing SBP-EGFP-ephrin-A1 (green) were co-cultured with HeLa cells expressing Dynamin-I-WT-CFP (blue, top panels) or Dynamin-I-K44A-CFP (blue, bottom panels). Cells were treated with biotin (90 min), fixed and stained with an antibody against active phosphorylated EphA receptors (pEphA, red). Right panels show high-magnification images of the boxed area. Arrows indicate examples of trans-endocytosed SBP-EGFP-ephrin-A1 clusters in an acceptor cell co-localizing with active EphA receptors.

(B) HeLa cells co-expressing SBP-Cherry-ephrin-A1 (red) and PM-EGFP (blue), under control conditions (upper panels) or after EphA2 signaling inhibition with Dasatinib (bottom panels). Cells were fixed after 90 min of biotin addition. Immuno-staining with an antibody against active phosphorylated EphA receptors (pEphA, green) are shown. Right panels show high-magnification images of donor/acceptor cell interfaces (boxed area in the left panel). Arrows indicate examples of trans-endocytosed SBP-Cherry-ephrin-A1 clusters in an acceptor cell co-localizing with active EphA receptors. PM-EGFP is show in inverted colors (right panels) to highlight the long donor filopodia inside the acceptor cell after EphA receptor inhibition.

(A-B) Scale bars, 10 μ m.



Supplementary Figure 4 Trans-endocytosed ephrin-A1 is co-transported with EphA2 and stored for a long time in acidic lysosomes in MDA-MB231 cells (Related to Figure 6)

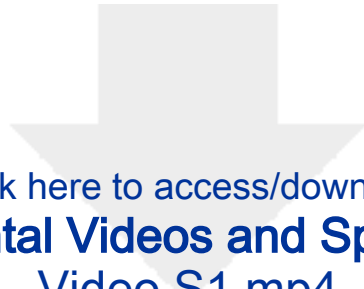
(A) Live-cell imaging of MDA-MB231 cells stably expressing the RUSH construct SBP-Cherry-ephrin-A1 (SBP-Cherry-ephrin-A1, red) co-cultured with MDA-MB231 cells expressing steady-state SBP-EGFP-EphA2 (SBP-EGFP-EphA2 in the absence of hook

expression, green). Lysosomes were stained with the SiR-lysosome dye (blue). High-magnification images are presented below in black and white: SBP-Cherry-ephrin-A1 (top row), SBP-EGFP-EphA2 (middle-upper row) and SiR-lysosome (middle-lower row). Merge images are shown (bottom row). Arrows indicate an example of a trans-endocytosed SBP-Cherry-ephrin-A1 cluster co-transported with SBP-EGFP-EphA2 towards a large lysosomal structure in an acceptor cell.

(B) Single frame from live-cell imaging of MDA-MB231 cells stably expressing the RUSH construct SBP-Cherry-ephrin-A1 (SBP-Cherry-ephrin-A1, red) co-cultured with wild-type MDA-MB231 cells (left panel). High magnification images of an acceptor cell are shown. Right panels shown the same cell after a short treatment with the acidotropic dye LysoTracker (blue). Arrows indicate trans-endocytosed SBP-Cherry-ephrin-A1 in acidic lysosomes.

(C) Live-cell imaging of MDA-MB231 cells stably expressing the RUSH construct SBP-Cherry-ephrin-A1 (SBP-Cherry-ephrin-A1, red) co-cultured with wild-type MDA-MB231 cells. Lysosomes were stained with the SiR-lysosome dye (blue). Degradative compartments were detected by the fluorogenic protease substrate DQ-BSA (green). High-magnification images are presented below in black and white: SBP-Cherry-ephrin-A1 (top row), DQ-BSA (middle-upper row) and SiR-lysosome (middle-lower row). Merge images are shown (bottom row). Arrows indicate trans-endocytosed SBP-Cherry-ephrin-A1 in degradative lysosomes.

(A-C) Scale bars, 10 μm .



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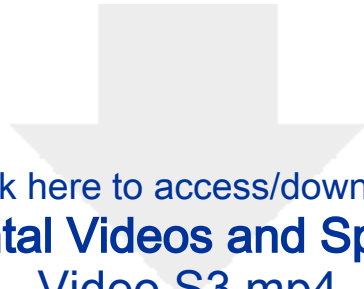




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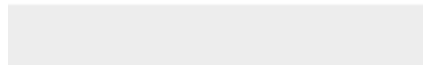
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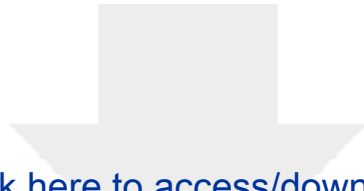




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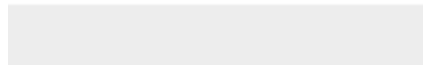




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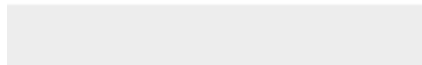
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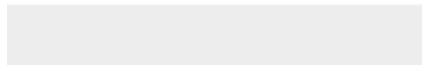
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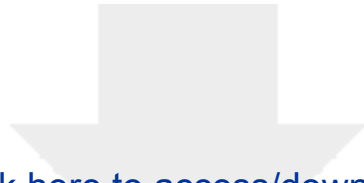




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Inventory of Supplemental Information

- **Valenzuela & Perez_Supplementary Materials** document
 - [Supplementary Figure 1](#): Synthetic sensor of ADAM protease-based trans-endocytosis of ephrin-A1 (Related to Figure 3)
 - [Supplementary Figure 2](#): Trans-endocytosed ephrin-A1 is sorted apart from Rab11-recycling endosomes (Related to Figure 4)
 - [Supplementary Figure 3](#): Activation of EphA receptors at the plasma membrane is necessary for ephrin-A1 trans-endocytosis (Related to Figure 5)
 - [Supplementary Figure 4](#): Trans-endocytosed ephrin-A1 is co-transported with EphA2 and stored for a long time in acidic lysosomes in MDA-MB231 cells (Related to Figure 6)

- **Supplementary movies**
 - [Supplementary Video 1](#) (Related to Figure 1) Partial sorting of EphA2 and ephrin-A1 at the Golgi apparatus
 - [Supplementary Video 2](#) (Related to Figure 1) Ephrin-A1 is exported from filopodia of ligand-producing cells
 - [Supplementary Video 3](#) (Related to Figure 2) Ephrin-A5 is transferred to neighboring cells from filopodia
 - [Supplementary Video 5](#) (Related to Figure 2) Actin-dependent trans-endocytosis of ephrin-A1 from filopodia
 - [Supplementary Video 6](#) (Related to Figure 3) Cleavage-independent trans-endocytosis of ephrin-A1
 - [Supplementary Video 7](#) (Related to Figure 4) Dynamin-dependent trans-endocytosis of ephrin A1
 - [Supplementary Video 8](#) (Related to Figure 5) Localized trans-endocytosis of ephrin-A1/EphA2 complexes
 - [Supplementary Video 9](#) (Related to Figure 5) Trans-endocytosis of ephrin-A1 is massive at steady state
 - [Supplementary Video 10](#) (Related to Figure 7) Inheritance of trans-endocytosed ephrin-A1 in cancer cells