

1 **Contact inhibitory Eph signaling suppresses EGF-promoted cell**  
2 **migration by decoupling EGFR activity from vesicular recycling**

3 Wayne Stallaert<sup>1</sup>, Ola Sabet<sup>1</sup>, Yannick Brüggemann<sup>1,2</sup>, Lisa Baak<sup>1</sup> and Philippe I.H.  
4 Bastiaens<sup>1,2\*</sup>

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6 **Affiliations**

7 <sup>1</sup>Department of Systemic Cell Biology, Max Planck Institute of Molecular Physiology, Otto-  
8 Hahn-Str.11, 44227 Dortmund, Germany

9 <sup>2</sup>Faculty of Chemistry and Chemical Biology, TU Dortmund, Otto-Hahn-Str. 6, 44227  
10 Dortmund, Germany

11 \*Correspondence to: [philippe.bastiaens@mpi-dortmund.mpg.de](mailto:philippe.bastiaens@mpi-dortmund.mpg.de)

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13

14 *Summary:*

15 Eph receptor activation generates context-dependent cellular responses to EGFR  
16 activation by altering its vesicular trafficking dynamics.

17

18

19 ***Abstract***

20 The ability of cells to adapt their behavior to growth factors in relation to their  
21 environment is an essential aspect of tissue development and homeostasis. Here we  
22 show that Eph receptor signaling from cell-cell contacts changes the cellular response to  
23 EGFR activation by altering its vesicular trafficking. Eph receptor activation traps EGFR  
24 in Rab5-positive early endosomes through an inhibition of Akt-dependent vesicular  
25 recycling. By altering the spatial distribution of EGFR activity during EGF stimulation,  
26 Eph receptor activation selectively suppresses migratory Akt signaling from the plasma  
27 membrane, while preserving proliferative ERK signaling from endosomes. We also show  
28 that soluble extracellular signals engaging the G-protein coupled receptor Kiss1  
29 similarly suppress vesicular recycling to alter EGFR signaling. The cellular environment  
30 can thus modulate EGFR vesicular trafficking dynamics to generate context-dependent  
31 responses to EGF stimulation.

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## 35 **Introduction**

36 Activation of epidermal growth factor receptor (EGFR) promotes a variety of cellular  
37 responses including cell growth, proliferation, survival, apoptosis, differentiation and  
38 migration (1), some of which are functionally opposed. To select among these diverse  
39 outcomes, the cell requires additional contextual information. This context can be  
40 intrinsic (e.g. cell type or cell cycle stage), or extrinsic, in the form of extracellular signals  
41 that provide information about the current (or past) environmental context.

42 Adaptability to a changing environment requires that extrinsic information be  
43 integrated through mechanisms that can transform the response to subsequent growth  
44 factor stimulation.

45

46 Local cell density is one such example of extrinsic context that can influence cellular  
47 activity to generate distinct functional states (2-4). The Eph family of receptor tyrosine  
48 kinases act as sensors of cell density, becoming activated at points of cell-cell contact  
49 through interactions with membrane bound ephrin ligands presented on the surfaces of  
50 adjacent cells (5). Eph receptors in many ways operate in functional opposition to EGFR,  
51 acting as tumour suppressors (5-11) and mediating contact inhibition of locomotion to  
52 suppress cellular migration and metastasis (12-15). Moreover, a functional coupling of  
53 EGFR and Eph receptor activity has been shown to control cell migration (15). Although  
54 the precise mechanism through which Eph receptors regulate EGF-promoted migration  
55 remains elusive, a convergence of receptor activity on phosphoinositide 3-kinase  
56 (PI3K)/Akt signaling was implicated.

57

58 Akt has been shown to regulate EGFR vesicular trafficking through the endosomal  
59 system (16). By stimulating the activity of the early endosomal effector PIKfyve (FYVE-

60 containing phosphatidylinositol 3-phosphate 5-kinase), Akt activity controls the  
61 transition of EGFR through early endosomes, regulating both its recycling back to the  
62 PM and its degradation in the lysosome. Thus, while endocytosis of cell surface  
63 receptors has traditionally been viewed as a mechanism to attenuate downstream  
64 signaling following ligand stimulation, the notion that signaling molecules downstream  
65 of cell surface receptors can, in turn, influence vesicular trafficking (16-21) generates a  
66 reciprocal relationship between receptor activation and vesicular dynamics whose role  
67 in shaping the cellular response to stimuli has only recently begun to garner attention  
68 (22). Furthermore, this bidirectional relationship could also allow the signaling activity  
69 of one receptor to influence the response properties of another through changes in its  
70 vesicular trafficking dynamics, generating context-dependent receptor activity.

71

72 In the current work, we show that Eph receptor activation at cell-cell contacts regulates  
73 the vesicular dynamics of EGFR through an inhibition of Akt-dependent trafficking. By  
74 modulating the spatial distribution of EGFR activity, Eph receptor activation alters the  
75 cellular response to EGF stimulation, selectively suppressing EGF-promoted migratory  
76 signaling while preserving its effect on proliferation.

77

## 78 **RESULTS**

### 79 ***Eph receptor activation affects EGFR vesicular trafficking***

80 Stimulation of endogenous Eph receptors in Cos-7 cells with a soluble, clustered  
81 ephrinA1-Fc (A1) ligand (23, 24) induced a reduction in EGFR abundance at the PM (**Fig.**  
82 **1A-B**). This decrease in PM EGFR abundance following Eph receptor activation was  
83 observed in various cell lines, including HEK293, NIH 3T3, MCF10A and MDA-MB-231  
84 cells (**Fig. S1A-D**), exhibiting a wide range of endogenous EGFR expression (**Fig. S1E**).



85 While EGFR internalization is a well-established consequence of growth factor-induced  
86 receptor activation, we observed that the loss of PM EGFR did not result from an Eph  
87 receptor-induced transactivation of EGFR (**Fig S1F**). Since EGFR also continuously  
88 recycles through the endosomal system in the absence of growth factor stimulation (25),  
89 we hypothesized that Eph receptor activation may reduce PM EGFR abundance by  
90 trapping constitutively recycling receptors in endosomes.

91

92 Eph receptor activation decreases the activity of Akt (**Fig. 1C**)(14) , a signaling effector  
93 previously demonstrated to promote EGFR vesicular recycling (16). Direct inhibition of  
94 Akt or its downstream early endosomal effector PIKfyve (16) indeed reduced PM EGFR  
95 abundance in Cos-7 cells (**Fig. 1D-E**). A1 stimulation or PIKfyve inhibition promoted  
96 similar decreases in PM EGFR abundance, as measured by a reduction in PM EGF-  
97 Alexa647 binding (**Fig. 1E**). Furthermore, the combination of Eph receptor activation  
98 with PIKfyve inhibition did not further reduce PM EGFR abundance (**Fig. 1E**), indicative  
99 of a shared molecular mechanism. Consistent with a suppression of constitutive EGFR  
100 recycling, we observed an endosomal accumulation of ectopically expressed EGFR-  
101 mCherry in live cells after Akt or PIKfyve inhibition (**Fig. 1F, Movie S1, Fig. S2A-B**).

102 Time lapse confocal imaging of Cos-7 cells expressing EGFR-mCherry and EphA2-  
103 mCitrine also revealed an endosomal accumulation of EGFR with time following soluble  
104 A1 stimulation (**Fig. 1G-I, Movie S2**) or upon presentation of ephrinA1 ligand on the  
105 membrane of adjacent cells at sites of cell-cell contact (**Movie S3**). This shift in the  
106 spatial distribution of EGFR from the PM to endosomes following A1 stimulation  
107 occurred primarily by trapping receptors in Rab5-positive early endosomes (**Fig. 1J**),  
108 consistent with an inhibition of Akt-dependent trafficking (16) (**Fig. 1J**). Thus, Eph  
109 receptor activation alters the subcellular distribution of EGFR prior to growth factor

110 stimulation by trapping constitutively recycling receptors in Rab5-positive early  
111 endosomes through an inhibition of Akt/PIKfyve-dependent vesicular recycling.  
112  
113 We next investigated how Eph receptor activation influences EGFR trafficking during  
114 EGF stimulation. The trafficking fate of EGFR through the endosomal system is  
115 determined by post-translational modifications, with receptor ubiquitination acting as a  
116 molecular switch that diverts EGFR to the lysosome for degradation (25). Since EGFR  
117 ubiquitination increases with EGF binding (26), saturating EGF concentrations (> 50  
118 ng/ml (27)) generate a finite temporal signaling response by progressively depleting PM  
119 EGFR through ubiquitin-dependent lysosomal degradation. Stimulation of endogenous  
120 receptors in Cos-7 cells with a saturating concentration of EGF (100 ng/ml) induced a  
121 ~40% reduction in total EGFR expression after 60 min of stimulation (**Fig. S2C**) and  
122 residual EGFR resided primarily in Rab7-positive late endosomes (**Fig. 1J**). A1  
123 pretreatment or direct Akt inhibition, in contrast, impaired Rab5-to-Rab7 endosomal  
124 maturation (28) (**Fig. 1J**), leading to a reduction in receptor degradation at saturating  
125 EGF concentrations ( $\geq 50$  ng/ml; **Fig. S2C**).  
126  
127 At lower, subsaturating EGF concentrations typically found in human tissue secretions  
128 (0.4-20 ng/ml)(29), only a fraction of receptors are ligand bound, receptor  
129 ubiquitination is reduced (26), and internalized receptors are preferentially recycled  
130 back to the PM (30). To assess whether Eph receptors inhibit EGFR recycling following  
131 subsaturating EGF stimulation, we pulsed endogenous receptors in Cos-7 cells with 10  
132 ng/ml EGF to induce EGFR endocytosis and measured its subsequent return to the PM  
133 following EGF washout (**Fig. 1K**). While we observed a complete recovery of PM EGFR

134 abundance in control cells following EGF washout, A1 pretreatment completely  
135 suppressed EGFR recycling.

136

137 Thus, Eph receptor activation suppresses EGFR trafficking through the early endosome  
138 during EGF stimulation; impairing the recycling of non-ubiquitinated receptors back to  
139 the PM as well as inhibiting the transition of ubiquitinated receptors to late endosomes.

140

#### 141 ***Eph receptor activation changes the spatial distribution of EGFR activity***

142 Many functional outcomes to EGFR activation, such as cellular migration, require that  
143 cells remain responsive to persistent growth factor stimulation. To ensure sensitivity to  
144 stimuli during long periods of exposure, the cell must maintain sufficient receptor  
145 abundance at the PM despite the continuous internalization of activated receptors. We  
146 therefore posed the following questions: Does Akt-dependent recycling help maintain  
147 cellular responsiveness to EGF during persistent, subsaturating stimulation? Can Eph  
148 receptor activation at cell-cell contacts change the response properties of EGFR by  
149 modulating its vesicular trafficking?

150

151 To address the impact of Akt-dependent recycling on EGFR activation, measurements of  
152 endogenous EGFR phosphorylation and trafficking in Cos-7 cells were obtained by  
153 immunofluorescence following subsaturating EGF stimulation in control cells and  
154 following Eph receptor activation or Akt/PIKfyve inhibition. Individual cells were  
155 radially segmented to quantify changes in the average spatial distribution of EGFR  
156 activity with time and visualized using 3-D spatial-temporal maps (**Fig. 2A**). Through an  
157 accumulation of EGFR in endosomal compartments during sustained EGF stimulation,  
158 cells pretreated with either A1 or an Akt inhibitor generated less EGFR phosphorylation

159 after 60 min of EGF stimulation relative to control cells (**Fig. 2A-B**). Decoupling Akt  
160 activation from its effect on trafficking by PIKfyve inhibition had indistinguishable  
161 effects from direct Akt inhibition or A1 pretreatment on EGFR phosphorylation and  
162 trafficking (**Fig. 2A-B**), indicating that Akt activity maintains EGFR activation at the PM  
163 during sustained, subsaturating EGF stimulation through its effects on vesicular  
164 recycling.

165  
166 To specifically quantify EGFR phosphorylation at the PM and on endosomes during  
167 sustained, subsaturating EGF stimulation, we employed fluorescence lifetime imaging  
168 microscopy (FLIM) to detect Förster resonance energy transfer (FRET) between EGFR-  
169 mCitrine and a phospho-tyrosine binding domain fused to mCherry (PTB-mCherry) (31)  
170 in Cos-7 cells (**Fig. 2C-F**). In control cells, EGFR-mCitrine remained highly  
171 phosphorylated at both the PM and in endosomes following 60 min of sustained EGF-  
172 Alexa647 stimulation. In cells pretreated with A1 or following Akt or PIKfyve inhibition,  
173 we observed reduced PM EGFR-mCitrine density (**Fig. 2D**) and EGF-Alexa647 binding  
174 (**Fig. 2E**), resulting in diminished EGFR-mCitrine phosphorylation specifically at the PM  
175 (**Fig. 2F**). In these conditions in which Akt-dependent recycling was suppressed, ligand-  
176 bound, active EGFR-mCitrine accumulated in endosomes, maintaining its  
177 phosphorylation in this compartment to the same extent as control cells (**Fig. 2C-F**).

178  
179 Eph receptor activation, therefore, by inhibiting Akt-dependent recycling, changes the  
180 spatial distribution of EGFR activity during sustained, subsaturating EGF stimulation,  
181 selectively reducing EGFR activation at the PM while preserving receptor activity in  
182 endosomes.

183

184 ***Eph receptor activation at cell-cell contact alters the EGFR signaling response***

185 Although EGFR continues to activate signaling effectors from endosomal membranes  
186 (32-40), Akt is preferentially activated at the PM (41, 42) (**Fig. S3**). We therefore  
187 investigated how Eph receptor activation, by changing the spatial distribution of EGFR  
188 activity, regulates its signaling response during EGF stimulation. By suppressing  
189 vesicular recycling and reducing EGFR activity at the PM, A1 pretreatment selectively  
190 inhibited Akt activation following subsaturating EGF stimulation of endogenous  
191 receptors in Cos-7 (**Fig. 3A top, Fig. S4A**) and HEK293 cells (**Fig. S4B**), while ERK  
192 activation, which can continue from endosomal membranes (37, 39, 43) (**Fig. S3**),  
193 remained intact (**Fig 3A bottom, Fig. S4**). To confirm that EphA2 inhibits EGF-promoted  
194 Akt activation by suppressing EGFR recycling and does not simply reflect the opposed  
195 regulation of Akt by EGFR and EphA2 (activation vs inhibition, respectively), we  
196 assessed whether EGFR trafficking was dispensable for the A1-induced suppression of  
197 EGF-promoted Akt activation. Cells were prestimulated with A1, followed by treatment  
198 with the dynamin inhibitor dynole 34-2 to block subsequent endocytosis, and then  
199 stimulated with EGF. When EGFR endocytosis was blocked (**Fig. S3C**), A1 pretreatment  
200 had no effect on EGF-promoted Akt activation (**Fig. 3B top**). Pretreatment with the  
201 negative control analogue dynole 31-2, to control for off-target effects, did not inhibit  
202 EGFR endocytosis (**Fig. S3C**), and had no effect on A1-induced suppression of EGF-  
203 promoted Akt activation (**Fig. 3B bottom**), corroborating that intact EGFR vesicular  
204 trafficking is required for the inhibitory effect of Eph receptors on EGFR signaling.

205

206 Increasing concentrations of A1 progressively inhibited EGF-mediated Akt activation  
207 (**Fig. 3C**), suggesting that the degree of cell-cell contact might determine the magnitude  
208 of Akt activation in response to a given concentration of EGF. To directly investigate the

209 influence of cell-cell contact on EGFR signaling, we obtained single cell measurements of  
210 Akt and ERK activation in Cos-7 with varying degrees of cell-cell contact. Homotypic cell-  
211 cell contact promotes Eph receptor activation through interactions with ephrins  
212 presented on neighboring cells (24) (**Fig. 3D**). Akt activation decreased with cell-cell  
213 contact both prior to and following EGF stimulation (**Fig. 3E**), demonstrating that  
214 increasing cell-cell contact reduces the magnitude of EGF-promoted Akt activation. ERK  
215 activation, on the other hand, was unaffected by cell-cell contact, with cells generating  
216 similar EGF-promoted increases in ERK activation irrespective of their degree of cell-cell  
217 contact (**Fig. 3F**).

218

### 219 ***Coupling EGFR activity to vesicular recycling generates a positive feedback***

220 While the inhibition of Akt-dependent recycling results in a reduction in PM EGFR  
221 abundance in Cos-7 cells (**Fig. 1A-K, Fig. S1A-D, Fig. S2A-B**), we also observed that an  
222 increase in cellular Akt activity through the inhibition of its negative regulator PP2A by  
223 okadaic acid resulted in a concomitant increase in PM EGFR (**Fig. 4A**). Since EGFR  
224 activation itself increases Akt activity in cells (**Fig. 3A-C, Fig. 3E, Fig. S3**), we next asked  
225 whether PM EGFR abundance is actively maintained during growth factor stimulation  
226 through an EGF-induced increase in Akt-dependent vesicular recycling. Using a  
227 fluorescence localization after photoactivation (FLAP) approach to quantify the  
228 vesicular trafficking of EGFR to the PM following photoactivation of EGFR-paGFP in  
229 endosomes (**Fig. 4B top**), we observed an increase in EGFR-paGFP recycling during EGF  
230 stimulation (**Fig. 4B bottom**). Akt inhibition completely suppressed this EGF-promoted  
231 increase in vesicular recycling (**Fig. 4B bottom**), further demonstrating the contribution  
232 of Akt-dependent recycling in sustaining PM EGFR activity (**Fig. 3A**). Thus, by

233 stimulating Akt-dependent recycling, EGFR activation generates a positive feedback that  
234 actively maintain its PM abundance during EGF stimulation.

235

236 Positive feedback in combination with inhibitory network motifs can convert graded  
237 inputs into switch-like, ultrasensitive signaling responses (44). Since Akt is  
238 preferentially activated at the PM (**Fig. S4**), the EGF-induced increase in EGFR vesicular  
239 recycling (**Fig. 4B**) could generate a positive feedback for Akt activation (**Fig. 4C**). To  
240 investigate if this positive feedback can generate a switch-like activation of Akt, we  
241 measured Akt phosphorylation in thousands of individual Cos-7 cells by flow cytometry  
242 following sustained stimulation with a range of EGF concentrations (**Fig. 4D-E**). Cells  
243 were stimulated in suspension to negate in situ cell-cell contact as an extrinsic source of  
244 variability in Akt activation (**Fig. 3E**). At concentrations  $\geq 1$  ng/ml, EGF stimulation  
245 produced a switch-like activation to a high Akt phosphorylation state in a subpopulation  
246 of cells, whose proportion increased with EGF concentration (**Fig. 4D-E top**). Decoupling  
247 EGFR activation from its effect on vesicular recycling by PIKfyve inhibition (**Fig. 4D-E**  
248 **middle**) or A1 pretreatment (**Fig. 4D-E bottom**) did not result in a global decrease in  
249 cellular Akt activation but rather reduced the proportion of cells generating a high Akt  
250 phosphorylation state, consistent with the inhibition of a positive feedback that  
251 produces this switch-like response. Intrinsic cell-to-cell variability in the EGF threshold  
252 required to stimulate Akt-dependent vesicular recycling, therefore, determines the  
253 proportion of cells that transition to a high Akt activity state at a given EGF  
254 concentration. Eph receptor activation, by decoupling EGFR activation from its effect on  
255 vesicular trafficking, reduces Akt activation within the population by decreasing the  
256 proportion of cells transitioning to a high Akt activity state during EGF stimulation.

257

258 *Eph activation at cell-cell contact suppresses the EGF-promoted transition to a migratory*  
259 *state*  
260  
261 EGFR signaling to effectors at the PM generates exploratory cellular behaviors (45-52)  
262 that must be maintained to induce a persistent migratory response. Given that contact  
263 inhibitory Eph receptor activation selectively suppresses PM signaling during sustained,  
264 subsaturating EGF stimulation (**Fig. 3A, 4D-E**), we investigated if cell-cell contact  
265 regulates EGF-promoted migration by inhibiting Akt-dependent recycling. Since Cos-7  
266 cells exhibit limited migratory behavior, we examined NIH 3T3 mouse embryonic  
267 fibroblast (MEF) cells, which generate a haptotactic migratory response to fibronectin  
268 that is enhanced by EGF through an increase in exploratory behavior (53). Similar to  
269 Cos-7 cells (**Fig. 1A-B**), these cells also exhibit a Eph-activity dependent depletion of PM  
270 EGFR abundance (**Fig. 5A-B**). Following stimulation with a subsaturating EGF  
271 concentration (20 ng/ml), we observed a significant increase in the proportion of  
272 migratory cells (**Fig. 5C top, Movie S4, Fig. S5**), but no change in the average distance  
273 travelled per cell (**Fig. 5C bottom**). This indicates that EGF promotes the transition of  
274 individual cells to a migratory state rather than increasing overall cellular motility. Since  
275 EGF binding promotes receptor ubiquitination and degradation, sustained stimulation  
276 with supraphysiological saturating EGF concentrations (100 ng/ml) induces a rapid loss  
277 in EGF sensitivity with time and thus did not significantly increase the proportion of  
278 migratory cells (**Fig. 5C top**). Decoupling EGFR activation from its effect on Akt-  
279 dependent recycling through the inhibition of PIKfyve or following Eph receptor  
280 activation decreased the proportion of migratory cells (**Fig. 5C top**). We observed  
281 further that increasing concentrations of A1 progressively decreased EGF-induced  
282 migration (**Fig. 5C top**), consistent with its concentration-dependent effect on EGF-



283 promoted Akt activation (**Fig. 3C**) and suggesting that the amount of ephrinA1-Eph  
284 receptor interactions at points of cell-cell contact may determine whether a cell initiates  
285 a migratory response to EGF. Indeed, we found that the number of migratory cells  
286 following EGF stimulation was inversely proportional to cell density (**Fig. 5D**) and that  
287 the increase in migration observed at low densities could be countered by treatment  
288 with soluble A1 to mimic Eph receptor contact inhibitory signaling (**Fig. 5D**). Thus,  
289 physiological Eph receptor activation at points of homotypic cell-cell contact suppresses  
290 EGF-promoted migration by inhibiting Akt-dependent vesicular recycling. However, by  
291 preserving endosomal ERK activation following EGF stimulation (**Fig. 3A bottom**), we  
292 found that neither PIKfyve inhibition nor A1 pretreatment led to a reduction in EGF-  
293 promoted cell proliferation (**Fig. 5E**). Thus, by altering the spatiotemporal distribution  
294 of EGFR activity, contact inhibitory signaling by Eph receptors influences the cellular  
295 outcome to EGF stimulation, preserving a proliferative response while suppressing cell  
296 migration.

297

298 *Modulation of vesicular dynamics as a general mechanism to produce context-dependent*  
299 *EGFR signaling*

300

301 To determine whether environmental signals other than cell-cell contact can influence  
302 EGFR signaling through changes in its vesicular trafficking, we investigated the effect of  
303 activation of the G protein-coupled receptor Kiss1 (Kiss1R), which, similar to Eph  
304 receptors, inhibits Akt (54) and suppresses cell migration and metastatic invasion (55).  
305 Stimulation with the soluble Kiss1R ligand kisspeptin-10 (Kp-10) reduced Akt activity in  
306 HEK293 cells and decreased PM EGFR abundance (**Fig. 6A**). Similar to the effect of cell-  
307 cell contact, pretreatment with Kp-10 selectively inhibited EGF-promoted Akt activation

308 **(Fig. 6B)**, while preserving ERK activation **(Fig. 6C)**. The modulation of EGFR vesicular  
309 trafficking dynamics could therefore provide a general mechanism to generate plasticity  
310 in the signaling response to EGFR activation, through which diverse environmental  
311 signals such as cell-cell contact or soluble stimuli like Kp-10 can influence the cellular  
312 response to EGF.

313

314

## 315 **DISCUSSION**

316

317 In this paper, we demonstrate that Eph receptor activation at cell-cell contacts can  
318 generate context-dependent cellular responses to EGF stimulation by modulating EGFR  
319 vesicular trafficking dynamics.

320

321 Chemotaxis requires that cells remain responsive to stimuli for prolonged periods of  
322 time as they migrate toward the chemotactic source. Through an increase in Akt-  
323 dependent recycling **(Fig. 4B)**, EGF stimulation maintains EGFR density at the PM during  
324 persistent, subsaturating stimulation. Since Akt itself is preferentially activated at the  
325 PM **(Fig. S3)**, the EGF-promoted increase in vesicular recycling generates a positive  
326 feedback that switches cells to a high Akt activation state **(Fig. 4D-E)**. Although Akt has  
327 been previously observed on endosomal membranes through interactions with the early  
328 endocytic adaptor protein APPL1 (56, 57), de novo activation of Akt by EGFR requires  
329 the production of PI(3,4,5)P<sub>3</sub>, which is impeded by the low abundance of PI(4,5)P<sub>2</sub> in  
330 endosomal membranes (41, 58). Although Akt activation may occur to some extent on  
331 endosomal membranes (59), since the coupling of active EGFR to Akt activation will be  
332 more efficient at the PM, any perturbations that influence the spatial distribution of

333 EGFR, such as Eph receptor activation, will influence the capacity of EGFR to activate Akt  
334 (**Fig. 3A, Fig. 6A-B, Fig. S3**).

335

336 We observed that the switch to a high Akt activity state only occurs in a proportion of  
337 cells, even in the absence of in situ cell-cell contacts, and increases with EGF  
338 concentration (**Fig. 4D-E**). Population heterogeneity in Akt activation has been  
339 previously attributed to cell-to-cell variation in PI3K expression (60). Our data suggest  
340 that intrinsic variability in the expression of signaling and/or trafficking effectors may  
341 determine, for a given cell, the EGF concentration required to stimulate Akt-dependent  
342 trafficking and engage the positive feedback that produces a high Akt activity state.

343 Small differences in EGF concentration substantially influence the proportion of cells  
344 generating a high Akt response (e.g. a shift from 5 to 10 ng/ml increases the proportion  
345 of cells from 43 to 85%, respectively, **Fig. 4D-E**). Perhaps it is not coincidental that the  
346 concentration range over which this switch occurs corresponds to the physiological  
347 range of EGF concentrations (29). By generating a sharp boundary for Akt activation  
348 within the physiological EGF concentration regime, even slight changes in the threshold  
349 of this switch could have profound implications for tissue dynamics (e.g. initiation of  
350 migration).

351

352 Eph receptor activation, for example, by suppressing EGFR recycling, decreased the  
353 proportion of cells generating a high Akt response from 85 to 41% in response to 10  
354 ng/ml EGF (**Fig. 4D-E**). The dependence of Akt activation on EGFR recycling allows the  
355 degree of cell-cell contact to regulate the proportion of cells generating a migratory  
356 response to EGF stimulation. PI3K/Akt signaling has previously been suggested as the  
357 point of convergence for EGFR/Eph control of cell migration (15); however, the

358 molecular mechanism underlying this oppositional relationship remained unclear. Our  
359 results indicate that Eph receptor activation inhibits EGF-promoted cell migration by  
360 suppressing Akt-dependent recycling, thus impeding the spatially-maintained positive  
361 feedback that generates a high Akt response and decreasing the sensitivity of cells to  
362 persistent EGF stimulation necessary to maintain exploratory behavior. However, by  
363 changing the spatial distribution of EGFR activity (**Fig. 2C-D**), Eph receptor activation  
364 selectively suppresses migratory signaling from the PM while leaving proliferative ERK  
365 signaling intact (**Fig. 3A, Fig. 3E-F, Fig. 5C-D**). This contextual plasticity generates two  
366 distinct cellular outcomes to EGF stimulation that may be important in physiological  
367 settings such as wound healing. At the tissue boundary, cells with reduced cell-cell  
368 contact would increase their exploratory behavior in response to EGF released at the  
369 site of the wound. Cells located deeper in the tissue, despite extensive cell-cell contact,  
370 would retain their proliferative response to extracellular EGF, and undergo mitosis to fill  
371 the vacant space created as exploratory cells migrate to occupy the wound area.

372

373 Our observations demonstrate that communication between receptors with opposed  
374 functionality can emerge through changes in vesicular trafficking dynamics rather than  
375 relying on direct interactions between the receptors or their respective effectors. Such a  
376 mechanism also allows different receptors with similar functional roles (e.g. EphA2 and  
377 Kiss1R) to alter the cellular response to stimuli without having to evolve distinct protein  
378 interaction domains to do so. The dependency of EGFR signaling on its vesicular  
379 dynamics could confer a general mechanism through which the cell can generate  
380 functional plasticity to growth factor stimulation while preserving specificity in cell-cell  
381 communication.

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386

387 **Materials and methods**

388

389 *Primary antibodies*

390 Mouse anti-Akt (2920, Cell Signaling Technology (CST), Danvers, MA, USA), mouse anti-  
391 Akt-Alexa488 (2917, CST), rabbit anti-phospho-Akt(Ser<sup>473</sup>) (4060, CST), rabbit anti-  
392 phospho-Akt (Ser<sup>473</sup>)-Alexa647 (4075, CST), mouse anti-HA (9658, Sigma-Aldrich,  
393 St.Louis, MO, USA), rabbit anti-EGFR (4267, CST), goat anti-EGFR (AF231, R&D Systems,  
394 Minneapolis, MN, USA), mouse anti-phospho-EGFR(Tyr<sup>845</sup>) (558381, BD Biosciences,  
395 Heidelberg, Germany), rabbit anti-phospho-EGFR(Tyr<sup>1045</sup>) (2237, CST), mouse anti-  
396 phospho-EGFR(Tyr<sup>1068</sup>) (2236, CST), goat anti-EphA2 (R&D Systems), rabbit anti-  
397 phospho-Eph(Tyr<sup>588/596</sup>) (Abcam, Cambridge, UK), mouse anti-ERK1/2 (4696, CST),  
398 rabbit anti-phospho-ERK(Thr<sup>202</sup>/Tyr<sup>204</sup>) (4370, CST), mouse anti-Rab5 (610724, BD  
399 Biosciences), rabbit anti-Rab7 (9367, CST), rabbit anti-phospho-Rb(Ser807/811, CST)  
400 mouse anti-tubulin (6074, Sigma-Aldrich)

401

402 *Secondary antibodies*

403 IRDye 680RD Donkey anti-Mouse (LI-COR Biosciences), IRDye 680RD Donkey anti-  
404 Rabbit (LI-COR Biosciences), IRDye 680RD Donkey anti-Goat (LI-COR Biosciences),  
405 IRDye 800CW, Donkey anti-Mouse (LI-COR Biosciences), IRDye 800CW Donkey anti-  
406 Rabbit (LI-COR Biosciences), IRDye 800CW Donkey anti-Rabbit (LI-COR Biosciences),  
407 AlexaFluor 405 goat anti-Mouse (Life Technologies), AlexaFluor 488 donkey anti-Goat  
408 (Life Technologies), AlexaFluor 546 donkey anti-rabbit (Life Technologies), AlexaFluor  
409 647 donkey anti-Rabbit (Life Technologies)

410

411 *Plasmids*

412 Generation of EGFR-mCitrine, EGFR-mCherry, EGFR-paGFP, PTB-mCherry, c-Cbl-BFP  
413 and HA-ubiquitin (25), as well as EphA2-mCitrine, SH2-mCherry and LIFEA2 (24) were  
414 previously described. pcDNA3.1-EphA2 was a gift from Tony Pawson.

415

#### 416 *Reagents*

417 AktVIII (sc-3513, Santa Cruz Biotechnology, Dallas, TX, USA), EGF (AF-100-15,  
418 Peptotech, Hamburg, Germany), okadaic acid (sc-3513, Santa Cruz Biotechnology),  
419 YM201636 (13576, Biomol GmbH, Hamburg, Germany), dynole 31-2 (ab120464,  
420 Abcam), dynole 34-2 (ab120463, Abcam), Kisspeptin-10 (445888, Merck Millipore).  
421 EGF-Alexa647 was prepared as previously described (25). EphrinA1-Fc (602-A1-200)  
422 was preclustered by incubating with chicken Anti-Fc (GW200083F, Sigma-Aldrich) at a  
423 ratio of 5:1 at room temperature for at least 30 min.

424

#### 425 *Cell culture*

426 Cos-7, HEK293 and NIH 3T3 cells were grown in Dulbecco's Modified Eagle's Medium  
427 (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1%  
428 non-essential amino acids (NEAA) and maintained at 37°C in 5% CO<sub>2</sub>. MCF10A cells  
429 were grown in DMEM/F12 supplemented with 5% horse serum, 20 ng/ml EGF, 500  
430 ng/ml hydrocortisone, 100 ng/ml cholera toxin and 10 µg/ml insulin and maintained at  
431 37°C in 5% CO<sub>2</sub>. MDA-MB-231 cells were grown in Leibowitz medium supplemented  
432 with 10% FBS and 2mM L-glutamine maintained at 37°C in 0% CO<sub>2</sub>. When required,  
433 transfection of cells was performed using FUGENE6 (Roche Diagnostics, Mannheim,  
434 Germany) or Lipofectamine 2000 (Life Technologies, Darmstadt, Germany) according to  
435 manufacturer's protocol. Approximately 16-18 hours prior to an experiment, cells were  
436 starved in DMEM containing 0.5% FBS, 2 mM L-glutamine and 1% NEAA. One hour

437 before stimulation, starvation media was changed to serum-free DMEM or DMEM  
438 without phenol red for live cell imaging. Unless explicitly stated in the figure legends, all  
439 experiments were performed with Cos-7 cells with endogenous expression of EGFR and  
440 Eph receptors.

441

#### 442 *In-cell westerns (ICW) and on-cell westerns (OCW)*

443 Cells were seeded on black, transparent bottomed 96-well plates (3340, Corning, Hagen,  
444 Germany) coated with poly-L-lysine (P6282, Sigma Aldrich). Cell were fixed with Roti-  
445 Histofix 4% (Carl Roth, Karsruhe, Germany) for 5 min at 37°C. For ICW, cells were  
446 permeabilized with 0.1% Triton X-100 (v/v) for 5 min at room temperature. For OCW,  
447 cells were not permeabilized. Samples were incubated in Odyssey TBS blocking buffer  
448 (LI-COR Biosciences, Lincoln, NE, USA) for 30 min at room temperature. Primary  
449 antibodies were incubated overnight at 4°C and secondary antibodies (IRDyes, LI-COR  
450 Biosciences) were incubated in the dark for 1 h at room temperature. All wash steps  
451 were performed with TBS (pH 7.4). Intensity measurements were made using the  
452 Odyssey Infrared Imaging System (LI-COR Biosciences). ICW/OCW were calibrated by  
453 Western blots to ensure accurate quantification. Quantification of the integrated  
454 intensity in each well was performed using the MicroArray Profile plugin (OptiNav Inc.,  
455 Bellevue, WA, USA) for ImageJ v1.47 (<http://rsbweb.nih.gov/ij/>). In each ICW or OCW,  
456 2-4 replicates per conditions were obtained per experiment, and all data presented  
457 represents means  $\pm$  s.e.m. from at least three independent experiments.

458

#### 459 *Immunofluorescence*

460 Cells were cultured on 4- or 8-well chambered glass slides (Lab-tek, Thermo Fisher  
461 Scientific, Waltham, MA) and fixed with 4% paraformaldehyde/PBS (w/v) for 10 min at



462 4°C. To measure PM EGFR, fixed, non-permeabilized samples were first incubated with  
463 primary antibody directed at an extracellular epitope of EGFR (AF231, R&D Systems,  
464 1:200) overnight at 4°C followed by secondary antibody for 1 h at room temperature.  
465 For all other immunofluorescence experiments, samples were permeabilized with 0.1%  
466 Triton X-100 (v/v) for 5 min at room temperature prior to incubation with primary  
467 antibodies. All wash steps were performed with TBS (pH 7.4). Fixed samples were  
468 imaged in PBS at 37°C. For all analysis, an initial background subtraction was performed  
469 on immunofluorescence images. To quantify the proportion of EGFR in Rab5 and Rab7  
470 compartments, binary masks were generated from intensity thresholded images of Rab5  
471 and Rab7 staining. To generate a mask of Rab5/Rab7 double positive endosomes, the  
472 product of their individual masks was used. The integrated fluorescence intensity of  
473 EGFR-mCherry was determined in each of the endosomal masks and divided by the total  
474 integrated EGFR fluorescence intensity of the cell. All analysis was performed using  
475 ImageJ. A cell segmentor tool was developed in-house in Anaconda Python (Python  
476 Software Foundation, version 2.7, <https://www.python.org/>) to quantify the spatial  
477 distribution of EGFR and pTyr<sup>845</sup>-EGFR in fixed cells. Cells were divided into 6 equally  
478 spaced radial bins emanating from the center of cell mass.

479

#### 480 *Confocal imaging*

481 Routinely, cells were cultured for live cell confocal imaging on 4- or 8-well chambered  
482 glass slides (Lab-tek) and transiently transfected as described above. Confocal images  
483 were recorded using an Olympus Fluoview FV1000 confocal microscope (Olympus Life  
484 Science Europa, Hamburg, Germany) or a Leica SP8 confocal microscope (Leica  
485 Microsystems, Wetzlar, Germany).

486

487 *Olympus Fluoview™ FV1000*

488 The Olympus Fluoview™ FV1000 confocal microscope was equipped with a temperature  
489 controlled CO<sub>2</sub> incubation chamber at 37°C (EMBL, Heidelberg, Germany) and a  
490 60x/1.35 NA Oil UPLSApo objective (Olympus, Hamburg, Germany). EphA2-mCitrine  
491 and EGFR-mCherry were excited using a 488 nm Argon-laser (GLG 3135, Showa  
492 Optronics, Tokyo, Japan) and a 561 nm DPSS laser (85-YCA-020-230, Melles Griot,  
493 Bensheim, Germany), respectively. Detection of fluorescence emission was restricted  
494 with an Acousto-Optical Beam Splitter (AOBS) for mCitrine @ 498-551 nm and mCherry  
495 @ 575-675 nm. In all cases, scanning was performed in frame-by-frame sequential mode  
496 with 2x frame averaging. The pinhole was set to 250 µm.

497

498 *Leica SP8*

499 The Leica TCS SP8 confocal microscope was equipped with an environment-controlled  
500 chamber (LIFE IMAGING SERVICES, Switzerland) maintained at 37°C and a HC PL APO  
501 CS2 1.4 NA oil objective (Leica Microsystems, Wetzlar, Germany). Alexa488-conjugated  
502 secondary antibodies, fluorescent fusion proteins containing mCitrine and mCherry, and  
503 EGF-Alexa647 were excited using a 470–670 nm white light laser (white light laser Kit  
504 WLL2, NKT Photonics, Denmark) at 488, 514 and 561 and 647 nm, respectively. PH-Akt-  
505 Cerulean was excited using an Argon Laser at 458 nm. Detection of fluorescence  
506 emission was restricted with an AOBS as follows: Cerulean @ 468-505 nm, Alexa488 @  
507 498-551 nm, mCitrine @ 525-570 nm, mCherry @ 570-650 nm and Alexa647 @ 654-  
508 754 nm. The pinhole was set to 250 µm and 12-bit images of 512x512 pixels were  
509 acquired in a frame-by-frame sequential mode.

510

511 *Analysis of time-lapse confocal imaging*

512

513 All analysis of live cell imaging data required an initial background subtraction for all  
514 images obtained.

515

516 To quantify the proportion of endosomal EGFR-mCherry or EphA2-mCitrine, binary  
517 masks of endosomes were generated from intensity thresholded images. The integrated  
518 fluorescence intensity of EGFR-mCherry and EphA2-mCitrine was determined in their  
519 corresponding endosomal masks and divided by the total integrated fluorescence  
520 intensity of the cell.

521

522 Fluorescence localization after photoactivation (FLAP) experiments were carried out at  
523 37°C on a Leica SP8. EGFR-mCherry was co-expressed to identify and select regions of  
524 endosomal EGFR for photoactivation. Background intensity of EGFR-paGFP prior to  
525 photoactivation was measured and subtracted from post-activation images.

526 Photoactivation of EGFR-paGFP was performed with the 405nm laser at 90% power.

527 Following photoactivation, fluorescence images of EGFR-paGFP were acquired every  
528 minute for a total of 15 minutes. PM EGFR-paGFP fluorescence was quantified as the  
529 integrated intensity in a 5-pixel ring of the cell periphery and, after subtracting pre-  
530 activation background intensity, was calculated as a proportion of total EGFR-paGFP  
531 intensity.

532

533 *Fluorescence lifetime imaging microscopy (FLIM)*

534 EGFR-mCitrine, PTB-mCherry and HA-c-Cbl-BFP were ectopically expressed in Cos-7  
535 cells. Fluorescence lifetime measurements of EGFR-mCitrine were performed at 37°C on  
536 a Leica SP8 equipped with a time-correlated single-photon counting module (LSM

537 Upgrade Kit, Picoquant, Berlin, Germany) using a 63x/1.4 NA oil objective. EGFR-  
538 mCitrine was excited using a pulsed WLL at a frequency of 20 MHz and fluorescence  
539 emission was restricted with an AOBS to 525-570 nm. Photons were integrated for a  
540 total of ~ 2 min per image using the SymPhoTime software V5.13 (Picoquant, Berlin,  
541 Germany). Data analysis was performed using custom software in Anaconda Python  
542 based on global analysis as described in (61).

543

544 Fluorescence lifetime measurements of LIFEA2 were performed and analyzed as  
545 previously described (24).

546

#### 547 *Flow cytometry*

548 Cells were detached using accutase, centrifuged at 200g for 5 min and resuspended in  
549 serum-free DMEM. Cells were fixed with 5% sucrose/Roti-Histofix (w/v) for 15 min at  
550 37°C. Ice-cold methanol was added to 90% (v/v) for 30 min on ice. Cells were rinsed  
551 once with 0.5% BSA/TBS (w/v) and incubated with Odyssey TBS blocking buffer (LI-  
552 COR Biosciences) for 30 min at room temperature. Anti-phospho-Akt(Ser<sup>473</sup>)-Alexa647  
553 (4075, Cell Signaling Technology) was added directly to blocking buffer and incubated  
554 overnight at 4°C. Anti-Akt-Alexa488 (2917, Cell Signaling Technology) was added for 2 h  
555 prior to measurement. Samples were analyzed using the LSRII flow cytometer (BD  
556 Biosciences). Alexa488 was excited with a 488nm laser and fluorescence emission was  
557 collected using a 505 nm LP dichroic and a 530/30 nm filter. Alexa647 was excited with  
558 633 nm lasers and fluorescence emission was collected using a 670/40 nm filter.  
559 Samples were analyzed using FlowJo v10 (FlowJo, LLC, Ashland, OR, USA) to obtain  
560 single cell intensity measurements of phospho- and total Akt. Population distributions of

561 log(phospho/total Akt) were fitted with a single Gaussian or a sum of two Gaussian  
562 distributions using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

563

#### 564 *Cell migration*

565 NIH 3T3 cells were seeded onto fibronectin-coated (F0895, Sigma, 1.25 ug/cm<sup>2</sup>) 12 well  
566 culture dishes (83.3921, Sarstedt, Nuembrecht, Germany) containing 2-well Culture-  
567 Inserts (80209, ibidi) to create a cell-free area. Immediately before stimulation, inserts  
568 were removed and cells were incubated with Hoechst to label nuclei. Wide field images  
569 were acquired using an Olympus IX81 inverted microscope (Olympus, Hamburg,  
570 Germany) equipped with a MT20 illumination system, a 4x/0.16 NA air objective and an  
571 Orca CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). Transmission and  
572 fluorescence images were acquired every 10 min for 16 h. The cell-free area created by  
573 the Culture-Insert was cropped using ImageJ and defined as the migration region.  
574 Individual cells were detected and tracked by their nuclear Hoechst staining as they  
575 travelled within the migration region using the TrackMate ImageJ plugin (62), and the  
576 total distance of each track was quantified.

577

#### 578 *Immunoprecipitation (IP) and western blotting*

579 Cells were lysed in TGH (150 mM NaCl, 2 mM EGTA/EDTA, 50 mM HEPES (pH 7.4), 1%  
580 Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM N-  
581 ethylmaleimide(NEM)) or RIPA (for immunoprecipitation; 50 mM Tris-HCl (pH 7.5),  
582 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate,  
583 0.2% SDS, 2.5 mM sodium pyrophosphate and 10 mM NEM), supplemented with  
584 Complete Mini EDTA-free protease inhibitor (Roche Applied Science, Heidelberg,  
585 Germany) and 100 µl phosphatase inhibitor cocktail 2 and 3 (P5726 and P0044, Sigma

586 Aldrich). Lysates were sonicated prior to centrifugation at 14 000 rpm for 10 min at 4°C  
587 to separate non-soluble material. For immunoprecipitation, cell lysates were incubated  
588 with 50 µl washed Protein G magnetic beads (10003D, Life Technologies) for 1 h at 4°C  
589 to pre-clear the samples from unspecific binding proteins. Supernatants were incubated  
590 with primary antibody alone for 2 h followed by the addition and overnight incubation  
591 with Protein G magnetic beads at 4°C with agitation. SDS-PAGE was performed using the  
592 X-cell II mini electrophoresis apparatus (Life Technologies) according to the  
593 manufacturer's instructions. Samples were transferred to preactivated PVDF  
594 membranes (Merck Millipore, Billerica, MA) and incubated with the respective primary  
595 antibodies at 4°C overnight. Detection was performed using species-specific secondary  
596 IR-Dye secondary antibodies (LI-COR Biosciences) and the Odyssey Infrared Imaging  
597 System (LI-COR Biosciences). The integrated intensity of protein bands of interest was  
598 measured using the ImageJ software and signals were normalised by dividing the  
599 intensities of phosphorylated protein by total protein intensities or by dividing  
600 intensities of co-immunoprecipitated proteins by the corresponding  
601 immunoprecipitated protein.  
602

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778

779 **Figure Legends:**

780 **Figure 1: Eph receptor activation affects Akt-dependent EGFR trafficking. (A,B)**

781 Representative immunofluorescence images (A) and quantification (B) of endogenous  
782 PM EGFR in Cos-7 cells ( $N = 16-29$  cells/condition) following stimulation with ephrinA1-  
783 Fc (A1, 2  $\mu\text{g/ml}$ ) for the indicated times (means  $\pm$  sd). **(C)** Quantification of Akt  
784 activation by In-cell Western (ICW) in Cos-7 cells following A1 stimulation (left: 2  
785  $\mu\text{g/ml}$ ; right: 15 min) (means  $\pm$  s.e.m.). **(D)** Quantification of endogenous PM EGFR  
786 abundance by On-cell Western (OCW) and Akt activation in Cos-7 cells following  
787 treatment with the Akt inhibitor AktVIII (10  $\mu\text{M}$ ) for the times indicated (means  $\pm$   
788 s.e.m.). **(E)** EGF-Alexa647 binding (200 ng/ml, 2 min) to endogenous EGFR in Cos-7 cells  
789 as a measure of PM EGFR abundance following 1 h pretreatment with A1 (2  $\mu\text{g/ml}$ ), the  
790 PIKfyve inhibitor YM201636 (YM, 200 nM) or both ( $N=33-59$  cells/condition) (means  $\pm$   
791 sd). **(F)** Representative confocal images of Cos-7 cells expressing EGFR-mCherry before  
792 (top left) and after (top right) treatment with AktVIII (10  $\mu\text{M}$ , 1 h) and quantification of  
793 the increase in endosomal EGFR-mCherry during AktVIII treatment (bottom,  $N = 6$  cells,  
794 means  $\pm$  sd). **(G)** Representative time-lapse confocal images of Cos-7 cells expressing  
795 EGFR-mCherry and EphA2-mCitrine following A1 stimulation (2  $\mu\text{g/ml}$ ). **(H)**  
796 Quantification of endosomal EGFR-mCherry and EphA2-mCitrine from time-lapse  
797 confocal imaging (G) during A1 stimulation ( $N = 7$  cells, means  $\pm$  sd). **(I)** Quantification  
798 of PM EGFR-mCherry and EphA2-mCitrine abundance by OCW during A1 stimulation (2  
799  $\mu\text{g/ml}$ ) (means  $\pm$  s.e.m.). **(J)** Immunofluorescence measurements of EGFR intensity in  
800 Rab5-, Rab5/Rab7- and Rab7-positive endosomal compartments in control, A1-(2  
801  $\mu\text{g/ml}$ , 1 h) and AktVIII-(10  $\mu\text{M}$ , 1 h) pretreated Cos-7 cells prior to (left) and following  
802 EGF stimulation (right, 100 ng/ml, 1 h). ( $N=6-11$  cells/condition). Data are represented  
803 by Tukey boxplots with the mean denoted as a cross and the median as a line. **(K)**

804 Measurements of EGFR recycling in control and A1-pretreated cells (2  $\mu\text{g}/\text{ml}$ , 1 h) by  
805 immunofluorescence prior to (pre), after EGF stimulation (10 ng/ml, 15 min) and 15  
806 min following EGF washout ( $N = 34\text{-}40$  cells/condition, means  $\pm$  sd). Statistical  
807 significance was determined in B, E, J and K using a one-way ANOVA with Sidak's *post-*  
808 *hoc* test (\*\*\*,  $p < 0.001$ ). Scale bars = 20  $\mu\text{m}$ .

809

810

811 **Figure 2: Eph receptor activation changes the spatial distribution of EGFR activity.**

812 **(A-B)** Average spatial-temporal maps (A) of endogenous EGFR abundance (top) and  
813 Tyr<sup>845</sup> phosphorylation (bottom) in radially segmented Cos-7 cells (plasma membrane,  
814 PM  $\rightarrow$  nuclear membrane, NM) prior to and during EGF stimulation (20 ng/ml for 5, 30  
815 and 60 min) in control, ephrinA1-Fc- (A1, 2  $\mu\text{g}/\text{ml}$ , 1 h), AktVIII- (10  $\mu\text{M}$ , 1 h) and  
816 YM201636- (YM, 200 nM, 1h) pretreated cells ( $N = 50\text{-}90$  cells per condition). Single cell  
817 measurements of EGFR pTyr<sup>845</sup> phosphorylation in the PM segment (B) during EGF  
818 stimulation (means  $\pm$  sd) **(C)** Phosphorylated fraction of EGFR-mCitrine as detected by  
819 FLIM-FRET ( $\alpha$ ) and representative images of EGFR-mCitrine and EGF-Alexa647  
820 fluorescence in control and A1- (2  $\mu\text{g}/\text{ml}$ , 1 h), AktVIII- (10  $\mu\text{M}$ , 1 h) and YM- (200 nM,  
821 1h) pretreated Cos-7 cells following 60 min of EGF-Alexa647 stimulation (20 ng/ml) ( $N$   
822 = 10-14 cells/condition). **(D-F)** Quantification of the PM:endosome ratio of (D) EGFR-  
823 mCitrine and (E) EGF-Alexa647 fluorescence intensity and **(F)** phosphorylated fraction  
824 of EGFR-mCitrine ( $\alpha$ ) at PM and endosomes (means  $\pm$  sd). Statistical significance was  
825 determined in B and D-F using a one-way ANOVA with Sidak's *post-hoc* test (\*\*\*,  $p <$   
826 0.001; \*\*,  $p < 0.01$ ; \*,  $p < 0.05$  ). Scale bars = 20  $\mu\text{m}$ .

827

828

829 **Figure 3: Eph receptor activation at cell-cell contact alters the EGFR signaling**  
830 **response. (A)** Quantification of Akt (top) and ERK (bottom) activation by ICW in control  
831 and ephrinA1-Fc-pretreated (A1, 2  $\mu$ g/ml, 1 h) Cos-7 cells endogenously expressing the  
832 receptors following EGF stimulation (1 ng/ml) (means  $\pm$  s.e.m.) **(B)** Quantification of Akt  
833 activation in control or A1-pretreated (2  $\mu$ g/ml, 1 h) HEK293 cells, followed by 30 min  
834 treatment with the dynamin inhibitor dynole 34-2 (100  $\mu$ M, top) or its negative control  
835 analog dynole 31-2 (100  $\mu$ M, bottom), then stimulated with EGF (1 ng/ml) for the times  
836 indicated (means  $\pm$  s.e.m.) **(C)** Quantification of EGF-promoted Akt activation in Cos-7  
837 cells following pretreatment with increasing concentrations of A1 (0.02, 0.2 and 2  
838  $\mu$ g/ml, 1 h) (means  $\pm$  s.e.m.) **(D)** Representative images of EphA2 activity using a FRET-  
839 based sensor (LIFEA2(24)), whereby a decrease in fluorescence lifetime ( $\tau$ , ns)  
840 represents an increase in activity, and fluorescence intensity measurements of LIFEA2-  
841 mCitrine and SH2-mCherry in Cos-7 cells. Scale bar = 20  $\mu$ m. **(E,F)** Single cell  
842 measurements of Akt (E) and ERK (F) activation versus cell-cell contact in 2-D cultures  
843 (% cell circumference) in unstimulated and EGF stimulated (20 ng/ml, 1 h) Cos-7 cells. A  
844 sum-of-squares F test was used to determine significance: Akt, unstimulated:  $F = 16.0$ ,  $p$   
845 = 0.001,  $r^2 = 0.432$ ; Akt, EGF:  $F = 21.4$ ,  $p < 0.001$ ,  $r^2 = 0.322$ ; ERK, unstimulated:  $F =$   
846 0.180,  $p = 0.673$ ,  $r^2 = 0.003$ ; ERK, EGF:  $F = 0.321$ ,  $p = 0.575$ ,  $r^2 = 0.009$ .

847

848 **Figure 4: Coupling EGFR activity to vesicular recycling generates a positive**  
849 **feedback. (A)** Quantification of endogenous PM EGFR abundance and Akt activation in  
850 Cos-7 cells by OCW and ICW, respectively, following treatment with the PP2A inhibitor  
851 okadaic acid (OA, 1  $\mu$ M, 2 h) (means  $\pm$  s.e.m.) **(B)** Representative images and  
852 quantification of EGFR-paGFP recycling to the PM following endosomal photoactivation  
853 in Cos-7 cells (top) in control, EGF (20 ng/ml, 15 min), AktVIII (10  $\mu$ M, 1 h) and AktVIII-



854 pretreated, EGF-stimulated (bottom,  $N = 6-10$  cells/condition) (means  $\pm$  s.e.m.) Scale bar  
855 = 20  $\mu\text{m}$  **(C)** Spatial network topology showing positive feedback generated by coupling  
856 PM EGFR activity and Akt-dependent vesicular recycling. PIKfyve inhibition by  
857 YM201636 (YM) decouples Akt activation from its effect on EGFR recycling. **(D-E)** Single  
858 cell measurements of Akt phosphorylation by flow cytometry in control (top), YM- (200  
859 nM, 1h, middle) and ephrinA1-Fc- (A1, 2  $\mu\text{g}/\text{ml}$ , 1h, bottom) pretreated Cos-7 cells  
860 endogenously expressing the receptors following stimulation with EGF concentrations  
861 indicated (ng/ml, 1 h). (D) Solid lines represent the sum of two Gaussian fits for data  
862 accumulated from at least 10 000 cells per condition in 3-4 independent experiments.  
863 (E) Quantification of Akt activation for increasing concentrations of EGF. Circle sizes  
864 represent the relative proportions of the low (gray) and high (red) Akt activity  
865 populations estimated from the Gaussian distributions derived for each EGF  
866 concentration shown in D (means  $\pm$  s.e.m.).

867  
868

869 **Figure 5: Eph activation at cell-cell contact suppresses the EGF-promoted**  
870 **transition to a migratory state. (A)** Representative immunofluorescence images and  
871 **(B)** quantification of endogenous PM EGFR abundance in NIH 3T3 cells following  
872 ephrinA1-Fc (A1, 2  $\mu\text{g}/\text{ml}$ ) stimulation (means  $\pm$  sd). Statistical significance was  
873 determined using a one-way ANOVA with Sidak's *post-hoc* test (\*\*\*,  $p < 0.001$ ; \*\*,  $p <$   
874 0.01). **(C)** Percent of NIH 3T3 cells initiating a migratory response (top, means  $\pm$  s.e.m)  
875 and the distance travelled by migratory cells (bottom, means  $\pm$  s.d.) following EGF  
876 stimulation. Cells were pretreated with vehicle (control), YM201636 (YM, 200 nM, 1 h),  
877 or ephrinA1-Fc (A1, 2  $\mu\text{g}/\text{ml}$ , 1h, green, or 0.02-2  $\mu\text{g}/\text{ml}$  as indicated, red) followed by  
878 EGF stimulation (0-100 ng/ml as indicated) for 16 h. **(D)** Percent of migrating NIH 3T3  
879 cells when seeded at low or high density in a single well following pretreatment with



880 vehicle or A1 (2  $\mu$ g/ml, 1 h) and stimulated with EGF (20 ng/ml) for 16 h (means  $\pm$   
881 s.e.m). Data in C and D were obtained from least three independent experiments,  
882 consisting of at least two replicates per experiment ( $N = 581$ – $1483$  cells/condition) and  
883 statistical significance was determined using an ordinary one-way ANOVA with Holm-  
884 Sidak's multiple corrections *post-hoc* test. **(E)** Quantification of retinoblastoma (Rb)  
885 phosphorylation by ICW for vehicle- (control), A1- (2  $\mu$ g/ml, 1 h) and YM- (200 nM, 1 h)  
886 pretreated NIH 3T3 cells following 24 h EGF stimulation at the concentrations indicated  
887 (means  $\pm$  s.e.m).

888  
889

890 **Figure 6: Modulation of vesicular dynamics as a general mechanism to produce**  
891 **context-dependent EGFR signaling. (A)** Quantification of Akt activation and PM EGFR  
892 abundance in HEK293 cells by ICW and OCW, respectively, following stimulation with  
893 kisspeptin-10 (Kp-10, 100 nM) (means  $\pm$  s.e.m). **(B,C)** Quantification of Akt and ERK  
894 activation by ICW in HEK293 cells for control and Kp-10-pretreated (100 nM, 1 h) cells  
895 following EGF stimulation (1 ng/ml) (means  $\pm$  s.e.m).

896

897 **Supplementary Materials**

898 Figure S1: Eph receptor activation reduces PM EGFR abundance.

899 Figure S2: Akt/PIKfyve regulates EGFR vesicular trafficking.

900 Figure S3. Akt is preferentially activated at the PM following EGFR stimulation.

901 Figure S4. EphrinA1-Fc pretreatment inhibits EGFR-promoted Akt activation.

902 Figure S5: Distributions of cell migration distances.

903 Movie S1: Akt inhibition induces EGFR endosomal accumulation.

904 Movie S2: EphrinA1-Fc stimulation induces EGFR endosomal accumulation.

905 Movie S3: EphrinA1-Fc:EphA2 interactions at cell-cell contact induces EGFR endosomal

906 accumulation.

907 Movie S4: EGF-promoted migration in NIH 3T3 cells.

908

909 **Supplementary Material Legends:**

910 **Figure S1: Eph receptor activation reduces PM EGFR abundance. (A-D)**

911 Quantification of endogenous PM EGFR abundance by ICW following ephrinA1-Fc

912 stimulation (A1, 2  $\mu\text{g}/\text{ml}$ ) of (A) HEK293, (B) NIH 3T3, (C) MCF10A and (D) MDA-MB-

913 231 cells. (means  $\pm$  s.e.m) (E) Single cell immunofluorescence measurements of EGFR

914 expression in the cell lines used in this study. (F-G) Cos-7 lysates immunoprecipitated

915 (IP) with anti-EGFR (left) or blotted for total proteins (right) following stimulation with

916 EGF (100 ng/ml) or A1 (2  $\mu\text{g}/\text{ml}$ ) for the indicated times. IP was probed with anti-HA (to

917 detect co-transfected HA-ubiquitin), anti-pTyr<sup>845</sup>, anti-pTyr<sup>1068</sup>, anti-pTyr<sup>1045</sup> (to detect

918 phosphorylated EGFR) and anti-EGFR. Total lysates were probed with anti phospho-Eph

919 (pEph), anti-EphA2, anti-pTyr<sup>1068</sup>, anti-EGFR and anti-tubulin. Shown are (F)

920 representative blots and (G) quantification of EGFR phosphorylation (pTyr<sup>845</sup>) from four

921 independent experiments (means  $\pm$  s.e.m). Statistical significance was determined using  
922 a one-way ANOVA with Sidak's *post-hoc* test (\*\*,  $p < 0.01$ ).

923

924 **Figure S2: Akt/PIKfyve regulates EGFR vesicular trafficking.** (A) Representative  
925 images and (B) quantification of endosomal EGFR-mCherry following treatment with the  
926 PIKfyve inhibitor YM201636 (YM, 200 nM, 1h) ( $N = 9$ , mean  $\pm$  s.e.m). Statistical  
927 significance was determined using a two-tailed Student's t test. Scale bar = 20  $\mu$ m. (C)  
928 ICW measurements of total EGFR abundance following EGF stimulation (1-200 ng/ml,  
929 30 min) in vehicle (control) and AktVIII (10  $\mu$ M, 1 h) pretreated cells (means  $\pm$  s.e.m).  
930 Statistical significance was determined using a two-way ANOVA with Sidak's multiple  
931 corrections *post-hoc* test (\*\*\*,  $p > 0.001$ ; \*\*,  $p > 0.01$ ).

932

933 **Figure S3. Akt is preferentially activated at the PM following EGFR stimulation.** (A)  
934 Akt rapidly translocates to regions of EGF-bound receptors at the PM, but is not  
935 recruited to endosomes following internalization of ligand-bound receptor. Shown are  
936 representative images of Cos-7 cells expressing EGFR-mCitrine and PH-Akt-Cerulean  
937 following stimulation with EGF-Alexa647 (100 ng/ml) for the indicated times. (B)  
938 Colocalization between EGF-Alexa647 and PH-Akt-Cerulean is highest immediately after  
939 stimulation (2 min) while most EGFR-mCitrine is localized at the plasma membrane. As  
940 EGF-Alexa647 accumulates in endosomes (20 and 40 min), colocalization with PH-Akt-  
941 Cerulean decreased. Quantification of colocalization by Mander's coefficient from EGF-  
942 Alexa647 intensity-dependent thresholded images (means  $\pm$  sd,  $N = 4$ ). (C) Inhibition of  
943 endocytosis increases EGF-promoted Akt activation, while ERK activation is decreased.  
944 PM EGFR abundance was quantified by OCW and Akt/ERK activation by ICW in HEK293  
945 cells stimulated with EGF (1 ng/ml) for the indicated times following pretreatment with

946 the dynamin inhibitor dynole 34-2 (100  $\mu$ M, 30 min) or its negative control analog  
947 dynole 31-2 (100  $\mu$ M, 30 min) (means  $\pm$  s.e.m). (D) Akt activation is rapidly terminated  
948 following EGF washout in Cos-7 cells, indicating the necessity of PM EGF binding for  
949 persistent Akt activation. ERK activation decays much slower following EGF removal  
950 through due to persistent activity of endosomal EGFR. Akt and ERK activation were  
951 measured by ICW following sustained EGF (1 ng/ml) stimulation or a 5 min EGF pulse  
952 and subsequent washout (means  $\pm$  s.e.m). Statistical significance was determined in B  
953 using a repeated measures one-way ANOVA with a Dunnett's *post-hoc* test, and in C  
954 using a two-way ANOVA with Sidak's *post-hoc* test. (\*\*\*,  $p < 0.001$ ; \*\*,  $p < 0.01$ ; \*,  $p <$   
955 0.05). Scale bars = 20  $\mu$ m

956  
957

958 **Figure S4. EphrinA1-Fc pretreatment inhibits EGFR-promoted Akt activation.** Akt  
959 and ERK activation were quantified by ICW in (A) Cos-7 cells ectopically expressing  
960 EGFR and EphA2, and in (B) HEK293 cells endogenously expressing the receptors. Cells  
961 were pretreated with vehicle (control) or ephrinA1-Fc (A1, 2  $\mu$ g/ml) for 1 h, followed by  
962 1 ng/ml EGF stimulation for the indicated times. Data represent means  $\pm$  s.e.m.

963

964 **Figure S5: Distributions of cell migration distances.** Migration distance was  
965 quantified for individual cells as described in *Methods*. Shown are the distributions of  
966 migration distance for (A) all tracked cells ( $N = 32\ 203$  cells) and (B) 20 ng/ml EGF  
967 treated cells ( $N = 2343$  cells). To distinguish migrating cells from those that are pushed  
968 into the cell-free area by population expansion due to cell division during the 16 h  
969 acquisition time, a minimal displacement distance of 0.01 cm was used as a threshold.

970

971 **Movie S1: Akt inhibition induces EGFR endosomal accumulation.** Treatment of Cos-  
972 7 cells ectotopically expressing EGFR-mCherry with the Akt inhibitor AktVIII (10  $\mu$ M)  
973 promotes an increase in endosomal EGFR. Scale bar = 20  $\mu$ m

974

975 **Movie S2: EphrinA1-Fc stimulation induces EGFR endosomal accumulation.**

976 Stimulation of Cos-7 cells ectotopically expressing EGFR-mCherry and EphA2-mCitrine  
977 with EphrinA1-Fc (2  $\mu$ g/ml) promotes an increase in endosomal EGFR. Scale bar = 20  
978  $\mu$ m

979

980 **Movie S3: EphrinA1-Fc:EphA2 interactions at cell-cell contact induces EGFR**

981 **endosomal accumulation.** HEK293 cells ectopically expressing EBFP-EphrinA1 in  
982 suspension were added to adherent Cos-7 cells ectopically expressing EGFR-mCherry  
983 and EphA2-mCitrine 30 min prior to imaging. Time lapse imaging begins as EBFP-  
984 EphrinA1-HEK293T cells make initial cell-cell contact with Cos-7 cells. Scale bar = 20  $\mu$ m

985

986 **Movie S4: EGF-promoted migration in NIH 3T3 cells.** Unstimulated (unstim), EGF (20

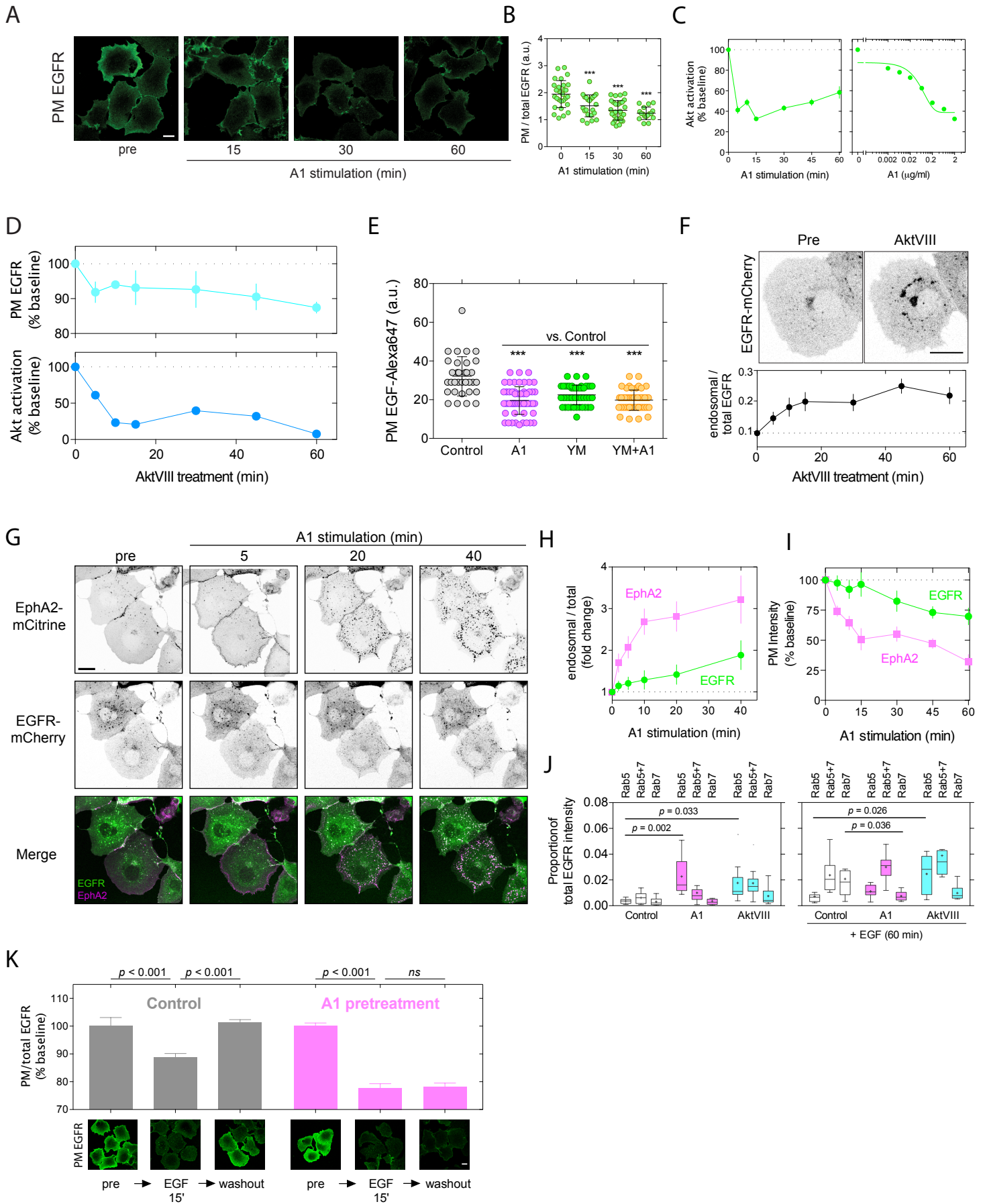
987 ng/ml), YM201636 (200 nM) followed by EGF (20 ng/ml) stimulation (YM) and

988 EphrinA1-Fc (2  $\mu$ g/ml) followed by EGF (20 ng/ml) stimulation (A1). Scale bar = 100

989  $\mu$ m

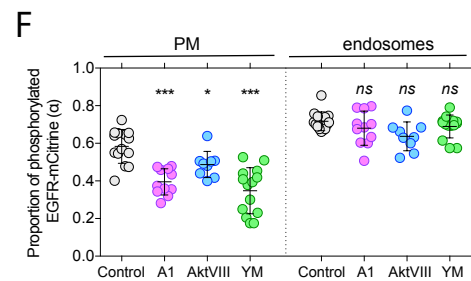
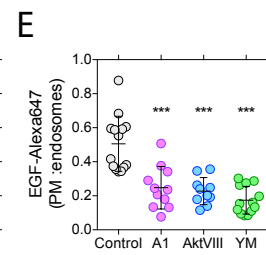
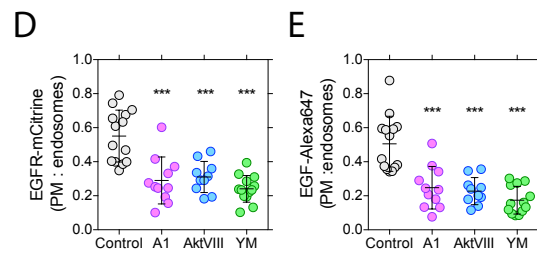
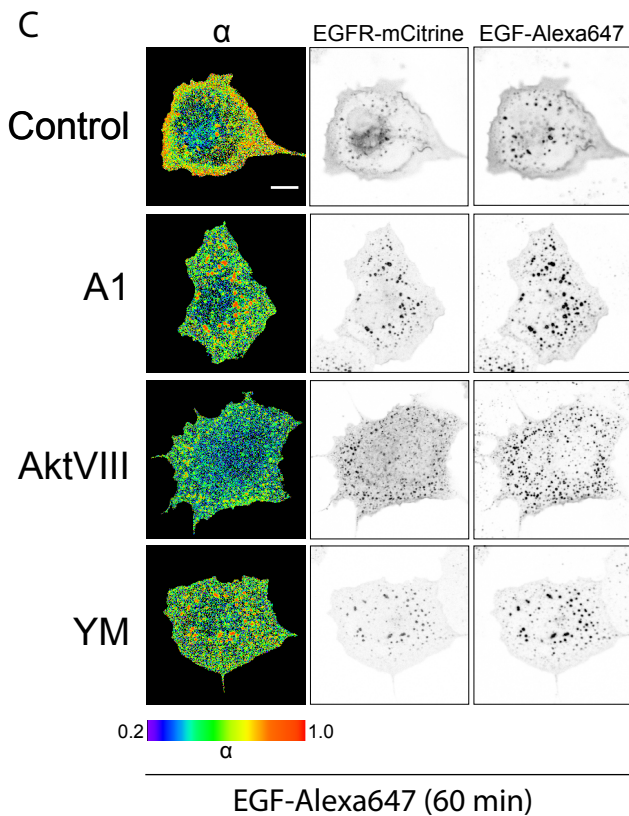
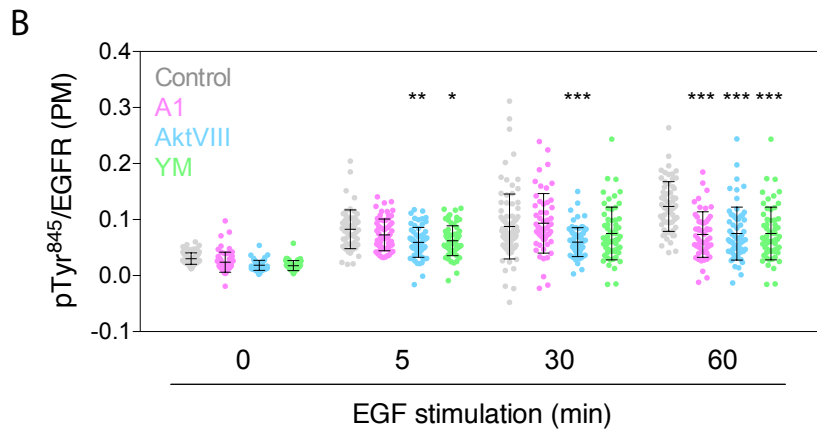
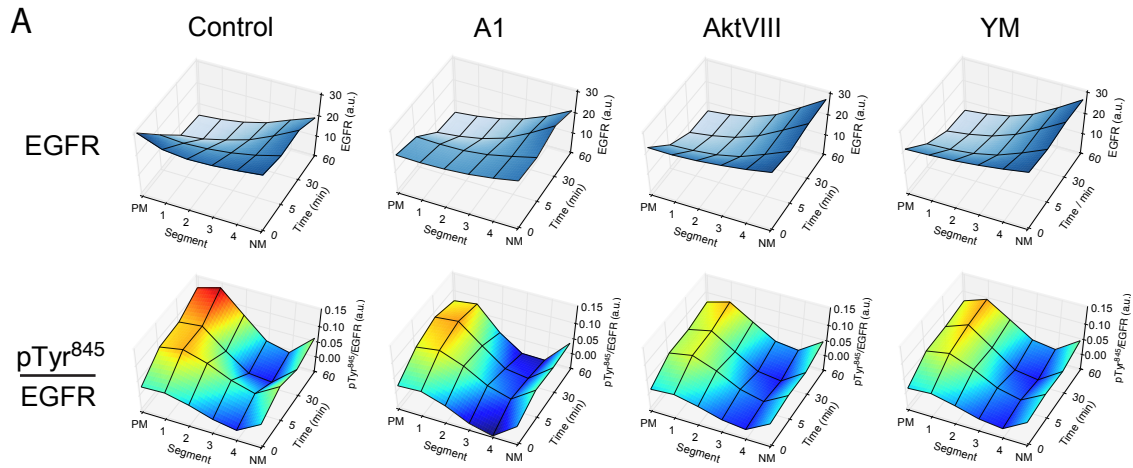
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# Figure 1

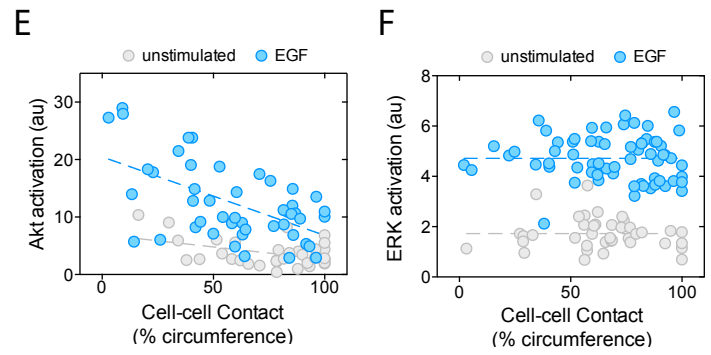
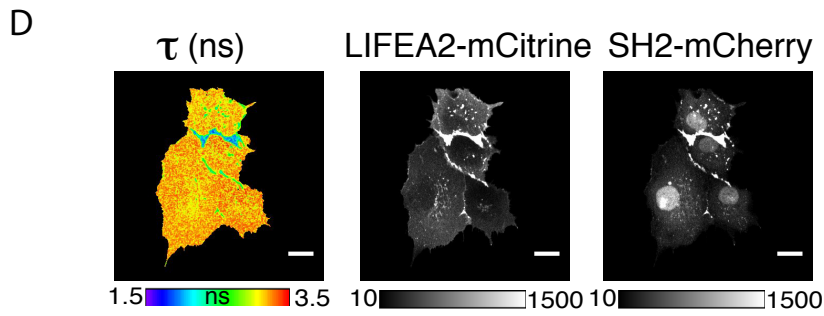
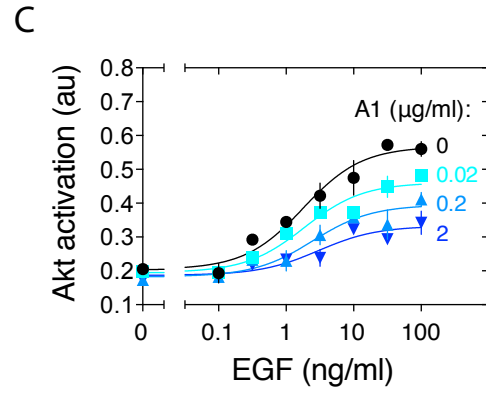
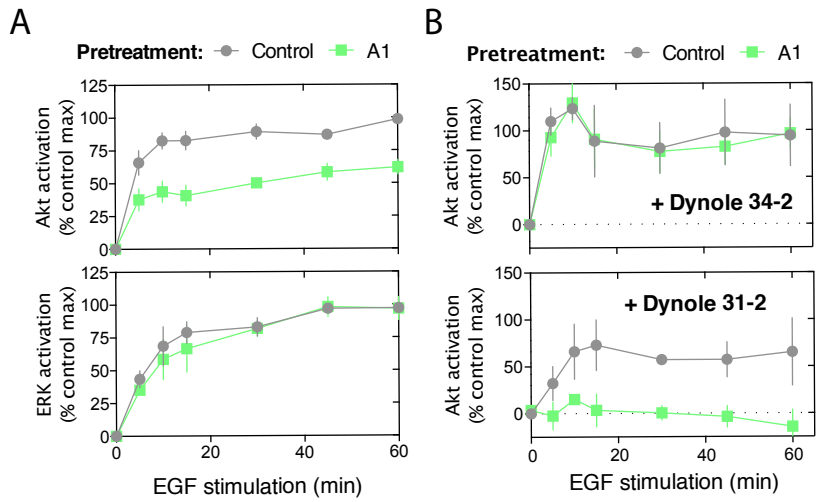




**Figure 2**

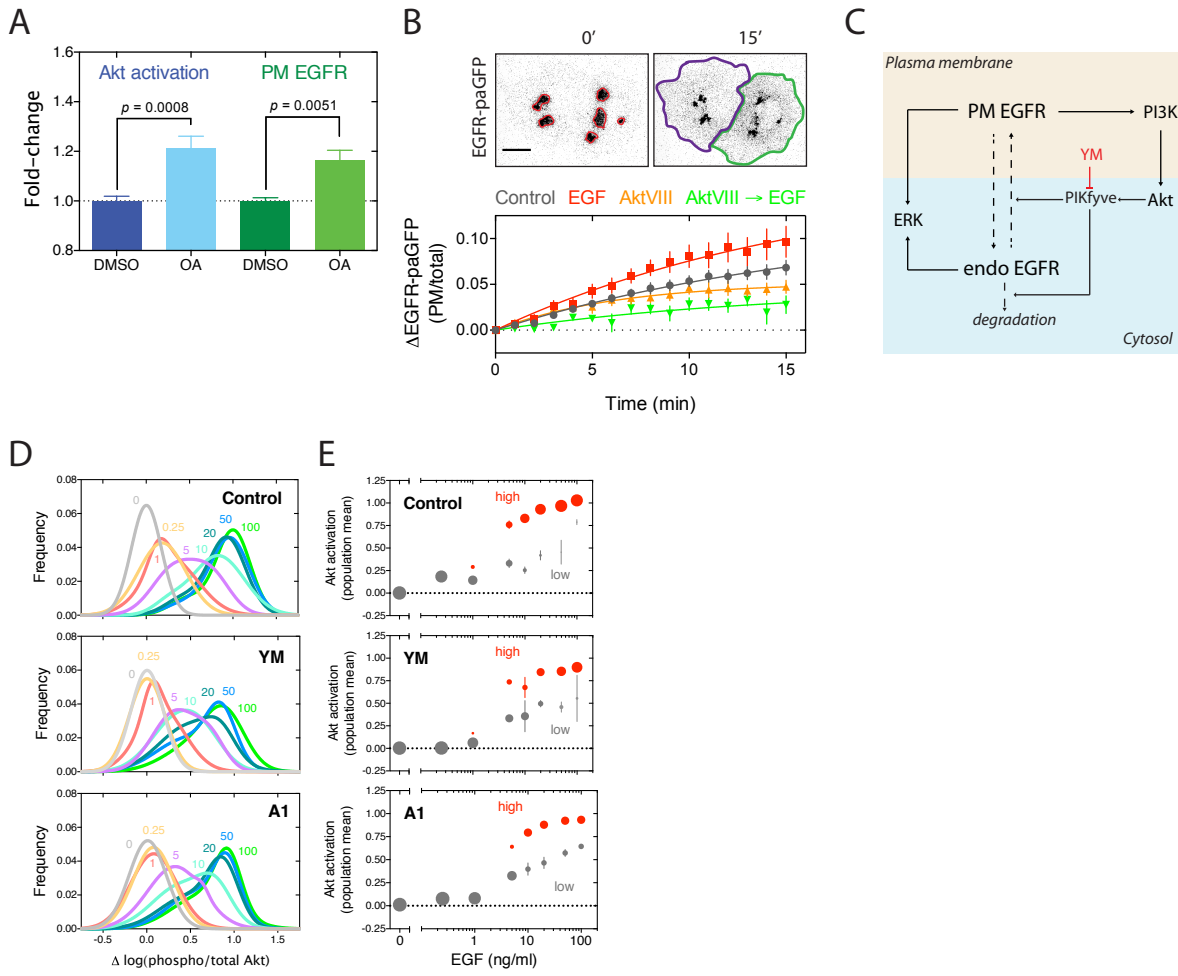


# Figure 3



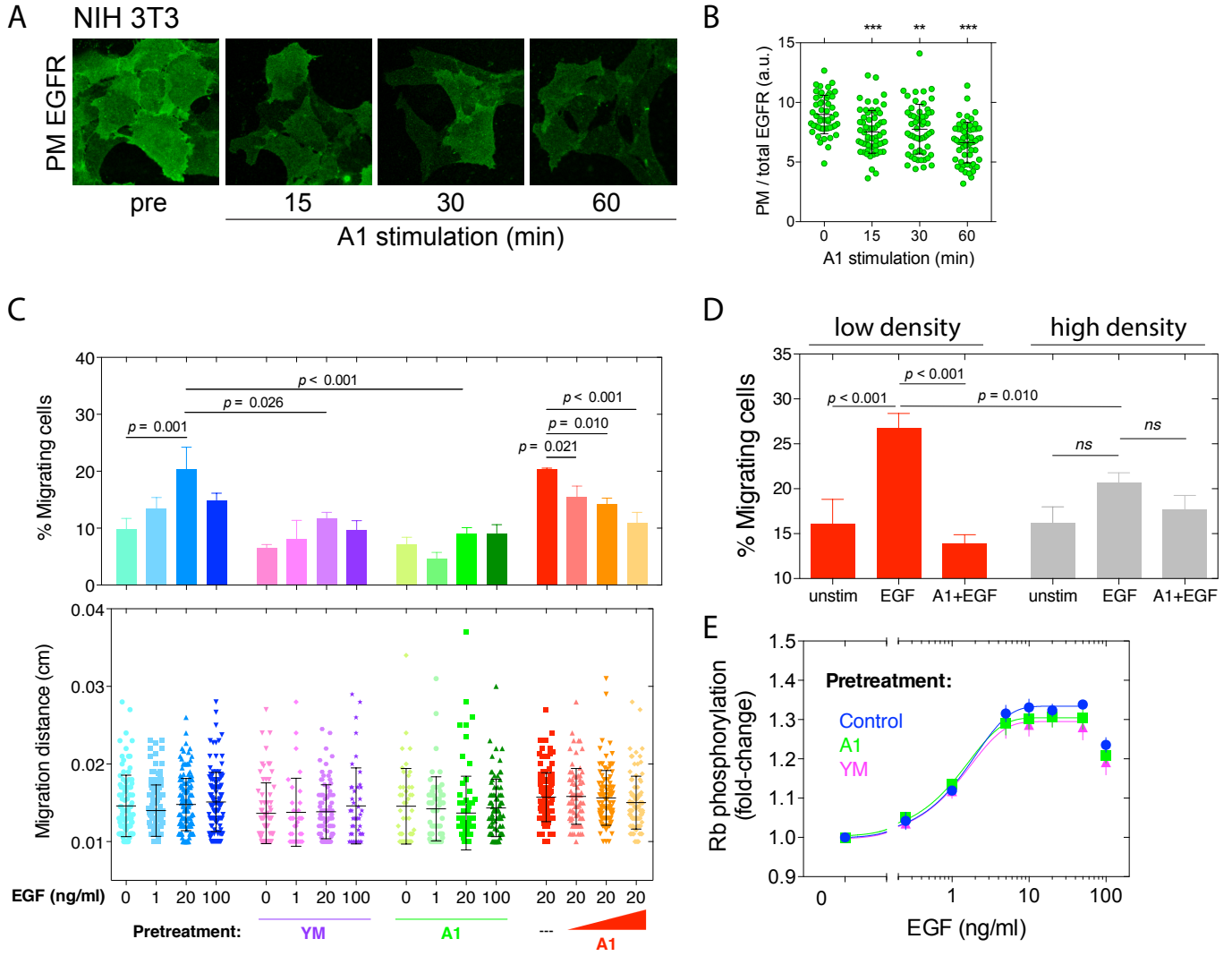


# Figure 4



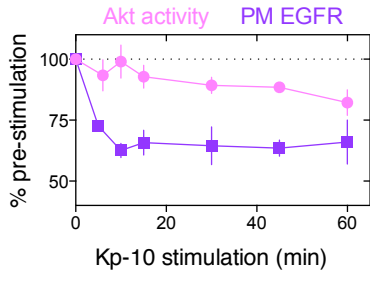
# Figure 5

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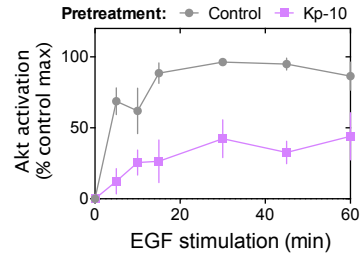


**Figure 6**

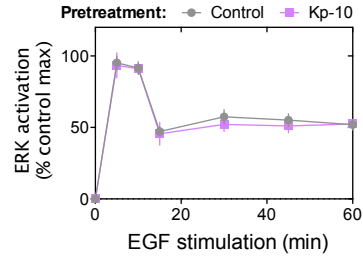
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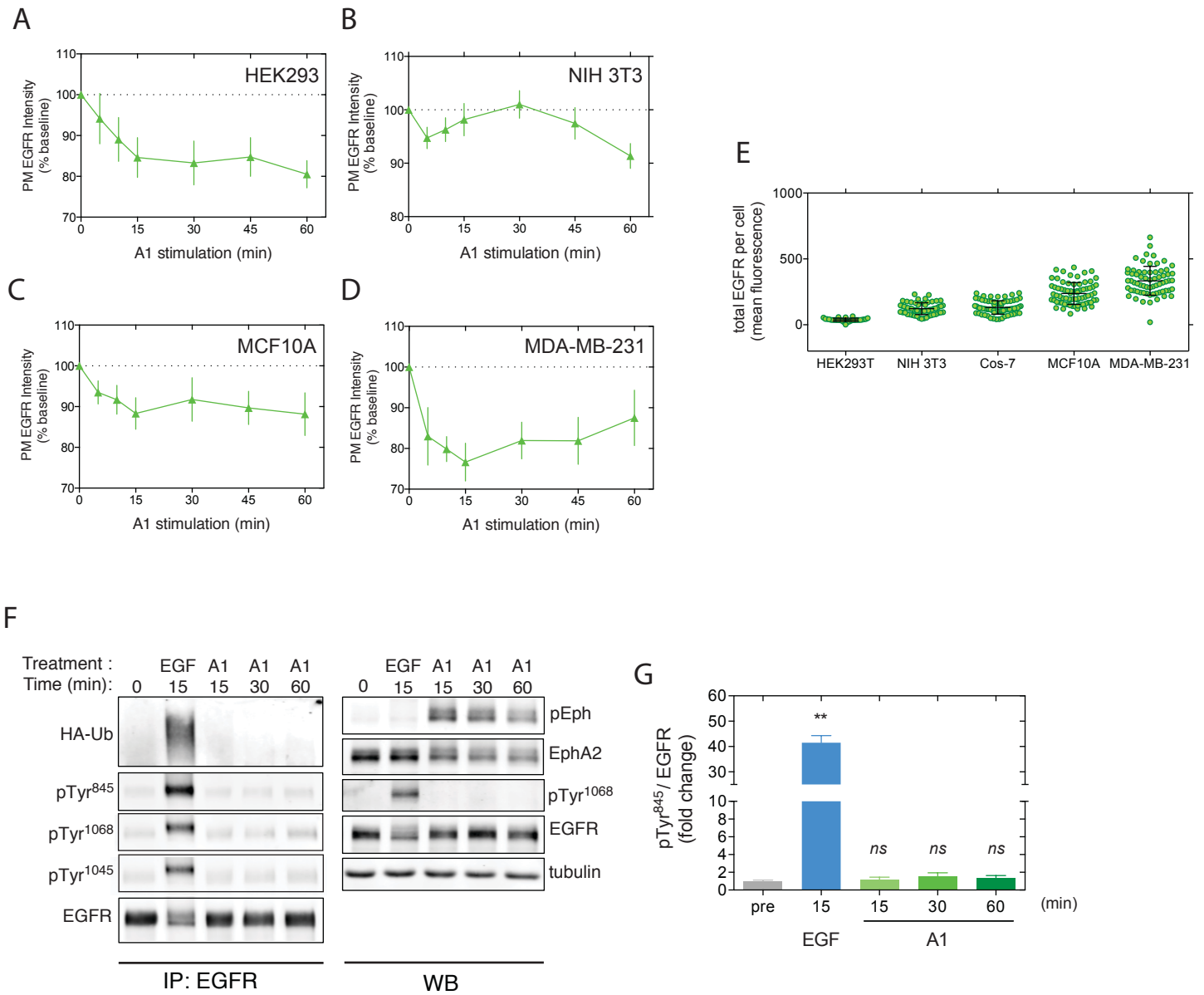
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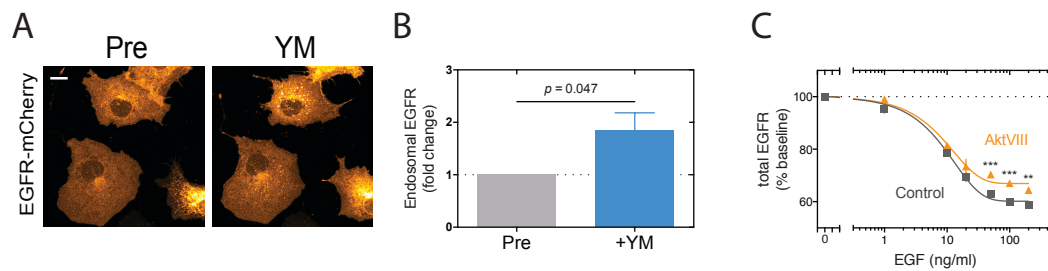
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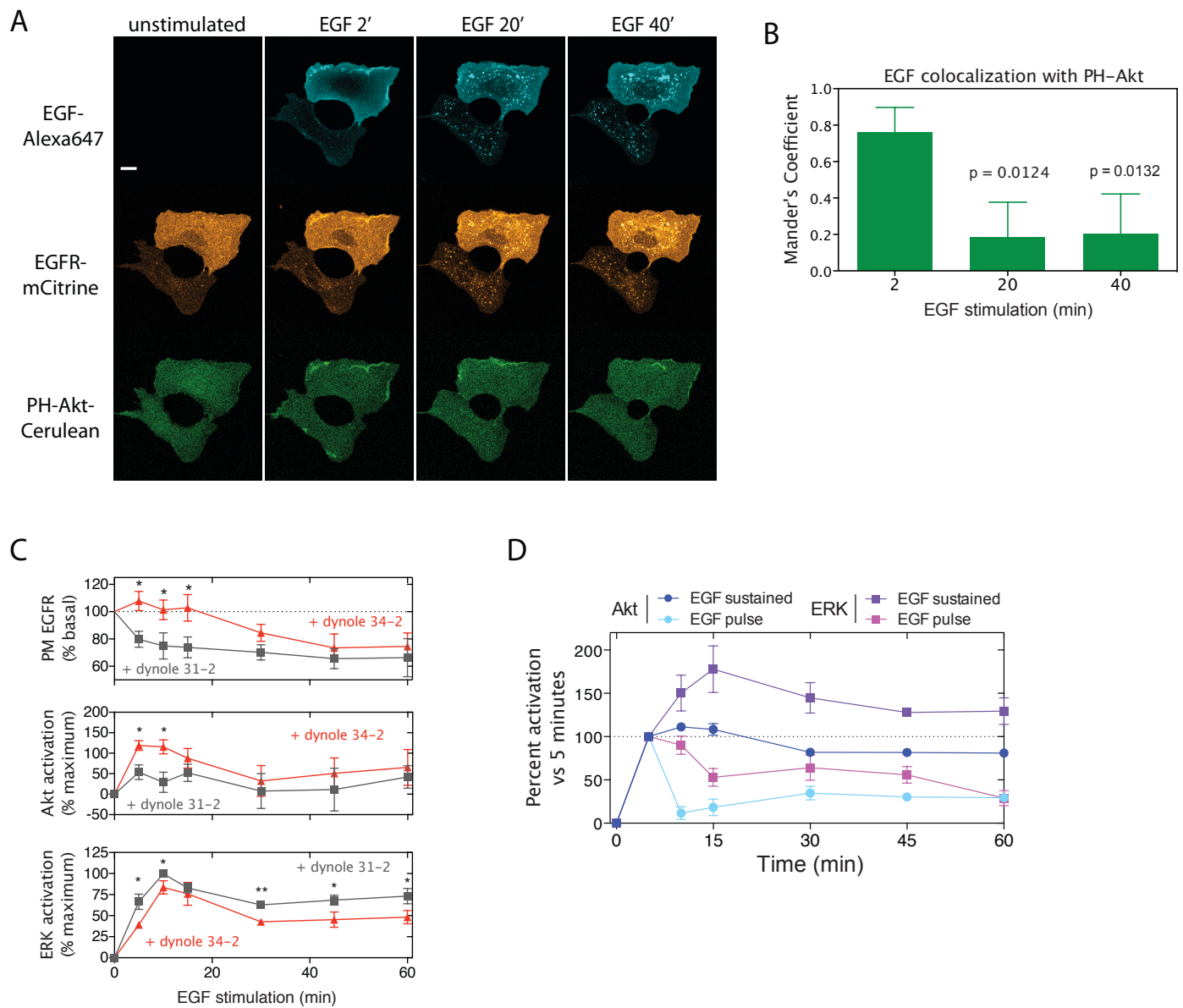
## Figure S1



## Figure S2



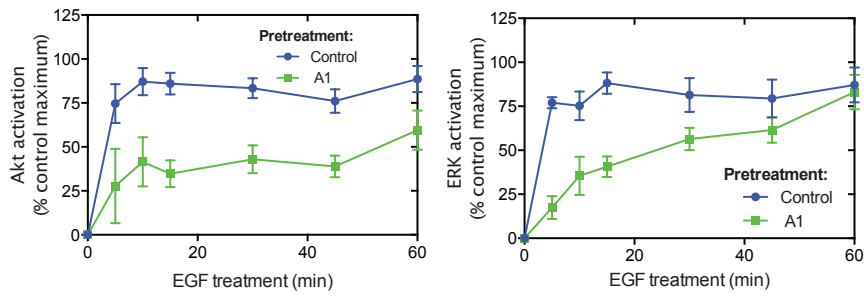
## Figure S3



## Figure S4

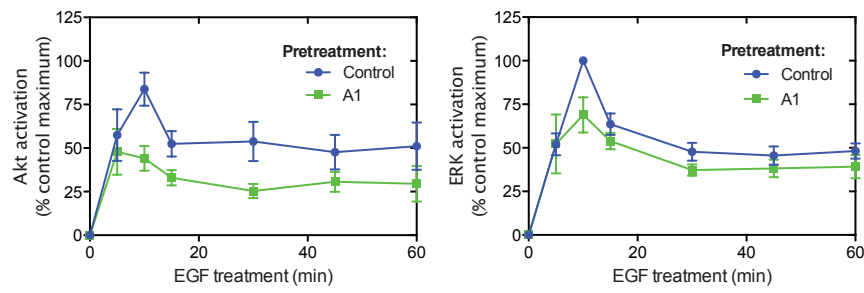
A

Cos-7 (+EGFR/EphA2)



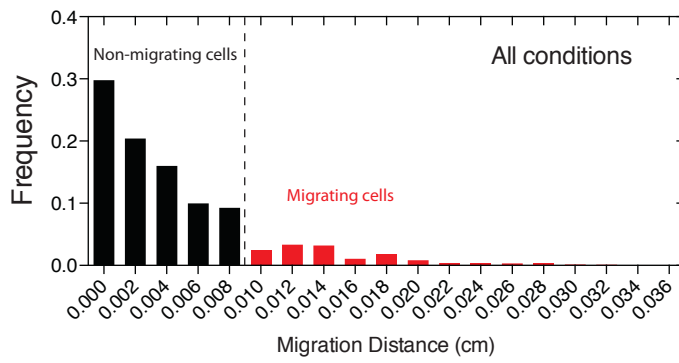
B

HEK293



## Figure S5

A



B

