Contact inhibitory Eph signalling decouples EGFR activity from vesicular recycling to generate contextual plasticity

Wayne Stallaert\textsuperscript{1}, Ola Sabet\textsuperscript{1}, Yannick Brüggemann\textsuperscript{1,2}, Lisa Baak\textsuperscript{1} and Philippe I.H. Bastiaens\textsuperscript{1,2*}

Affiliations

\textsuperscript{1}Department of Systemic Cell Biology, Max Planck Institute of Molecular Physiology, Otto-Hahn-Str.11, 44227 Dortmund, Germany

\textsuperscript{2}Faculty of Chemistry and Chemical Biology, TU Dortmund, Otto-Hahn-Str. 6, 44227 Dortmund, Germany

*Correspondence to: philippe.bastiaens@mpi-dortmund.mpg.de

Abstract

The ability of cells to adapt their behaviour to growth factors in relation to their environment is an essential aspect of tissue development and homeostasis. Here we show that cell-cell contact can change the outcome of EGFR activation by altering its vesicular trafficking. EGFR promotes Akt-mediated vesicular recycling to maintain its plasma membrane (PM) abundance during EGF stimulation. This self-sustained vesicular recycling of EGFR generates a positive feedback that maintains PM Akt signalling and promotes migration. By decoupling EGFR activation from its vesicular recycling, Eph activity at cell-cell contacts impedes the positive feedback that maintains PM signalling and traps EGFR in endosomes. Through this change in the spatial distribution of EGFR activity, cell-cell contact selectively suppresses migratory PI3K/Akt signalling from the PM, while preserving proliferative ERK signalling from endosomes. Thus, by altering the vesicular trafficking of EGFR, the cellular environment can modulate its signalling to generate diverse outcomes to EGF stimulation.
Introduction

Activation of epidermal growth factor receptor (EGFR) promotes a variety of cellular responses including cell growth, proliferation, survival, apoptosis, differentiation and migration (Yarden and Sliwkowski 2001), some of which are functionally opposed. To select among these diverse outcomes, the cell requires additional contextual information. This context can be intrinsic (e.g. cell type or cell cycle stage), or extrinsic, in the form of extracellular signals that provide information about the current (or past) environmental context. Adaptability to a changing environment requires that extrinsic information be integrated through mechanisms that can transform the response to subsequent EGF stimulation.

Vesicular trafficking allows the cell to enact a precise and coordinated response to extracellular signals by dynamically regulating the spatial distribution of cell surface receptors between the plasma membrane (PM) and functionally heterogeneous endosomal compartments (Baumdick et al. 2015). Recycling of unliganded, monomeric EGFR through the protein tyrosine phosphatase (PTP)-rich perinuclear environment safeguards against autonomous receptor activation and regulates the cellular responsiveness to both new and sustained stimuli by maintaining PM receptor abundance (Baumdick et al. 2015). EGF binding promotes receptor ubiquitination (Sigismund et al. 2013), which re-routes internalized EGFR to lysosomal degradation (Baumdick et al. 2015). Therefore, at subsaturating EGF concentrations typically found in human tissue secretions (0.4-20 ng/ml)(Konturek et al. 1989), the cell maintains its sensitivity to persistent stimulation by continually recycling unliganded, non-ubiquitinated receptors to the PM. Saturating EGF concentrations (> 50 ng/ml)(Macdonald and Pike 2008), on the other hand, generate a finite temporal response by progressively depleting PM and total EGFR abundance through ubiquitin-dependent lysosomal degradation.
Although endocytosis reduces receptor activation and signalling at the PM, many receptors continue to signal from endosomes following internalization (Villaseñor, Kalaidzidis, and Zerial 2016; Sorkin and von Zastrow 2009; Daaka et al. 1998; Wouters and Bastiaens 1999; DeFea et al. 2000; Maminska et al. 2016; Ménard, Parker, and Kermorgant 2014; Sadowski et al. 2013; Howe et al. 2001; Verma et al. 2015). The signalling repertoire of these endosomal receptors can differ from that at the PM if biochemical components of the transduction machinery are not equally accessible in these distinct subcellular environments. EGFR can continue to activate ERK1/2, for example, by scaffolding components of this kinase cascade on endosomes (Guglielmo et al. 1994; Teis et al. 2006; Nada et al. 2009; Fortian and Sorkin 2014; Villaseñor et al. 2015). De novo activation of Akt by endosomal EGFR, on the other hand, is limited by a deficiency of the phosphoinositide PI(4,5)P$_2$ in endosomal membranes (Matsuda et al. 2001; Haugh and Meyer 2002), which is phosphorylated to PI(3,4,5)P$_3$ through the EGFR-mediated activation of phosphoinositide 3 kinase (PI3K) and acts as a docking site for Akt and its upstream kinase PDK1. Consequently, sustained activation of Akt requires a continuous recycling of internalized receptors to the PI(4,5)P$_2$-rich environment of the PM (Nishimura et al. 2015). Any changes in EGFR trafficking dynamics which alter the distribution of receptors between the PM and endosomal compartments might therefore change the signalling response to EGF stimulation. Furthermore, recent evidence suggests that certain signalling molecules downstream of cell surface receptors can, in turn, influence vesicular trafficking (Er et al. 2013; Laketa et al. 2014), generating a reciprocal relationship between receptor activation and vesicular dynamics whose role in shaping the cellular response to stimuli has recently begun to garner attention (Schmid 2017).

Given the role of trafficking in the regulation of EGFR activation and signalling, changes in its vesicular dynamics may represent a mechanism through which the environmental context can influence the cellular response to EGF stimulation. In the current work, we reveal that cell-cell contact, through the activation of Eph receptors, selectively suppresses EGF-promoted migratory signalling while preserving its proliferative response by altering EGFR vesicular trafficking dynamics.
Results

Akt-dependent recycling of EGFR sustains its autocatalytic activation at the PM

In the absence of EGF stimulation, constitutive vesicular recycling of EGFR acts as a safeguard against autonomous receptor activation both by reducing PM abundance and continuously recycling receptors through the PTP-rich perinuclear compartment to dephosphorylate the autocatalytic Y845 (Baumdick et al. 2015). We observed that this constitutive recycling of unliganded EGFR is dependent on cellular Akt activity (Fig. 1a-d).

Endosomal accumulation of EGFR (Fig. 1a, Supplementary Movie 1) and a decrease in PM EGFR abundance (Fig. 1b top) were observed following pharmacological inhibition of Akt (Fig. 1b bottom) in Cos-7 cells. Consistent with a previous report, we found that Akt inhibition traps endocytic EGFR in Rab5-positive early endosomes (Fig. 1c)(Er et al. 2013). Conversely, an increase in Akt activation following inhibition of its negative regulator, PP2A, by okadaic acid promoted a coincidental increase in the abundance of PM EGFR (Fig. 1d), demonstrating that the PM density of EGFR is positively regulated by Akt activity.

EGF binding increases the probability of EGFR ubiquitination (Sigismund et al. 2013), which redirects liganded, internalized receptors toward the Rab7-positive late endosomal compartment en route to degradation in the lysosome, while unliganded, non-ubiquitinated receptors are recycled back to the PM (Baumdick et al. 2015). Since EGFR activation itself increases Akt activity in cells (Supplementary Fig. 1), we asked whether the vesicular recycling of unliganded receptors is increased during subsaturating EGF stimulation. By measuring the accumulation of EGFR-paGFP at the PM following photoactivation in endosomes (Fig. 1e top), we observed that EGF stimulation increased EGFR recycling (Fig. 1e bottom), while Akt inhibition suppressed EGFR recycling both prior to and during EGF stimulation (Fig. 1e). Thus, through the activation of Akt, EGFR actively maintains its PM abundance during persistent, subsaturating stimulation by increasing vesicular recycling. Furthermore, by trapping internalized EGFR in Rab5-positive early endosomes, Akt inhibition also impaired the transition of EGFR to Rab7-positive late endosomes (Poteryaev et al. 2009) in EGF-stimulated cells (Fig. 1c), leading to a decrease in receptor degradation.
Therefore, by impeding trafficking from the early endosomal compartment, Akt regulates both the recycling of unliganded, non-ubiquitinated receptors to the PM and the degradation of liganded, ubiquitinated receptors in the lysosome.

We next investigated how these effects of Akt on EGFR vesicular trafficking influence receptor response properties during EGF stimulation. By decreasing PM receptor abundance prior to and during EGF stimulation (Fig. 1b,e), pretreatment with an Akt inhibitor significantly decreased the proportion of phosphorylated receptors at the autocatalytic Y845, the effector docking site Y1068 and the Cbl-mediated ubiquitination regulatory site Y1045 (Baumdick et al. 2015) following 30 min of sustained EGF stimulation (Fig. 1g). At higher EGF concentrations (≥ 50 ng/ml), Akt inhibition also suppresses receptor degradation (Fig. 1f), trapping internalized EGFR in endosomal compartments accessible to ER-bound PTPs with high activity (Yudushkin et al. 2007; Haj et al. 2002), further decreasing the proportion of phosphorylated receptors in the entire cell (Fig. 1g).

At subsaturating, physiological EGF concentrations, EGFR phosphorylation is propagated at the PM through the transphosphorylation of unliganded receptors by liganded receptors, generating an autocatalytic activation of EGFR (Reynolds et al. 2003; Ibach et al. 2015; Masip et al. 2016, Stanoev et al. 2017). Once unliganded receptors are phosphorylated on Y845, which stabilizes their active conformation (Baumdick et. al, 2017), autocatalytic activation can further propagate to additional unliganded EGFR. This autocatalytic component in EGFR activation increases non-linearly with PM receptor density. We therefore hypothesized that Akt activity might regulate EGFR autocatalysis by controlling receptor abundance at the PM. As receptor occupancy increases at higher EGF concentrations, EGFR phosphorylation becomes less dependent on autocatalysis. Therefore, to specifically measure the effect of Akt on EGFR autocatalytic activation, we stimulated cells with increasing, subsaturating concentrations of EGF-Alexa647 (1, 3, 10 and 20 ng/ml) and simultaneously measured EGF binding and EGFR activation (by translocation of the EGFR phospho-tyrosine binding (PTB) domain) at the PM in individual cells by time lapse imaging (Fig. 1h-j, Supplementary Fig. 2). Compared to control cells (Fig. 1h), pretreatment with an Akt inhibitor decreased both EGF binding and EGFR
phosphorylation (Fig. 1i), consistent with a reduction in PM receptor abundance prior to EGF stimulation (Fig. 1b,e). Plotting the relationship between the number of liganded receptors (i.e. EGF-Alexa647 binding) and the extent of EGFR phosphorylation revealed that phosphorylation increases non-linearly with EGF binding above a certain threshold in control cells, consistent with an autocatalytic activation (Fig. 1j). Pretreatment with an Akt inhibitor, on the other hand, generated less PM EGFR phosphorylation for equivalent numbers of liganded receptors by impeding its autocatalytic activation (Fig. 1j). Therefore, by promoting the constitutive recycling of EGFR to maintain its PM density prior to EGF stimulation (Fig. 1a-e), Akt activity fosters the autocatalytic activation of EGFR at subsaturating EGF concentrations.

Since EGF stimulation itself increases Akt-dependent vesicular recycling (Fig. 1e), we further reasoned that EGFR might self-sustain its activation during persistent, subsaturating EGF stimulation. To further investigate how Akt-dependent recycling influences PM EGFR autocatalytic activation during EGF stimulation, measurements of EGFR phosphorylation and trafficking were obtained by immunofluorescence prior to and following subsaturating EGF stimulation (20 ng/ml) for 5, 30 and 60 min. Cells were radially segmented to quantify changes in the spatial distribution of EGFR with time and visualized using 3D spatial-temporal maps (Fig. 1k, Stanoev et al. 2017). By reducing PM receptor density prior to EGF stimulation (Fig. 1k, top), pretreatment with an Akt inhibitor decreased the proportion of phosphorylated EGFR at the PM after 5 min of EGF stimulation (Fig. 1k, bottom), consistent with reduced EGFR autocatalysis (Fig. 1j). By continually delivering internalized, non-ubiquitinated receptors to the PM to both bind additional EGF and act as a substrate for autocatalysis, the EGF-induced increase in vesicular recycling (Fig. 1e), maintains a high proportion of phosphorylated receptors at the PM during sustained stimulation (Fig. 1k, bottom). Akt inhibition, on the other hand, by suppressing the EGF-induced increase in receptor recycling (Fig. 1e) also reduced EGFR autocatalytic activation at the PM at later time points (Fig. 1k, bottom) by trapping internalized receptors in endosomes (Fig. 1k, top). Akt promotes vesicular trafficking through the activation of the Rab5 effector PIKfyve (Er et al. 2013). PIKfyve inhibition led to an endosomal accumulation of EGFR (Supplementary Fig. 3a,b) and decreased PM EGFR phosphorylation.
abundance (Fig. 1k, top), consistent with a suppression of Akt-dependent recycling. Decoupling Akt activation from its effect on trafficking by PIKfyve inhibition had indistinguishable effects from direct Akt inhibition on EGFR phosphorylation and trafficking (Fig. 1l), further validating that Akt activity sustains autocatalytic EGFR activation at the PM through its effects on vesicular recycling.

Therefore, by stimulating an increase in Akt-dependent recycling, EGFR maintains its sensitivity to EGF, effectively generating a positive feedback that sustains autocatalytic EGFR activation at the PM during persistent, subsaturating stimulation (Fig. 1l).

Eph receptor activation regulates EGFR vesicular trafficking

The Eph family of receptor tyrosine kinases is activated at points of cell-cell contact through interactions with membrane bound ephrin ligands presented on the surfaces of adjacent cells (Pasquale 2010). Eph receptors, such as EphA2, in many ways operate in functional opposition to EGFR, acting as a tumour suppressor (Miao et al. 2001; Batlle et al. 2005; Merlos-Suárez and Batlle 2008; Cortina et al. 2007; Li et al. 2009; Pasquale 2010; Boyd, Bartlett, and Lackmann 2013) and mediating contact inhibition of locomotion to suppress cellular migration and metastasis (Miao et al. 2009; Astin et al. 2010; Li et al. 2012; Lin et al. 2015). Indeed, a functional coupling of EGFR and Eph receptor activity has recently been shown to regulate EGF-promoted migration (Lin et al. 2015). Since the activation of Eph receptors decreases cellular Akt activity (Supplementary Fig. 4a-b) (Li et al. 2012), we hypothesized that Eph receptors might regulate the cellular response to the persistent, subsaturating EGF stimulation typical of pro-migratory signals by altering EGFR vesicular trafficking.

Stimulation with soluble, clustered ephrinA1-Fc (A1) (Davis et al. 1994; Sabet et al. 2015), led to Rab5-positive early endosomal accumulation of EGFR (Fig. 2a-c, Supplementary Movie 2) and a reduction in PM EGFR abundance in Cos-7 (Fig. 2d, Supplementary Fig. 4c-d), HEK293 (Supplementary Fig. 4e), MCF10A (Supplementary Fig. 4f) and MDA-MB-231 cells (Supplementary Fig. 4g). EGFR also accumulated in endosomes upon
physiological presentation of ephrinA1 ligand at sites of cell-cell contact (Supplementary Movie 3). Consistent with the delayed endosomal accumulation of EGFR relative to EphA2 (Fig. 2b,d), we confirmed that the loss of PM EGFR does not result from the increased formation of a heterodimer (Supplementary Fig. 5) or through the transactivation of EGFR by EphA2 (Fig 2e). To directly assess whether Eph receptors decrease PM EGFR through an inhibition of Akt-mediated EGFR recycling, we stimulated cells for 15 min with a subsaturating concentration of EGF to induce EGFR endocytosis and measured the subsequent recycling of internalized receptors back to the PM after EGF washout (Fig. 2f).

In control cells, the PM abundance of EGFR recovered to pre-EGF levels following washout, while in A1-pretreated cells, EGFR recycling was significantly inhibited (Fig. 2f), consistent with an EphA2-mediated inhibition of Akt-dependent recycling.

Cell-cell contact alters the spatial distribution of EGFR activity

We next assessed whether prior activation of Eph receptors regulates the subsequent cellular response to EGF by suppressing its vesicular recycling. Similar to the effect of Akt and PIKfyve inhibition (Fig. 1k), A1 pretreatment reduced autocatalytic activation of EGFR at the PM during persistent, subsaturating EGF stimulation, while preserving EGFR phosphorylation in endosomes (Fig. 3a). Although EGFR can continue to activate some signalling effectors from endosomal membranes (Villaseñor, Kalaidzidis, and Zerial 2016; Sorkin and von Zastrow 2009), Akt itself is preferentially activated at the PM (Supplementary Fig. 1)(Haugh and Meyer 2002; Nishimura et al. 2015). By suppressing the positive feedback that maintains EGFR activation at the PM, A1 pretreatment selectively inhibited EGF-promoted Akt activation in both Cos-7 and HEK293 cells (Fig. 3b top, Supplementary Fig. 6), while ERK activation, which can continue from endosomal membranes (Supplementary Fig. 1c-d)(Guglielmo et al. 1994; Nada et al. 2009; Villaseñor et al. 2015), remained intact (Fig 3b bottom, Supplementary Fig. 6). To confirm that EphA2 inhibits EGF-promoted Akt activation by suppressing EGFR recycling and does not simply reflect the opposed regulation of Akt by EGFR and EphA2 (activation vs inhibition, respectively), we assessed whether EGFR trafficking was dispensable for the A1-induced
suppression of EGF-promoted Akt activation. Cells were prestimulated with A1 for 30
minutes, followed by treatment with the dynamin inhibitor dynole 34-2 to block
subsequent endocytosis, and then stimulated with EGF. When EGFR endocytosis was
blocked (Supplementary Fig. 1c), A1 pretreatment had no effect on EGF-promoted Akt
activation (Fig. 3c top), while the negative control analogue, dynole 31-2, which does not
inhibit EGFR endocytosis (Supplementary Fig. 1c), had no effect on A1-induced
suppression of EGF-promoted Akt activation (Fig. 3c bottom), corroborating that intact
EGFR vesicular trafficking is required for the inhibitory effect of Eph receptors on EGFR
signalling.

Increasing concentrations of A1 progressively inhibited EGF-mediated Akt activation (Fig.
3d), suggesting that the amount of ephrinA1:Eph receptor interactions at points of cell-cell
contact might determine the magnitude of Akt activation in response to a given
concentration of EGF. To directly investigate the influence of cell-cell contact on EGFR
signalling, we obtained single cell measurements of Akt and ERK activation in cells with
varying degrees of cell-cell contact (Fig. 3e,f). Akt activation decreased with cell-cell
contact both prior to ($F = 16.0, p = 0.001, r^2 = 0.432$) and following EGF stimulation ($F =
21.4, p < 0.001, r^2 = 0.322$) (Fig. 3e), demonstrating that increasing cell-cell contact reduces
the magnitude of EGF-promoted Akt activation. ERK activation, on the other hand, was
unaffected by cell-cell contact (unstimulated: $F = 0.180, p = 0.673, r^2 = 0.003$; EGF: $F =
0.321, p = 0.575, r^2 = 0.009$), with cells generating similar EGF-promoted increases in ERK
activation irrespective of their degree of cell-cell contact (Fig. 3f).

To determine whether environmental signals other than cell-cell contact can influence
EGFR signalling through changes in its vesicular trafficking, we investigated the effect of
prior activation of the G protein-coupled receptor Kiss1 (Kiss1R), which, similar to EphA2,
inhibits Akt and acts as a tumour and metastasis suppressor (Navenot, Fujii, and Peiper
2009). Stimulation with the Kiss1R ligand kisspeptin-10 (Kp-10) reduced Akt activity and
decreased PM EGFR abundance (Fig. 3g). Similar to the effect of cell-cell contact,
pretreatment with Kp-10 selectively inhibited EGF-promoted Akt activation (Fig. 3h),
while preserving ERK activation (Fig. 3i). Changes in EGFR vesicular trafficking dynamics,
therefore, provides a general mechanism to generate plasticity in the signalling response to EGFR activation, through which diverse environmental signals such as cell-cell contact or soluble stimuli like Kp-10 can influence the cellular response to EGF.

Positive feedback in combination with inhibitory network motifs can convert graded inputs into switch-like, ultrasensitive signalling responses (Ferrell and Ha 2014). Since Akt is preferentially activated at the PM (Supplementary Fig. 1), the EGF-induced increase in EGFR vesicular recycling (Fig. 1e) should generate a positive feedback for Akt activation (Fig. 1l). While population-based measurements of Akt activation do not demonstrate ultrasensitivity (Fig. 3d, Hill coefficient of 0.975±0.215 for control cells), if cells possess intrinsic variability in the EGF concentration required to stimulate Akt-dependent recycling, ultrasensitivity in individual cells will be averaged out at the population level to produce a graded response (Ferrell and Machleder 1998). To investigate if this positive feedback can generate a switch-like activation of Akt at the single cell level, we measured the Akt response in thousands of individual cells by flow cytometry following sustained stimulation (60 min) with a range of EGF concentrations (0.25-100 ng/ml) (Fig. 3j-k).

Additionally, cells were stimulated in suspension to negate the effect of in situ cell-cell contact as an extrinsic source of variability in Akt activation (Fig. 3e). At concentrations ≥ 1 ng/ml, EGF stimulation produced a switch-like activation to a high Akt phosphorylation state in a subpopulation of cells, whose proportion increased with EGF concentration (Fig. 3j-k, top). Decoupling EGFR activation from its effect on vesicular recycling by PIKfyve inhibition (Fig. 3j-k, middle) or A1 pretreatment (Fig. 3j-k, bottom) did not result in a global decrease in Akt activation but rather reduced the proportion of cells generating a high Akt response, consistent with an inhibition of the positive feedback that produces this switch-like response. Intrinsic cell-to-cell variability in the EGF threshold required to stimulate Akt-dependent vesicular recycling, therefore, determines the proportion of cells that transition to a high Akt activity state at a given EGF concentration.

If the transition to a high Akt activity state is dependent on the positive feedback produced by coupling EGFR activation to vesicular recycling, we should also observe similar cell-to-
cell variability in PM EGF binding during sustained stimulation. We therefore performed single cell time lapse imaging and quantified PM EGF binding during persistent stimulation with a subsaturating concentration of EGF-Alexa647 (20 ng/ml) (Fig. 3l-n). Cells that successfully engage Akt-dependent recycling should exhibit a more sustained EGF binding at the PM during stimulation. Cells were grouped by their rate of loss in PM EGF binding using k-means clustering into two groups exhibiting a slow (Group 1) or fast (Group 2) loss of PM EGF during stimulation (Fig. 3l-m). While cells of both types were observed in control and A1 pretreated samples, in control conditions we observed an enrichment of cells with sustained binding (Group 1; 28%) versus cells pretreated with A1 (10.5%) (Fig. 3n). Although the percent of cells generating sustained EGF binding in control cells (Fig. 3n) is less than the proportion producing a high Akt response in flow cytometry experiments (Fig. 3j-k, top), in situ cell-cell contact during time lapse imaging should decrease the number of cells stimulating Akt-dependent recycling, consistent with the observed effect of Eph receptor activation (Fig. 3j-k, bottom).

Thus, cells possess intrinsic variability to engage Akt-dependent recycling and preserve PM EGF binding during sustained stimulation. Eph receptor activation, by decoupling EGFR activation from its effect on vesicular trafficking, reduces Akt activation within the population by decreasing the proportion of cells transitioning to a high Akt activity state during persistent EGF stimulation.

\textit{Cell-cell contact changes the cellular outcome of EGF stimulation}

EGFR signalling to effectors at the PM generates exploratory cellular behaviours (Chinkers, McKanna, and Cohen 1979; Ridley and Hall 1992; Chan et al. 1998; Segall et al. 1996; Bailly et al. 1999; Harms et al. 2005; Yip et al. 2007; El-Sibai et al. 2007) that must be maintained to induce a persistent migratory response. Given that contact inhibitory Eph receptor activation selectively suppresses PM signalling during persistent, subsaturating EGF presentation (Fig. 3b,e,f), we investigated if cell-cell contact regulates EGF-promoted migration by inhibiting Akt-dependent recycling. NIH 3T3 mouse embryonic fibroblast (MEF) cells were grown on fibronectin in the presence of a silicone barrier that, once
removed, creates a cell-free area into which individual cells can be tracked following uniform stimulation with EGF. When grown on fibronectin in 2-D cultures, fibroblasts generate an autonomous, haptotactic migratory response which is enhanced by an EGF-induced increase in their exporatory behaviour (Li et al. 1999). Following stimulation with subsaturating EGF concentrations (20 ng/ml), we observed a significant increase in the proportion of migratory cells (Fig. 4a, top, Supplementary Movie 4, Supplementary Fig. 7), but no change in the average distance travelled by migrating cells (Fig. 4a, bottom), indicating that EGF promotes the transition of individual cells to a migratory state rather than increasing overall cellular motility. Since ligand binding promotes receptor ubiquitination and degradation, persistent stimulation with saturating, supraphysiological EGF concentrations (100 ng/ml) induces a loss in EGF sensitivity with time and thus does not significantly increase the proportion of migratory cells (Fig. 4a, top). Decoupling EGFR activation from its effect on trafficking through the PIKfyve inhibition or A1 pretreatment decreased the proportion of migratory cells (Fig. 4a), demonstrating that the positive feedback generated by the EGF-induced increase in Akt-dependent recycling promotes the transition to a migratory state. We observed further that increasing concentrations of A1 progressively decreased EGF-induced migration (Fig. 4a), consistent with its concentration-dependent effect on EGF-promoted Akt activation (Fig. 3d) and suggesting that the amount of ephrinA1-Eph receptor interactions at points of cell-cell contact may determine whether a cell initiates a migratory response to EGF. Consistent with this observation, we found that the number of migratory cells following EGF stimulation was inversely proportional to cell density (Fig. 4b) and that the increase in migration observed at low densities could be countered by treatment with soluble A1 to mimic Eph receptor contact inhibitory signalling (Fig. 4b). Thus, physiological Eph receptor activation at points of cell-cell contact suppresses EGF-promoted migration by inhibiting Akt-dependent vesicular recycling. However, by preserving endosomal ERK activation following EGF stimulation (Fig. 3b, bottom), we found that neither PIKfyve inhibition nor A1 pretreatment led to a reduction in EGF-promoted cell proliferation (Fig. 4c). Thus by altering the spatiotemporal distribution of EGFR activity, contact inhibitory signalling by Eph receptors determines the cellular outcome to EGF stimulation, biasing cells to a proliferative instead of a migratory response.
DISCUSSION

In this paper, we demonstrate that cell-cell interactions can generate context-dependent cellular outcomes to EGF stimulation by modulating EGFR vesicular trafficking dynamics. Akt-dependent recycling promotes EGFR activation both by enriching the concentration of potential EGF binding sites at the PM but also by increasing the autocatalytic activation of unliganded receptors by transphosphorylation. By increasing Akt activity, EGF stimulation generates a positive feedback that maintains EGFR density at the PM by increasing the vesicular recycling of non-ubiquitinated receptors during persistent, subsaturating stimulation (Fig. 1e). Saturating, supraphysiological EGF concentrations, on the other hand, increase the proportion of liganded, ubiquitinated receptors that unidirectionally traffic, in an Akt-dependent manner, from early to late endosomes to be degraded in lysosomes (Fig. 1c, f) (Baumdick et al. 2015), resulting in a depletion of PM EGFR abundance with time.

Chemotaxis requires that cells remain responsive to stimuli for prolonged periods of time as they migrate toward the chemotactic source. Akt-dependent trafficking, therefore, determines the concentration range at which EGF produces a migratory response. By increasing both the vesicular recycling of unliganded, non-ubiquitinated receptors and the degradation of ubiquitinated receptors, Akt activation maintains cellular sensitivity to persistent, subsaturating EGF concentrations, while limiting the duration of receptor activation to saturating concentrations.

Since Akt itself is preferentially activated at the PM (Supplementary Fig. 1), the EGF-promoted increase in vesicular recycling generates a positive feedback that switches cells to a high Akt activation state (Fig. 3j-k). Although Akt has been previously observed on endosomal membranes through interactions with the early endocytic adaptor protein APPL1 (Schenck et al. 2008; Ebner et al. 2017), de novo activation of Akt by EGFR requires the production of PI(3,4,5)P₃, which is impeded by the low abundance of PI(4,5)P₂ in endosomal membranes (Matsuda et al. 2001; Haugh and Meyer 2002). Although Akt
activation may occur to some extent on endosomal membranes (Jethwa et al. 2015), since the coupling of active EGFR to Akt activation will be more efficient at the PM, any perturbations that influence the spatial distribution of EGFR will influence the capacity of EGFR to activate Akt (Fig. 3b, Supplementary Fig. 1c).

We observed that the switch to a high Akt activity state only occurs in a proportion of cells, which increases with EGF concentration (Fig. 3j-k). Population heterogeneity in Akt activation has been previously attributed to cell-to-cell variation in PI3K expression (Yuan et al. 2011). Our data suggest that intrinsic variability in the expression of signalling and/or trafficking effectors may determine, for a given cell, the EGF concentration required to stimulate Akt-dependent trafficking and engage the positive feedback that produces a high Akt activity state. Small differences in EGF concentration substantially influence the proportion of cells generating a high Akt response (e.g. a shift from 5 to 10 ng/ml increases the proportion of cells from 43 to 85%, respectively, Fig. 3j-k). Perhaps it is not coincidental that the concentration range over which this switch occurs corresponds to the physiological range of EGF concentrations in human tissue secretions (Konturek et al. 1989). By generating a sharp boundary for Akt activation within the physiological EGF concentration regime, even slight changes in the threshold of this switch could have profound implications for tissue dynamics (e.g. initiation of migration).

Eph receptor activation, for example, by suppressing EGFR recycling, decreased the proportion of cells generating a high Akt response from 85 to 41% in response to 10 ng/ml EGF (Fig. 3j-k). The dependence of Akt activation on EGFR recycling allows the degree of cell-cell contact, or other environmental stimuli that influence vesicular dynamics, to regulate proportion of cells generating a migratory response to EGF stimulation. PI3K/Akt signalling has previously been suggested as the point of convergence for EGFR/EphB2 control of cell migration (Lin et al. 2015); however, the molecular mechanism underlying this oppositional relationship remained unclear. Our results indicate that Eph receptor activation inhibits EGF-promoted cell migration by suppressing Akt-dependent recycling, thus impeding the positive feedback that generates a high Akt response and decreasing the sensitivity of cells to persistent EGF stimulation necessary to maintain exploratory
behaviour. However, by changing the spatial distribution of active EGFR, Eph receptor
activation selectively suppresses migratory signalling from the PM while leaving
proliferative ERK signalling intact (Fig. 3b,e-f, Fig. 4)(Li et al. 2012). This contextual
plasticity generates two distinct cellular outcomes to EGF stimulation that may be
important in physiological settings such as wound healing. At the tissue boundary, cells
with reduced cell-cell contact would increase their exploratory behaviour in response to
EGF released at the site of the wound. Cells located deeper in the tissue, despite extensive
cell-cell contact, would retain their proliferative response to environmental EGF, and
undergo mitosis to fill the vacant space created as exploratory cells migrate to occupy the
wound area.

Our results suggest that the spatial component of the positive feedback generated by Akt-
dependent recycling may also explain how EGF can act as a positional chemotactic cue (e.g.
during Drosophila border cell migration)(Duchek and Rørth 2001). Given the small
difference in EGF concentration between the leading edge and the tail of a migrating cell in
a shallow EGF gradient, preferential trafficking of receptors to the PM region of highest
EGFR activity would enhance local signalling (Bailly et al. 2000) to enrich exploratory
behaviour in the direction of the EGF source (Jékely et al. 2005; Arrieumerlou and Meyer
2005), leading to directed movement.

Our observations demonstrate that crosstalk between receptors with opposed functionality
can emerge through changes in vesicular trafficking dynamics rather than relying on direct
interactions between the receptors or their respective effectors. Such a mechanism also
allows different receptors with similar functional roles (e.g. EphA2 and Kiss1R) to alter the
cellular response to stimuli without having to evolve distinct protein interaction domains
to do so. The dependency of EGFR signalling on its vesicular dynamics thus confers a
universal mechanism through which the cell can generate functional plasticity to growth
factor stimulation while preserving specificity in cell-cell communication through the
coexpression of distinct but functionally-related cell surface receptors.

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Author contributions

P.I.H.B. and W.S. conceived the project. W.S. performed and analysed most experiments. O.S. contributed to the migration experiments, Y.B. contributed to the time lapse imaging of EGF binding, L.B. contributed to the ICW and immunoprecipitation experiments. W.S. wrote the manuscript with assistance from P.I.H.B.

Competing financial interests

The authors declare that no competing interests exist.
Methods

Primary antibodies

Mouse anti-Akt (2920, Cell Signalling Technology (CST), Danvers, MA, USA), mouse anti-Akt-Alexa488 (2917, CST), rabbit anti-phospho-Akt(Ser473) (4060, CST), rabbit anti-phospho-Akt (Ser473)-Alexa647 (4075, CST), mouse anti-HA (9658, Sigma-Aldrich, St.Louis, MO, USA), rabbit anti-EGFR (4267, CST), goat anti-EGFR (AF231, R&D Systems, Minneapolis, MN, USA), mouse anti-phospho-EGFR(Tyr845) (558381, BD Biosciences, Heidelberg, Germany), rabbit anti-phospho-EGFR(Tyr1045) (2237, CST), mouse anti-phospho-EGFR(Tyr1068) (2236, CST), goat anti-EphA2 (R&D Systems), rabbit anti-phospho-Eph(Tyr588/596) (Abcam, Cambridge, UK), mouse anti-ERK1/2 (4696, CST), rabbit anti-phospho-ERK(Thr202/Tyr204) (4370, CST), mouse anti-Rab5 (610724, BD Biosciences), rabbit anti-Rab7 (9367, CST), rabbit anti-phospho-Rb(Ser807/811, CST) mouse anti-tubulin (6074, Sigma-Aldrich)

Secondary antibodies

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

Plasmids

Generation of EGFR-mCitrine, EGFR-mCherry, EGFR-paGFP, PTB-mCherry and HA-ubiquitin (Baumdick et al. 2015), and EphA2-mCitrine (Sabet et al. 2015) were previously described. pcDNA3.1-EphA2 was a gift from Tony Pawson.
Reagents

AktVIII (sc-3513, Santa Cruz Biotechnology, Dallas, TX, USA), EGF (AF-100-15, Peprotech, Hamburg, Germany), okadaic acid (sc-3513, Santa Cruz Biotechnology), YM201636 (13576, Biomol GmbH, Hamburg, Germany), dynole 31-2 (ab120464, Abcam), dynole 34-2 (ab120463, Abcam), Kisspeptin-10 (445888, Merck Millipore). EGF-Alexa647 was prepared as previously described (Baumdick et al. 2015). EphrinA1-Fc (602-A1-200) was preclustered by incubating with chicken Anti-Fc (GW200083F, Sigma-Aldrich) at a ratio of 5:1 at room temperature for at least 30 min.

Cell culture

Cos-7, HEK293 and NIH 3T3 cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and 1% non-essential amino acids (NEAA) and maintained at 37°C in 5% CO₂. MCF10A cells were grown in DMEM/F12 supplemented with 5% horse serum, 20 ng/ml EGF, 500 ng/ml hydrocortisone, 100 ng/ml cholera toxin and 10 µg/ml insulin and maintained at 37°C in 5% CO₂. MDA-MB-231 cells were grown in Leibowitz medium supplemented with 10% FBS and 2 mM L-glutamine maintained at 37°C in 0% CO₂. When required, transfection of cells was performed using FUGENE6 (Roche Diagnostics, Mannheim, Germany) or Lipofectamine 2000 (Life Technologies, Darmstadt, Germany) according to manufacturer’s protocol. Approximately 16-18 hours prior to an experiment, cells were starved in DMEM containing 0.5% FBS, 2 mM L-glutamine and 1% NEAA. One hour before stimulation, starvation media was changed to serum-free DMEM or DMEM without phenol red for live cell imaging. Unless explicitly stated in the figure legends, all experiments were performed with Cos-7 cells with endogenous expression of EGFR and Eph receptors.

In-cell westerns (ICW) and on-cell westerns (OCW)

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

**Immunofluorescence**

Cells were cultured on 4-well chambered glass slides (Lab-tek, Thermo Fisher Scientific, Waltham, MA) and fixed with 4% paraformaldehyde/PBS (w/v) for 10 min at 4°C. To measure PM EGFR, fixed, non-permeabilized samples were first incubated with primary antibody directed at an extracellular epitope of EGFR (AF231, R&D Systems, 1:200) overnight at 4°C followed by secondary antibody for 1 h at room temperature. For all other immunofluorescence experiments, samples were permeabilized with 0.1% Triton X-100 (v/v) for 5 min at room temperature prior to incubation with primary antibodies. All wash steps were performed with TBS (pH 7.4). Fixed samples were imaged in PBS at 25°C. For all analysis, an initial background subtraction was performed on immunofluorescence images. To quantify the proportion of EGFR in Rab5 and Rab7 compartments, binary masks were generated from intensity thresholded images of Rab5 and Rab7 staining. To generate a mask of Rab5/Rab7 double positive endosomes, the product of their individual masks was used. The integrated fluorescence intensity of EGFR-mCherry was determined in each of the endosomal masks and divided by the total integrated EGFR fluorescence intensity of the cell. All analysis was performed using ImageJ. A cell segmentor tool was developed in-house in Anaconda Python (Python Software Foundation, version 2.7, https://www.python.org/) to quantify the spatial distribution of EGFR and pY845-EGFR in fixed cells. Cells were divided into 6 equally spaced radial bins emanating from the centre of cell mass.
Confocal imaging

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

Olympus Fluoview™ FV100
The Olympus Fluoview™ FV1000 confocal microscope was equipped with a temperature controlled CO₂ incubation chamber at 37°C (EMBL, Heidelberg, Germany) and a 60x/1.35 NA Oil UPLSApo objective (Olympus, Hamburg, Germany). EphA2-mCitrine and EGFR-mCherry were excited using a 488 nm Argon-laser (GLG 3135, Showa Optronics, Tokyo, Japan) and a 561 nm DPSS laser (85-YCA-020-230, Melles Griot, Bensheim, Germany), respectively. Detection of fluorescence emission was restricted with an Acousto-Optical Beam Splitter (AOBS) for mCitrine @ 498-551 nm and mCherry @ 575-675 nm. In all cases, scanning was performed in frame-by-frame sequential mode with 2x frame averaging. The pinhole was set to 250 μm.

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

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

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

Quantification of PM EGF-Alexa647 binding and PTB-mCherry translocation was performed using the cell segmentation tool described above. To quantify PTB-mCherry translocation to the PM, the mean intensity of the outermost, PM segment was divided by the mean nuclear intensity. To determine the proportion of phosphorylated EGFR at the PM, PM translocated PTB-mCherry was divided by the PM intensity of EGFR-mCitrine.

Photoactivation experiments were carried out at 37°C on a Leica SP8. EGFR-mCherry was co-expressed to identify and select regions of endosomal EGFR for photoactivation. Background intensity of EGFR-paGFP prior to photoactivation was measured and subtracted from post-activation images. Photoactivation of EGFR-paGFP was performed with the 405nm laser at 90% power. Following photoactivation, fluorescence images of EGFR-paGFP were acquired every minute for a total of 15 minutes. PM EGFR-paGFP fluorescence was quantified as the integrated intensity in a 5-pixel ring of the cell periphery and, after subtracting pre-activation background intensity, was calculated as a proportion of total EGFR-paGFP intensity.

To measure changes PM EGF-Alexa647 binding with time, binary masks of endosomal EGF-Alexa647 were generated from intensity thresholded images and PM EGF-Alexa647 intensity was calculated by subtracting endosomal EGF-Alexa647 from total EGF-Alexa647 fluorescence intensity. The percent of PM EGF-Alexa-647 was calculated by dividing PM-
EGF-Alexa647 by total fluorescence intensity. Curves were fit with a one-phase exponential decay in GraphPad Prism v6 (GraphPad Software, La Jolla, CA, USA) and grouped by their rates of loss in PM EGF binding using the k-means clustering package in R (www.r-project.org).

*Flow cytometry*

Cells were detached using accutase, centrifuged at 200g for 5 min and resuspended in serum-free DMEM. Cells were fixed with 5% sucrose/Roti-Histofix (w/v) for 15 min at 37°C. Ice-cold methanol was added to 90% (v/v) for 30 min on ice. Cells were rinsed once with 0.5% BSA/TBS (w/v) and incubated with Odyssey TBS blocking buffer (LI-COR Biosciences) for 30 min at room temperature. Anti-phospho-Akt(Ser473)-Alexa647 (4075, Cell Signalling Technology) was added directly to blocking buffer and incubated overnight at 4°C. Anti-Akt-Alexa488 (2917, Cell Signalling Technology) was added for 2 h prior to measurement. Samples were analyzed using the LSRII flow cytometer (BD Biosciences). Alexa488 was excited with a 488nm laser and fluorescence emission was collected using a 505 nm LP dichroic and a 530/30 nm filter. Alexa647 was excited with 633 nm lasers and fluorescence emission was collected using a 670/40 nm filter. Samples were analyzed using FlowJo v10 (FlowJo, LLC, Ashland, OR, USA) to obtain single cell intensity measurements of phospho- and total Akt. Population distributions of log(phospho/total Akt) were fitted with a single Gaussian or a sum of two Gaussian distributions using GraphPad Prism.

*Cell migration*

MEF cells were seeded onto fibronectin-coated (F0895, Sigma, 1.25 ug/cm²) 12 well culture dishes (83.3921, Sarstedt, Nuembrecht, Germany) containing 2-well Culture-Inserts (80209, ibidi) to create a cell-free area. Immediately before stimulation, inserts were removed and cells were incubated with Hoechst to label nuclei. Wide field images were acquired using an Olympus IX81 inverted microscope (Olympus, Hamburg, Germany) equipped with a MT20 illumination system, a 4x/0.16 NA air objective and an Orca CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). Transmission and fluorescence images were acquired every 10 min for 16 h. The cell-free area created by the Culture-Insert was cropped using ImageJ and defined as the migration region. Individual cells were
detected and tracked by their nuclear Hoechst staining as they travelled within the
migration region using the TrackMate ImageJ plugin (Tinevez et al. 2016), and the total
distance of each track was quantified.

*Immunoprecipitation (IP) and western blotting*

Cells were lysed in TGH (150 mM NaCl, 2 mM EGTA/EDTA, 50 mM HEPES (pH 7.4), 1%
Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 mM N-
ethylmaleimide (NEM)) or RIPA (for immunoprecipitation; 50 mM Tris–HCl (pH 7.5), 150
mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate, 0.2% SDS,
2.5 mM sodium pyrophosphate and 10 mM NEM), supplemented with Complete Mini
EDTA-free protease inhibitor (Roche Applied Science, Heidelberg, Germany) and 100 µl
phosphatase inhibitor cocktail 2 and 3 (P5726 and P0044, Sigma Aldrich). Lysates were
sonicated prior to centrifugation at 14,000 rpm for 10 min at 4°C to separate non-soluble
material. For immunoprecipitation, cell lysates were incubated with 50 µl washed Protein
G magnetic beads (10003D, Life Technologies) for 1 h at 4°C to pre-clear the samples from
unspecific binding proteins. Supernatants were incubated with primary antibody alone for
2 h followed by the addition and overnight incubation with Protein G magnetic beads at 4°C
with agitation. SDS–PAGE was performed using the X-cell II mini electrophoresis apparatus
(Life Technologies) according to the manufacturer’s instructions. Samples were transferred
to preactivated PVDF membranes (Merck Millipore, Billerica, MA) and incubated with the
respective primary antibodies at 4°C overnight. Detection was performed using species-
specific secondary IR-Dye secondary antibodies (LI-COR Biosciences) and the Odyssey
Infrared Imaging System (LI-COR Biosciences). The integrated intensity of protein bands of
interest was measured using the ImageJ software and signals were normalised by dividing
the intensities of phosphorylated protein by total protein intensities or by dividing
intensities of co-immunoprecipitated proteins by the corresponding immunoprecipitated
protein.
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Figure Legends:

**Figure 1: Akt-dependent recycling of EGFR sustains its autocatalytic activation at the PM.** (a) Representative images of Cos-7 cells expressing EGFR-mCherry before (top left) and after (top right) treatment with the Akt inhibitor AktVIII (10 µM, 1 h) and quantification of the increase in endosomal EGFR-mCherry during AktVIII treatment (N = 6). See also Supplementary Movie 1. (b) Quantification of PM EGFR abundance by On-cell Western (OCW, top) and Akt activation by In-cell Western (ICW, bottom) following treatment with AktVIII for the times indicated. (c) Immunofluorescence measurements of EGFR intensity in Rab5-, Rab5/Rab7- and Rab7-positive endosomal compartments as a proportion of total EGFR intensity in DMSO (control, white, N = 6) and AktVIII (orange, N = 11) treated conditions, as well as DMSO- (red, N = 9) and AktVIII-pretreated (green, N = 8) conditions followed by EGF stimulation (100 ng/ml, 1 h). (d) Quantification of PM EGFR abundance and Akt activation by OCW and ICW, respectively, following treatment with the PP2A inhibitor okadaic acid (OA, 1 µM, 2 h). (e) Measurements of EGFR-paGFP recycling to the PM following photoactivation in endosomes (top) in control (gray, N = 10), EGF (20 ng/ml, 15 min, red, N = 6), AktVIII (orange, N = 9) and AktVIII-pretreated, EGF-stimulated (green, N = 8). (f,g) ICW measurements of total EGFR abundance (f) and phosphorylation (g) following EGF stimulation (1-200 ng/ml, 30 min) in DMSO (control, gray) and AktVIII (orange) pretreated cells. EGFR phosphorylation was measured using antibodies recognizing phosphorylated Y845 (g, top), Y1068 (g, middle) and Y1045 (g, bottom). (h,i) Live cell measurements of EGF-Alexa647 binding to the PM (green) and PTB-mCherry translocation to the PM (magenta) with increasing EGF-Alexa647 concentrations (1, 3, 10 and 20 ng/ml, as indicated) for 2 min each in control (h, N = 9) and AktVIII-pretreated (i, N = 7) cells. (j) Comparison of PTB-mCherry translocation for equivalent numbers of liganded receptors for data presented in h,i. (k) Spatial-temporal maps of EGFR abundance (top) and Y845 phosphorylation (bottom) obtained by immunofluorescence in radially segmented cells (plasma membrane, PM → nuclear membrane, NM) prior to and after EGF stimulation (20 ng/ml for 5, 30 and 60 min) in control (left), AktVIII- (middle) and YM201636- (YM, 200 nM, 1 h) pretreated cells (N = 50-90 cells per condition).
network topology showing positive feedback generated by coupling PM EGFR activity and 
Akt-dependent vesicular trafficking. PIKfyve inhibition by YM decouples Akt activation 
from its effect on EGFR recycling. All experiments were performed in Cos-7 cells. Data in c 
are represented by Tukey boxplots with the mean denoted as a cross and the median as a 
line. All other data represents means ± s.e.m. Statistical significance was determined in c 
using an ordinary one-way ANOVA with Sidak’s multiple corrections post-hoc test, in d 
using a Student’s t test and in f,g using a two-way ANOVA with Sidak’s multiple corrections 
post-hoc test (***, p > 0.001; **, p > 0.01; *, p > 0.05). Scale bars = 20 µm
Figure 2: Eph receptor activation regulates EGFR vesicular trafficking. (a)
Representative images of Cos-7 cells expressing EGFR-mCherry and EphA2-mCitrine
(green and magenta in merged images, respectively) following stimulation with EphrinA1-Fc (A1, 2 µg/ml). See Supplementary Movie 2. (b) Quantification of endosomal EphA2-mCitrine and EGFR-mCherry from time-lapse confocal imaging during A1 stimulation (N = 7). (c) Immunofluorescence measurements of EGFR intensity in Rab5-, Rab5/Rab7- and Rab7-positive endosomal compartments as a proportion of total EGFR intensity in control (white, N = 6) and A1 treated (60 min, magenta, N = 9) (d) Quantification of PM EphA2 and EGFR abundance by OCW during A1 treatment. (e) Cos-7 lysates immunoprecipitated (IP) with anti-EGFR (left) or blotted for total proteins (right) following stimulation with EGF (100 ng/ml) or A1 for the indicated times. IP was probed with anti-HA (to detect co-transfected HA-ubiquitin), anti-pY845, anti-pY1068, anti-pY1045 and anti-EGFR. Lysates were probed with anti phospho-Eph (pEph), anti-EphA2, anti-pY1068, anti-EGFR and anti-tubulin. Shown are representative blots from three independent experiments. (f) Measurements of EGFR recycling in control (gray) and A1-pretreated cells (green). PM/total EGFR was quantified by immunofluorescence prior to (pre), after 15 min EGF stimulation (10 ng/ml) and 15 min following EGF washout (N = 34-40 cells/condition). All experiments were performed in Cos-7 cells. Data in c are represented by Tukey boxplots with the mean denoted as a cross and the median as a line. All other data represents means ± s.e.m. Statistical significance was determined in c using an ordinary one-way ANOVA with Sidak’s multiple corrections post-hoc test and in d using a two-way ANOVA with Dunnett’s multiple corrections post-hoc test. Scale bars = 20 µm.
**Figure 3:** Cell-cell contact alters the spatial distribution of EGFR activity. (a) Spatial-temporal maps of EGFR abundance (top) and Y\textsubscript{845} phosphorylation (bottom) in radially segmented cells (plasma membrane, PM → nuclear membrane, NM) obtained by immunofluorescence prior to and after EGF stimulation (20 ng/ml for 5, 30 and 60 min) in control (left) and EphrinA1-Fc (A1, 2 µg/ml, 1 h) pretreated Cos-7 cells (N = 53-90 cells per condition). Control data previously presented in Figure 1k. (b) Quantification of Akt and ERK activation by ICW in control (gray) and A1-pretreated (1 h, green) Cos-7 cells following EGF stimulation (1 ng/ml). (c) Quantification of Akt activation by ICW in HEK293 cells pretreated for 1 h with vehicle (control, gray) or A1 (green), followed by 30 min treatment with the dynamin inhibitor dynole 34-2 (100 µM, 30 min, top) or its negative control analog dynole 31-2 (bottom), then stimulated with EGF (1 ng/ml) for the times indicated. (d) Quantification of EGF-promoted Akt activation by ICW following pretreatment with increasing concentrations of A1 (0.02, 0.2 and 2 µg/ml, 1 h) in Cos-7 cells. (e,f) Single cell measurements of Akt (e) and ERK (f) activation versus cell-cell contact in 2-D cultures (% cell circumference) in control (gray) and EGF stimulated (20 ng/ml, 1 h, blue) Cos-7 cells. (g) Quantification of Akt activation (pink) and PM EGFR abundance (magenta) in HEK293 cells by ICW and OCW, respectively, following stimulation with kisspeptin-10 (Kp-10, 100 nM). (h,i) Quantification of Akt and ERK activation by ICW for control (gray) and Kp-10-pretreated (1 h, magenta) HEK293 cells following EGF stimulation (1 ng/ml). (j,k) Single cell measurements of Akt phosphorylation by flow cytometry in control (top), YM201636- (YM, 200 nM, 1h, middle) and EphrinA1-Fc- (A1, 2 µg/ml, 1h, bottom) pretreated Cos-7 cells following stimulation with the EGF concentrations indicated (ng/ml, 1 h). (j) Solid lines represent the sum of two Gaussian fits for data accumulated from at least 10 000 cells per condition in 3-4 independent experiments. (k) Quantification of Akt activation for increasing concentrations of EGF. Circle sizes represent the relative proportions of the low (gray) and high (red) Akt activity populations from the Gaussian distributions derived for each EGF concentration shown in j. (l) Quantification of PM EGF as a percent of total EGF intensity from time lapse imaging. Individual Cos-7 cells (thin lines) were clustered by their rate of PM EGF loss into two distinct groups (1, green; 2, magenta; group mean represented by thick line). (m) Rate of
loss of PM EGF binding for individual cells in each group (mean ± s.d.). All other data
represents means ± s.e.m. Statistical significance was determined using a Student’s t test.

(n) Percent of cells in group 1 (green) and 2 (magenta) for control and A1-pretreated
conditions.
Figure 4: Cell-cell contact changes the cellular outcome of EGF stimulation. (a) Percent of NIH 3T3 cells initiating a migratory response (top, means ± s.e.m) and the distance travelled by migratory cells (bottom, means ± s.d.) following EGF stimulation. Cells were pretreated with vehicle (control, blue), YM201636 (YM, 200 nM, 1 h, purple), or EphrinA1-Fc (A1, green: 2 µg/ml, 1h, or red: 0.02-2 µg/ml as indicated) followed by EGF stimulation (0-100 ng/ml as indicated) and tracked for 16 h. See Supplementary Movie 4. (b) NIH 3T3 cells were seeded at low and high density in a single well, separated by a removable insert. Following pretreatment with vehicle or A1 (2 µg/ml, 1 h), cells were stimulated with EGF (20 ng/ml) and tracked for 16 h (means ± s.e.m). (c) Quantification of retinoblastoma (Rb) phosphorylation by ICW for vehicle- (control, blue), A1- (green) and YM- (magenta) pretreated NIH 3T3 cells following 24 h EGF stimulation at the concentrations indicated (means ± s.e.m). Data in a and b were obtained from least three independent experiments, consisting of at least two replicates per experiment (N = 581 – 1483 cells/condition). Statistical significance in a and b was determined using an ordinary one-way ANOVA with Holm-Sidak’s multiple corrections post-hoc test.
Figure 1: Akt-dependent recycling of EGFR sustains its autocatalytic activation at the PM.
Figure 2: Eph receptor activation regulates EGFR vesicular trafficking.
Figure 3: Cell-cell contact alters the spatial distribution of EGFR activity.
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