Cell adhesion defines the topology of endocytosis and signaling

Jean-Philippe Grossier¹, Georgia Xouri², Bruno Goud¹,* & Kristine Schauer¹,**

Abstract

Preferred sites of endocytosis have been observed in various cell types, but whether they occur randomly or are linked to cellular cues is debated. Here, we quantified the sites of endocytosis of transferrin (Tfn) and epidermal growth factor (EGF) in cells whose adhesion geometry was defined by micropatterns. 3D probabilistic density maps revealed that Tfn was enriched in adhesive sites during uptake, whereas EGF endocytosis was restricted to the dorsal cellular surface. This spatial separation was not due to distributions of corresponding receptors but was regulated by uptake mechanisms. Asymmetric uptake of Tfn resulted from the enrichment of clathrin and adaptor protein 2 at adhesive areas. Asymmetry in EGF uptake was strongly dependent on the actin cytoskeleton and led to asymmetry in EGF receptor activation. Mild alteration of actin dynamics abolished asymmetry in EGF uptake and decreased EGF-induced downstream signaling, suggesting that cellular adhesion cues influence signal propagation. We propose that restriction of endocytosis at distinct sites allows cells to sense their environment in an "outside-in" mechanism.

Keywords cell asymmetry; hot spots; micropatterns; spatial memory

Subject Categories Membrane & Intracellular Transport; Signal Transduction

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Introduction

Endocytosis is a fundamental process that allows eukaryotic cells to internalize macromolecules from their environment. Many endocytic mechanisms exist, either through the well-characterized clathrin-dependent uptake or through alternative clathrin-independent pathways that have been studied to a lesser extent (Doherty & McMahon, 2009). Despite a large body of publications, only a few studies have focused on the spatial organization of endocytosis. It has been shown in some specialized cells that endocytosis does not occur randomly along the entire cell surface but is restricted to discrete sites. For instance, hepatocytes internalize trophic receptors at endocytic 'hot spots' that are defined by large membrane invaginations at the ventral side of the cell (Cao et al, 2011). In neurons, 'hot spots' of endocytosis have been observed in close proximity to exocytic sites during high-frequency stimulation (Gaffield et al, 2009). Predefined sites at which clathrin-coated pits nucleate repeatedly have also been observed in various cell lines (Gaidarov et al, 1999; Ehrlich et al, 2004; Kirchhausen, 2009; Saffarian et al, 2009; Batchelder & Yarar, 2010; Nunez et al, 2011; Taylor et al, 2011). Some of these studies argue for the existence of defined endocytic platforms at the plasma membrane (Gaidarov et al, 1999; Nunez et al, 2011). The plasma membrane could compartmentalize endocytosis by differential sorting of cargo molecules through the action of adaptor proteins that are present at specific and limited loci (Gaidarov et al, 1999; Lakadamyali et al, 2006; Leonard et al, 2008). Against the existence of pre-existing sites, it has been suggested that clustered endocytic events occur by random initiation and stabilization of clathrin-coated pits (Ehrlich et al, 2004).

Another hypothesis that has not been directly tested is that the presence of predefined sites of endocytosis is linked to cellular adhesion cues. To address this issue, we sought to exploit an approach developed in our laboratory that combines normalization of cellular adhesion geometry by micropatterning (Thery et al, 2006b) with quantification of spatial organization of trafficking molecules by density mapping (Schauer et al, 2010). Micropatterns of extracellular matrix proteins that impose adhesive and non-adhesive areas to cultured cells, allow the control of internal cell organization, mimicking the microenvironment found in tissues (Gumbiner, 1996). To measure spatial organization of labeled intracellular structures in micropatterned cells, the corresponding fluorescent signals are transformed into probabilistic, three-dimensional density maps (supplementary Fig 1A). Here, we analyze the intracellular distribution of endocytosed transferrin (Tfn) and epidermal growth factor (EGF), two classical ligands of receptor-mediated endocytosis, in micropatterned cells in order to establish the role of cellular adhesion on uptake mechanisms and to address the potential function of predefined sites of endocytosis.

Results

Pulse-chase experiments were performed on crossbow-shaped human retinal pigment epithelial (RPE-1) cells using fluorescently
labeled Tfn-conjugate at 20 µg/ml and EGF-conjugate at 1 µg/ml to visualize their uptake by light microscopy (Fig 1A). Of note, the EGF-conjugate being a complex of biotin-EGF and labeled streptavidin, the effective concentration of EGF was 100 ng/ml. Fluorescent microscopy images of Tfn/EGF taken at various time points were segmented and the 3D spatial coordinates of the structures were replaced by kernels and summed, revealing their underlying density throughout the cell. The reproducible cellular orientation induced by the micropattern allows the collation of several tens of cells and the construction of average density maps for the cell population analyzed. In contrast to classical, single particle-based, eulerian approaches, probabilistic mapping is a population based, lagrangian approach.

Figure 1. Uptake of transferrin (Tfn) and epidermal growth factor (EGF) in micropatterned cells.

A Maximum intensity projection (MIP) of fluorescently marked Tfn (upper panel) and EGF (lower panel) in single crossbow-shaped micropatterned cells 5 and 10 min after ligand addition. Scale bars, 10 µm.

B Corresponding 3D density maps of the 50% probability contour of Tfn (upper panel) and EGF (lower panel) at 5 and 10 min for n cells. Density maps are shown in two different views and represent the smallest regions in which 50% of fluorescent structures are found. For ease of visualization, the height (z axis) has been stretched fivefold.

C MIPs and merged images of fluorescently marked Tfn and EGF in single crossbow-shaped (upper panel), disc-shaped (middle panel) and ring-shaped (lower panel) cells after 1 min of ligand addition. Corresponding y–z projections (of the yellow region) are shown at the bottom. Scale bars, 10 µm.

D Corresponding 3D density maps of the 50% probability contour of Tfn (green) and EGF (red) for n cells.

Source data are available online for this figure.
method that focuses on aggregated system behavior. Thus, density maps provide information about the behavior of the majority of endocytosed molecules but give less accurate knowledge of individual uptake events. We calculated the 50% density contours that represent the smallest intracellular volume where 50% of total Tfn or EGF signals are found. These maps showed that Tfn and EGF accumulated at the cell periphery at 5 min post-pulse and further concentrated in the cell center at 10 min post-pulse (Fig 1B). Object-based colocalization analysis of endosomal marker proteins in micropatterned cells showed that both cargos were first shuttled to Rab5-positive early endosomes before Tfn concentrated in the Rab11-positive recycling compartment and EGF concentrated in the Rab7-positive late endosomal compartment (supplementary Fig 1B). Hence, trafficking dynamics of Tfn and EGF were in agreement with previous experiments performed in non-patterned cells (Leonard et al., 2008).

Intriguingly, when density maps of Tfn and EGF were compared at 1 min post-pulse, a strong and unexpected difference in their distributions was observed (Fig 1C,D). The Z-projection of the fluorescence images of single cells (Fig 1C) as well as quantitative density maps (Fig 1D) revealed that Tfn was densest at adhesive areas at the bottom of the cells matching the fibronectin micropattern geometry. A correlation between Tfn uptake topology and cell adhesion sites was further observed using different micropattern shapes such as a ring and a disc (Fig 1C,D, middle and lower level). In contrast, EGF was concentrated on the upper, dorsal surface of the cell. To verify this observation, we additionally calculated the 75 and 90% density maps. Although Tfn uptake was densest above the micropattern, Tfn uptake was also detected on the dorsal part of the cell as judged by the 90% contour (supplementary Fig 1C). In contrast, EGF uptake was restricted to the dorsal part even when taking into account the 90% contour, with no EGF density at the ventral side. In non-patterned RPE-1 cells (either seeded on uncoated or fibronectin-coated coverslips), Tfn also concentrated at the cell bottom and EGF was densest at the upper side (supplementary Fig 1D), although this was more difficult to monitor than in patterned cells. Similar results were obtained with HeLa cells and cells that form polarized epithelia, LLC-PK1 and Caco-2 cells, although we noticed for these cell lines more permeabilization of the RPE-1 cells (either seeded on uncoated or fibronectin-coated coverslips) than in patterned cells (Leonard et al., 2008).

The EGF-conjugate used in this study is about the same size (MW = 68 kDa) as the Tfn-conjugate (MW = 80 kDa), suggesting that the difference between EGF and Tfn uptake topology cannot be explained by a lack of accessibility of EGF to the ventral part of the cell. In addition, rhodamine-labeled EGF (MW = 6.5 kDa) displayed the same behavior as the EGF-conjugate (supplementary Fig 3A). Then, we investigated the cellular distribution of the corresponding receptors in non-permeabilized micropatterned cells (Fig 2A,B). Tfn receptors (TfnR) were mainly localized at the bottom of the cell where they were distributed throughout the entire ventral side (Fig 2A, green). Different from Tfn, TfnR distribution did not exactly match the pattern shape. More surprisingly, EGF receptors (EGFR) showed uniform distribution on the entire cell surface, including the bottom of the cell, where EGF was absent (Fig 2B, green). The distributions of the TfnR and EGFR did not change in the presence and absence of their ligands. These results demonstrated that receptor distribution did not explain the topology of Tfn and EGF endocytosis.

Tfn is exclusively taken up by clathrin-dependent endocytosis and requires the clathrin adaptor protein AP2 (Smythe et al., 1992). Thus, we investigated the localization of clathrin and AP2 in micropatterned cells. The characteristic density maps of clathrin and AP2 showed a crossbow shape similar to the micropattern and the endocytosed Tfn density map after 1 min pulse (Fig 2C). The distribution of clathrin was similar in the presence and absence of Tfn. As expected, gene silencing of clathrin heavy chain reduced the number of Tfn structures by 90% (Fig 2D,E). These results suggest that concentration of Tfn uptake above the micropattern is due to the selective recruitment of AP2 and clathrin to adhesive areas.

Epidermal growth factor uptake involves different mechanisms depending on EGF concentration and cell types (Yamazaki et al., 2002; Orth et al., 2006; Sigismund et al., 2008, 2012). At high concentrations (>100 ng/ml) as used in this study for fluorescent visualization, it has been shown that a substantial proportion of EGF (≥50%) is taken up by clathrin-independent mechanisms. In RPE-1 cells, EGF uptake was not altered by clathrin knock down but was strongly inhibited by filipin treatment (the number of EGF structures was reduced by 80%, Fig 2D–F). Filipin binds specifically to cholesterol and has been shown to inhibit clathrin-independent endocytosis of EGF (Sigismund et al., 2008). Notably, the characteristic density map of endocytosed EGF did not correlate with those of clathrin and AP2 (compare Fig 1D with Fig 2C). To better understand the mechanisms of EGF uptake in RPE-1 cells, we varied the EGF concentration and performed co-localization analysis of EGF and clathrin. Decreasing the effective EGF concentration to 10 ng/ml, which should favor clathrin-dependent uptake, did not modify the density maps, indicating that EGF uptake was still predominately restricted to the dorsal cell surface (supplementary Fig 3B). Co-localization analysis of EGF and clathrin showed that at high concentration of EGF (100 ng/ml), a substantial portion of the EGF co-localized with clathrin (14 ± 3%) (Fig 2G). Moreover, at low EGF concentration (10 ng/ml), the percentage of co-localization increased to 20 ± 6%. It is likely that in RPE-1 cells EGF uptake is partly clathrin-dependent at the upper cell surface, but that this process can be fully compensated upon clathrin depletion.

The cellular cytoskeleton organization intimately depends on the adhesion geometry of a cell (Thery et al., 2006a). We therefore asked if cytoskeleton networks could define the topology of uptake. Actin fibers were disrupted with Cytochalasin D (CD) and microtubules were depolymerized with Nocodazole (NZ). Remarkably, a mild treatment with CD redistributed Tfn as well as AP2 from a ventral localization matching the micropattern to the entire lower cell surface (Fig 3A,B middle panels). Although this change in Tfn/AP2 topology was subtle, it demonstrated that concentration of Tfn uptake/AP2 at cell-matrix interaction sites is dependent on the actin cytoskeleton. However, Tfn uptake remained quantitatively the same as the number of Tfn structures (Fig 3C) and the colocalization with the early endosome marker Rab5 at 5 min post-pulse (supplementary Fig 3C) was comparable to that in control cells. This is in agreement with published data showing that disruption of the actin network does not significantly affect Tfn uptake (Gaidarov et al., 1999). In contrast, treatment with NZ did not change the topology of either Tfn or AP2 (Fig 3A,B right panels).

Epidermal growth factor uptake topology was also modified due to actin disruption but not upon microtubule depolymerization (Fig 3E). Treatment with several drugs that either destabilized (CD, Latrunculin A) or stabilized (Jasplakinolide) actin fibers or inhibited acto-myosin contraction (Blebbistatin), redistributed EGF uptake uniformly all around the cell (Fig 3E and supplementary Fig 3D).
Figure 2. 3D distribution of Tfn receptor (TfnR) and EGF receptor (EGFR) in crossbow-shaped micropatterned cells and the role of clathrin in Tfn and EGF uptake.

A–C MIPs and merged images of single crossbow-shaped micropatterned cells (upper panel), and corresponding 3D density maps of the 50% probability contours for n cells (lower panel). Density maps are shown in two different views. Fluorescently marked Tfn and TfnR (without permeabilization) after 1 min of ligand addition (A), fluorescently marked EGF and EGFR (without permeabilization) after 1 min of ligand addition (B) and immunostained clathrin heavy chain and AP2 (C) are shown. Scale bars, 10 μm.

D Immunoblot of cells transfected with siRNA against luciferase or clathrin heavy chain (CHC) for 72 h.

E Normalized count of Tfn and EGF structures per cell after 72 h of transfection with siRNA against luciferase (control) or clathrin heavy chain (CHC). Results are from three independent experiments and 80 cells per condition.

F Normalized count of EGF structures per cell after 1 h of treatment with DMSO or filipin. Results are from two independent experiments and 54 cells per condition.

G Co-localization analysis of EGF at different concentrations with clathrin, for ≥ 40 cells per condition from two independent experiments.

Data information: In (E), (F) and (G), error bars are s.d.
Similar changes upon mild disruption of the actin cytoskeleton were obtained with rhodamine-coupled EGF and EGF at 10 ng/ml (supplementary Fig 3D). Depletion of the actin nucleation regulatory protein complex Arp2/3 (Rottner et al, 2010) led to a similar effect (supplementary Fig 3E). The number of EGF structures was comparable to that in control cells under all tested conditions (Fig 3D and supplementary Fig 3F) and the EGF uptake was not inhibited as revealed by colocalization analysis with the early endosome marker Rab5 (supplementary Fig 3C).

Tfn and EGF uptake assays are often performed by incubating the cells at 4°C with saturating amounts of ligands followed by washes and transfer to 37°C. Under these conditions, Tfn was internalized from the entire lower surface and EGF internalization was observed all around the cell, similar to conditions in which actin was disrupted and similar to the corresponding receptor distributions (supplementary Fig 4 A,B). Interestingly, incubation of cells at 4°C altered the actin cytoskeleton, reinforcing the hypothesis that actin dynamics play an important role in the topology of endocytosis (supplementary Fig 4C).

We additionally monitored the dynamics of Tfn and EGF uptake at the adhesive plasma membrane by total internal reflection fluorescence microscopy (TIRFM) (Fig 4 A and supplementary mov-
Cells stably expressing EGFP-tagged clathrin heavy chain were imaged after 1 min of addition of Tfn or EGF. In agreement with results obtained in fixed cells, Tfn was present at the lower plasma membrane and showed colocalisation with EGFP-tagged clathrin heavy chain (supplementary movie 1), whereas EGF (supplementary movie 2) was almost not detectable at the bottom of the cell by TIRFM (Fig 4A, left panels). Similarly, mild disruption of the actin cytoskeleton with CD changed uptake topology, as Tfn (supplementary movie 3) and EGF (supplementary movie 4) were both detected at the lower plasma membrane (Fig 4A, right panels). This demonstrates that spatial separation of Tfn and EGF uptake on micropatterned cells is consistent under different experimental conditions.

We noticed in TIRFM that mild disruption of the actin cytoskeleton led to higher co-localization between EGF and clathrin (Fig 4A, lower panels, Fig 4B). To further investigate this observation we analyzed the co-localization between Tfn or EGF and endogenous clathrin (Fig 4C). In the presence of CD, EGF co-localized more with endogenous clathrin (21 ± 8%) than in control conditions. On the other hand, the extent of co-localization was not changed between Tfn and clathrin under the same conditions. Concomitantly, co-localization between EGF and Tfn increased substantially upon actin disruption (using different drugs, supplementary Fig 3G). These results question whether depolymerization of the actin cytoskeleton led to a switch in the uptake mechanism of EGF requiring careful analysis in future studies.

Next, we investigated the effects of the disruption of the actin cytoskeleton in HeLa, LLC-PK1 and Caco-2 cells. As observed in RPE-1 cells, the treatment of HeLa and LLC-PK1 cells with CD...
Adhesion cues place signaling into the cellular space

Jean-Philippe Grossier et al.

Eukaryotic cells display an intrinsic internal polarity relying on the anisotropy of the actin and microtubule cytoskeleton that is most obvious when cells migrate or form epithelia. Studies using fibronectin micropatterns have illustrated that cell internal polarity responds to the geometry of cellular adhesion cues (Thery et al., 2006b). We have previously found that trafficking compartments such as multivesicular bodies/lysosomes show an anisotropic distribution in cells grown on polarizing micropatterns (Schauer et al., 2010). The present study reveals an unexpected dorsal/ventral asymmetry in both clathrin-dependent and clathrin-independent endocytosis that predefines uptake of Tfn and EGF at distinct cellular sites.

We found that the distribution of both TfnR and EGFR does not define the sites of endocytosis, indicating that the presence of receptors at the plasma membrane is not sufficient to initiate endocytosis after cargo binding. This suggests that additional mechanisms such as receptor activation and/or the types of endocytosis need to be considered. In the case of TfnR, endocytosis is likely initiated by a specific recruitment of AP2 and clathrin to adhesive sites, possibly by SNX9 that was found to organize nucleation of clathrin coated pits (Nunez et al., 2011). Our results show that Tfn, and by extension, clathrin-dependent endocytosis is enriched at cell-ECM adhesions. In migrating cells, a significant accumulation of clathrin was found at the leading edge (Rappoport & Simon, 2003), which corresponds to the bow front of the micropattern. In addition, clathrin-dependent endocytosis plays a major role in the maturation, the dynamics and disassembly of focal adhesions that accumulate on the edges of the micropattern (Thery et al., 2006b; Ezratty et al., 2009; Stehbens & Wittmann, 2013). Note that although clathrin-dependent endocytosis was enriched above the micropattern, it is not restricted to these sites (supplementary Fig 1C). Indeed, clathrin is an abundant protein that is detected on the entire plasma membrane.

For EGFR, mechanisms of receptor activation and the type of endocytosis seem to be important. It was initially proposed that binding of EGF to EGFR results in the dimerization of the receptor, its activation by trans-phosphorylation and its subsequent internalization. However, the picture became far more complex through high-resolution studies visualizing receptor dimerization occurring continuously and reversibly even in the absence of ligand (Chung et al., 2010). Interestingly, Chung et al have shown that dimer formation of EGFR is not homogeneous at the (ventral) plasma membrane but occurs more prevalent at the cell periphery in agreement with our results that EGF uptake from the ventral plasma membrane is limited. The internalization mechanism of EGF has long been a subject of debate. Although EGFR has an YXXXφ motif that binds AP-2, it has been shown that the depletion thereof does not affect the internalization of EGF (Motley et al., 2003). We found that in patterned RPE-1 cells, EGF internalization was mostly clathrin-independent at the dorsal plasma membrane. This internalization was Filipin sensitive as previously reported (Sigismund et al., 2008). However, it is likely that a small portion of EGF is internalized by a clathrin-dependent mechanism, because decreasing the EGF concentration increased co-localization with clathrin. RPE-1 cells seem to fully compensate this pathway with clathrin-independent ones after

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CytoSkelton may explain the lack of effect of CD on EGF uptake in reflecting the round cellular form. This heterogeneity in the actin constrast, the Caco-2 cell line showed a strong morphological heteroge-neity in its actin cytoskeleton. Some cells formed stress fibers at the surface of the micropattern but others did not, and bundles of stress fibers were often observed along the edges of the micropattern, reflecting the round cellular form. This heterogeneity in the actin cytoskeleton may explain the lack of effect of CD on EGF uptake in Caco-2 cells.

Epidermal growth factor endocytosis has been shown to trigger signaling pathways that regulate cell proliferation, differentiation, motility and cell death (Roberts & Der, 2007; Sigismund et al., 2012). Therefore, we investigated whether EGF-induced signal transduction through the mitogen-activated protein kinase (MAPK) pathways (Seger & Krebs, 1995) was altered upon changes in EGF uptake topology. To redistribute EGF uptake throughout the entire cell, the actin cytoskeleton was disrupted with CD and immuno-blot analysis was performed against phosphorylated EGFR (p-EGFR) and downstream signaling molecules, p-Akt and p-ERK1/2, at different time points after EGF stimulation. Whereas phosphorylation of EGFR was not significantly changed after EGF addition, the amount of p-Akt and p-ERK1/2 was significantly decreased in cells treated with CD (Fig 5A,B), indicating that downstream EGF-mediated signaling has been altered. Similar results were obtained after inhibition of the actomyosin network with Blebbistatin (supplementary Fig 5A).

Finally, we asked whether asymmetry in EGF uptake is translated into asymmetry in signal transduction. For this, EGF-induced signal initiation was directly monitored in living cells using a fluorescence resonance energy transfer (FRET)-based probe. Micropatterned cells stably expressing a modified FLAME probe (Offterdinger et al., 2004) that monitors phosphorylation of the EGFR due to changes in FRET ratio (Fig 5C,D and supplementary Fig 5B) were analyzed. Ratiometric image analysis was performed at several z-planes for each cell studied and at different areas (central versus peripheral areas) for each z-plane (Fig 5C and supplementary Fig 5C). We found that addition of EGF decreased FRET ratio at the cell periphery at the upper and middle part of the cells but did not change FRET ratio at the central region at the bottom of the cells (Fig 5D, left panel). This demonstrates that asymmetry in EGFR endocytosis leads to asymmetric EGFR phosphorylation in micropatterned cells. Similar analyses were performed in cells whose actin cytoskeleton was disrupted by a mild treatment with CD (Fig 5D, right panel). Under this condition, EGFR stimulation was detected in all parts of the cell. These results directly demonstrate that actin-dependent asymmetry in EGF uptake leads to asymmetry in EGFR activation.
depletion of clathrin. Moreover, we found that depolymerization of the actin cytoskeleton increased co-localization between EGF and clathrin, especially at the lower plasma membrane. The actin network is critical to define and maintain asymmetry in EGF and Tfn uptake. In accordance to the ‘fence and picket’ model, the actin cortex partitions the plasmalemma into membrane domains with potentially variable molecular properties (Kusumi & Sako, 1996). Our results show that the actin cytoskeleton integrates the cell with its extracellular environment using a delicate balance between restriction and propagation of endocytosis. As a consequence, differences in the actin cytoskeleton observed in distinct cell lines may lead to subtle differences in ligand internalization. This seems to be particularly important for ligands that can enter the cell by different uptake mechanisms, like EGF. Tfn endocytosis is less restricted to adhesive sites when the actin cytoskeleton is disturbed, in agreement with previous studies showing that preformed sites of internalization are defined by the presence of cortical actin (Gaida-rov et al., 1999; Nunez et al., 2011). The role of actin in the distribution of clathrin may be more indirect, for instance destabilizing adhesion complexes, which would be no longer able to recruit AP2.
and clathrin. As the actin cytoskeleton shows strong variations between different cell lines, uptake topology of ligands may vary substantially between different cell lines.

Together our results suggest that preformed sites of internalization are spatially organized by cell adhesion and are specific for ligands. We demonstrate that the cell surface is functionally organized and that this organization is defined by an ‘outside-in’ mechanism where cellular adhesion cues permit endocytosis at distinct sites.

A tight coupling between the endocytic and signaling systems has been recognized during recent years (Scita & Di Fiore, 2010; Sigismund et al., 2012). It has been proposed that triggering internalization of EGF by clathrin-independent pathways (at 100 ng/ml) leads to the quick degradation of the EGFR, therefore attenuating signaling (Sigismund et al., 2008). Contrary, during clathrin-dependent endocytosis (at <20 ng/ml), the EGFR is recycled, which sustains signaling. We found that mild disruption of the actin cytoskeleton increased co-localization between EGF and clathrin, but reduced Akt and ERK1/2 phosphorylation indicating that additional mechanisms of regulation might exist.

We found that actin-dependent restriction of EGF endocytosis to the dorsal site was accompanied by spatial restriction of EGFR activation. It is interesting to note that a recent study described a dorsal activation of EGFR (Endres et al., 2013). Treatment with CD led to a homogenous EGFR activation without decreasing total p-EGFR levels. One possible interpretation is that, upon actin depolymerization, effectors downstream of p-EGFR are recruited to a larger surface on the plasma membrane, thus decreasing their local concentration. We propose that polarized endocytosis of ligands determines the spatial distribution of downstream signaling gradients, thus propagating signals from the extracellular into the intracellular space and allowing cells to sense their environment (Fig 5E). It has been suggested that partitioning the plasma membrane into domains, will allow cells to keep the balance between responding to an actual signal and restrict spurious events induced by noise (Grecco et al., 2011). Our observation that the cell surface is functionally organized could provide a cellular mechanism for the maintenance of spatial memory: allowing cells to sense their environment and to dynamically respond to growth factor stimulation in a polarized fashion. However, we cannot exclude that actin has a more indirect effect on downstream effectors by inhibiting their proper recruitment. Future studies will aim at addressing this issue.

In conclusion, our results highlight that endocytosis from the cell surface is asymmetric and that spatial restriction of endocytic sites plays a crucial role in signal transduction. They point out for a cellular mechanism responsible for context-dependent signaling responses.

Materials and Methods

Cells and reagents

Human Retinal Pigment Epithelial (RPE-1) cells (Invitrogen, Eugene, OR, USA) were grown in DMEM/F12 medium supplemented with 10% FBS and 1% ampicillin-streptomycin. EGFP-Rab7A, EGFP-Rab11A, EGFP-clathrin heavy chain (CHC) and FLAME—expressing stable cell lines were generated by transfection of the plasmids pEGFP-FP-Rab7Aw, pEGFP-Rab11Aw, pEGFP-clathrin heavy chain and pFLAME into RPE-1 cells and selection with 500 μg/ml geneticin. The pFLAME construct was a modified FLAME probe that contains mCitrine and mCherry as FRET donor and acceptor (Offerdinger et al., 2004). Pattern production and cell plating conditions were as previously described (Azouine et al., 2009). EGF (biotinylated and complexed to Alexa Fluor® 555 streptavidin or a tetramethylrhodamine conjugate), Tfn (from human serum, Alexa fluoro 488 conjugate, Invitrogen, Eugene, OR, USA) and phalloidin (Alexa Fluor® 350 Phalloidin) were from Invitrogen. To depolymerize microtubules, Nocodazole (NZ) was added to a final concentration of 20 μM and cells were left at 4°C for 60 min. Cells were then transferred to 37°C and incubated for 1 h. To disrupt the actin cytoskeleton, Cytochalasin D (CD) was added to a final concentration of 0.5 μM and cells were incubated at 37°C for 1 h. Latrunculin A (0.5 μM), Blebbistatin (50 μM), Jasplakinolide (1 μM) and Filipin (1 μM) were used following the same procedure. To dephosphorylate EGFR, AG1478 was added to cells at a final concentration of 100 μg/ml (Calbiochem, Darmstadt, Germany). Antibodies: anti-Rab5 (BD Biosciences, Franklin Lakes, NJ, USA), anti-Clathrin Heavy Chain (sc6579, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-EGFR (#3777, Cell Signaling Technology, Danvers, MA, USA), anti-p-ERK1/2 (#4370, Cell Signaling Technology, Danvers, MA, USA), anti-p- Akt (#4060, Cell Signaling Technology, Danvers, MA, USA), anti-p-ERK1/2 (#4370, Cell Signaling Technology, Danvers, MA, USA).

Pulse-chase experiments

Cells were seeded on micropatterned coverslips and serum starved for 2 h at 37°C in DMEM/F12 medium supplemented with 20 nM HEPS and 0.1% BSA. Cells were pulsed with fluorescently marked EGF (1 μg/ml) and Tfn (20 μg/ml) for 1 min at 37°C, then quickly washed and fixed directly in 4% PFA or chased at 37°C in DMEM/F12 medium supplemented with 20 nM HEPS for various time points and fixed. A mild acidic wash with acidic salt buffer (0.2 M sodium acetate, 0.5 M NaCl, pH 4.5) was performed to discriminate points and fixed. To dephosphorylate EGFR, AG1478 was added follow-

Immunofluorescence

PFA-fixed cells were quenched with 0.05 M NH₄Cl, washed three times with PBS and either permeabilized in PBS with 2% BSA or only incubated in PBS with 2% BSA. Cells were incubated with Alexa Fluor 488- or Cy3 coupled secondary antibod-

Image acquisition and processing

Images were acquired with an upright widefield microscope from Leica equipped with 10× UPlanApo VC oil objective, a piezoelectric motor and highly sensitive cooled interline charge-coupled device (CCD) camera (CoolSnap Hq2, Photometrics, Tucson, AZ, USA) or an inverted widefield Deltavision Core Microscope.
RPE-1 cells were seeded (300,000 cells/well) into fibronectin-coated conditions, the mean number of fluorescent objects per cell detected by applying an average intensity projection of the most in-focus image. Density map estimation was performed using the kS library in R (R Development Core Team, 2013) as previously described (Schauer et al., 2010). To visualize density maps, the extension libraries mvtnorm, rgl, miscd, ks were used.

Image analysis
For the counting of fluorescent TfR/EGF structures under different conditions, the mean number of fluorescent objects per cell detected by MIA was calculated for each condition (>20 cells per condition) and normalized to control. Co-localization analysis was performed with an in house object-based macro in ImageJ as in (Bolte & Cordelieres, 2006). Briefly, two structures were considered co-localized if the distance between their centroids (MIA segmentation output) were found inferior to the optical resolution limit. Co-localization results are given as the percentage of co-localized structures over the total number of structures. At least 20 cells per condition were analyzed. Average intensity maps of the actin distribution were obtained by applying an average intensity projection of the most in focus plane of the phalloidin staining for >20 aligned micropatterned cells.

siRNA gene silencing
Cells (10,000) were transfected with 20 pmol siRNA using standard protocols and incubated for 72 h prior further manipulations. The target sequence for clathrin heavy chain was 5′-UAAUCCAAUUUAGCAACCAAU-3′ and those for luciferase was 5′-CGUA CGCGGAUACUUGCA-3′. Arp2/3 was silenced using siRNA as in (Missetlwitz et al, 2011).

Immunoblotting
RPE-1 cells were seeded (300,000 cells/well) into fibronectin-coated 6-well plates (Millipore) and starved overnight in DMEM/F12 medium supplemented with 20 mM HEPES. Cells were treated with DMSO or Cytochalasin D for 1 h and pulsed with EGF (1 μg/ml) at 37°C for different time points. After one wash in ice-cold PBS, cells were harvested in Laemmli buffer and subjected to immuno-blotting using standard protocols. Quantification of the ECL signals from three independent experiments was performed with ImageJ.

Total Internal Reflection Fluorescence Microscopy (TIRFM)
Micropatterned EGFP-clathrin heavy chain RPE-1 cells were imaged using a Nikon Eclipse Ti Inverted Microscope (Nikon Instruments Europe BV, Amsterdam, Netherlands) equipped with a 100X/NA1.49 TIRF objective, a TIRF arm and a QuantEM EMCCD camera (Roper Scientific, Evry, France). GFP and Cy3 were excited with a 491 and 560 nm laser, respectively (100 mW, Roper Scientific, Evry, France). The system was driven by MetaMorph (Molecular Devices, Sunnyvale, CA, USA). Image sequences were recorded for 5 min at a 1 s frame rate 1 min after ligand addition.

FRET analysis of the FLAME probe
Ratiometric imaging was performed using an inverted widefield Del- tavision Core Microscope (Applied Precision, Issaquah, WA, USA) equipped with a 60X/1.4NA oil immersion objective. Z-dimension series of images were taken every 2 μm. Micropatterned FLAME RPE-1 cells were illuminated by a xenon arc lamp through a 490/20 excitation filter and imaged sequentially through 525/36 (GFP) and 605/52 (cherry) emission filters with a coolSnap Hq2 camera set to 2 × 2 binning. After background subtraction, images were corrected for photobleaching using a mono-exponential curve fitting. Ratios were calculated by pixel-wise divisions of the images in the cherry and GFP channels. Quantification was performed on every z section in two regions of interest (ROI) defined at the center and the periphery of the cell. At least three cells per condition were analyzed.

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Author contributions
BG and KS: designed the research, J-PG and KS: performed experiments and analysis. GX: provided reagents. J-PG, BG and KS: wrote the manuscript.

Conflict of interest
The authors declare that they have no conflict of interest.
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Jean-Philippe Grossier et al

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