# Endocytic trafficking of activated EGFR is AP-2 dependent and occurs through preformed clathrin spots

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## Summary

**The removal of the epidermal growth factor receptor (EGFR) from the cell surface by endocytosis is triggered by receptor activation, but many facets of EGFR trafficking remain unresolved. We employed total internal fluorescence microscopy to elucidate the dynamics of activated EGFR at the cell surface through live-cell imaging. The results of these studies demonstrate that: (1) EGFR does not localize to caveolae in live cells either before or after activation; (2) EGFR does localize to clathrin-coated pits, but only after activation; (3) activation does not result in the formation of new clathrin-coated pits; (4) activated EGFR clusters at sites of preformed clathrin lattices; (5) The AP-2 complex is involved in the internalization of activated EGFR. Using imaging techniques to show the endocytic sorting of activated EGFR for the first time in live cells, these studies suggest a refinement of the model for EGFR entry.**

Supplementary material available online at
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Key words: EGF receptor, Total internal reflection fluorescence microscopy, Endocytosis

## Introduction

The epidermal growth factor receptor (EGFR) is a frequently studied canonical member of the receptor tyrosine kinase superfamily (Atalay et al., 2003). EGFR family members play roles in important physiological processes such as wound healing (Vermeer et al., 2003). EGFR is also a target of study for cancer biologists. EGFR is an oncogene, increased EGFR signaling is associated with many cancers (Arteaga, 2002; Khazaie et al., 1993) and several anti-cancer drugs function through attenuation of EGFR signaling (Atalay et al., 2003). Additionally, high EGFR expression correlates with resistance of estrogen receptor-positive breast cancer to anti-estrogen therapy (Lichtner, 2003), and metastatic cancer cells migrate towards sources of EGF (Bailly et al., 2000). EGFR is removed from the cell surface by endocytosis, which is triggered by receptor activation (Wiley, 2003). During internalization, EGFR signaling persists, even after entry into the endosomal system (Sorkin, 2001).

Some reports have indicated that EGFR can enter the endocytic system via caveolae (Orlichenko et al., 2006; Sigismund et al., 2005; Zhu et al., 2005). This conclusion is based upon studies employing concentrations of EGF of >20 ng/ml. Other reports have concluded that even at concentrations as high as 100 ng/ml EGFR solely enters through clathrin-mediated endocytosis (Kazazic et al., 2006). Evidence supporting the hypothesis that EGFR employs a clathrin-mediated pathway includes fluorescence imaging studies, as well as those in which inhibitors of this route reduced EGFR uptake (Benesch et al., 2005; Benmerah et al., 1999; Huang et al., 2004). Some studies have tried to reconcile the conflicting data between clathrin- and caveolae-mediated EGFR entry. One hypothesis states that inactive EGFR resides in caveolae until ligand binding stimulates exit from this compartment followed by clathrin-dependent entry (Mineo et al., 1999). In some studies that have concluded that EGFR enters through clathrin, it has been reported that the activated EGFR induces formation of new clathrin-coated pits formed de novo (Johannessen et al., 2006; Puri et al., 2005; Wilde et al., 1999).

Another unresolved issue is whether there is a role for the clathrin adaptor AP-2 (also known as AP2A) in the trafficking of activated EGFR (Hinrichsen et al., 2003; Huang et al., 2004; Motley et al., 2003). Two studies employing very similar methods suggested that AP-2 is dispensable for EGFR entry (Hinrichsen et al., 2003; Motley et al., 2003). However, a subsequent report suggested that these results might actually have been experimental artifacts (Huang et al., 2004). Specifically, the studies that suggested the AP-2 is dispensable for EGFR entry employed pre-binding of EGF at 4°C, whereas the one that showed a role for AP-2 demonstrated that continuous incubation at 37°C was required for this effect to be measured.

We set out to image the dynamics of EGFR at the cell surface. Our results demonstrate that (1) activated EGFR enters through clathrin-coated vesicles and not caveolae; (2) following activation, EGFR clusters at the sites of preformed clathrin lattices; and (3) the AP-2 complex plays a role in EGFR trafficking.

## Results and Discussion

To image the dynamics of activated EGFR, HeLa cells were transfected with a construct encoding EGFP-tagged EGFR (EGFR-EGFP), shown previously to retain functionality (Carter and Sorkin, 1998), and imaged with total internal reflection fluorescence microscopy (TIR-FM). Based upon pilot studies aimed at determining conditions by which endocytic sorting and internalization of activated EGFR could be studied, we pre-

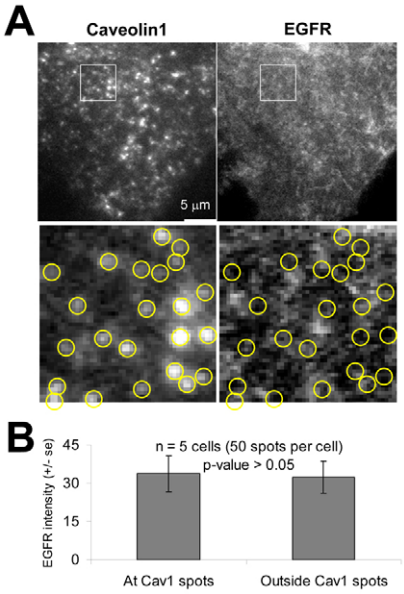


**Fig. 1.** EGFR does not reside in caveolae prior to EGF stimulation. (A) TIR-FM and (B) quantification demonstrates that prior to addition of EGF, EGFR does not colocalize with caveolin 1. Fluorescence of EGFR at sites of caveolae is nearly identical to the intensity outside caveolin 1 spots.

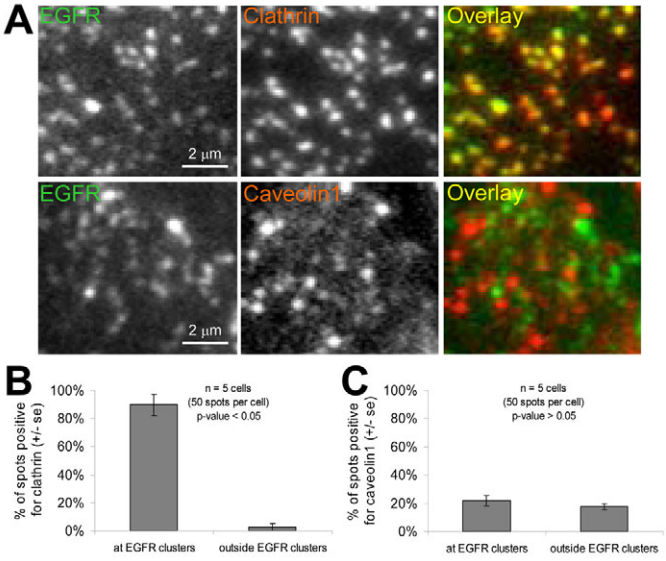


**Fig. 2.** Activated EGFR colocalizes with clathrin, and not caveolin 1. (A) EGFR-EGFP and clathrin-dsRed, or caveolin 1-mRFP, were imaged by simultaneous two-color TIR-FM 10 minutes after addition of EGF. Colocalization of EGFR and clathrin appears yellow in overlay . (B,C) Quantification demonstrates colocalization of clathrin at the sites of EGFR clusters (B) but not caveolin 1 (C).

incubated cells in serum-free medium for 15 minutes on the microscope stage at 37°C. Under these conditions, the EGFR-EGFP showed a diffuse fluorescence over the cell surface (Fig. 1). It has been previously proposed that EGFR resides in a caveolar compartment until the receptor is activated, at which point it exits this compartment (Mineo et al., 1999). To test this we quantified the fluorescence of EGFR at caveolin 1 sites before the addition of EGF (Fig. 1). Circles were drawn around 250 regions of caveolin 1 and 250 regions devoid of caveolin 1. The EGFR fluorescence intensity within the 250 caveolin 1 sites was nearly identical to that outside of the caveolin 1 sites. Thus our analysis suggests that in cells continuously incubated at 37°C, EGFR is not significantly accumulated at caveolae prior to stimulation with EGF.

After addition of EGF (100 ng/ml) to the bath, the EGFR-EGFP on the cell surface redistributed into discrete spots (supplementary material Fig. S1). Individual spots of tagged EGFR were visible as early as 1-2 minutes after ligand addition and the clustering process was complete in 6 minutes. Clusters of EGFR seemed to form more rapidly and to a greater extent at the cell periphery rather than towards the center of the adherent plasma membrane. This could reflect increased accessibility of receptors to ligand near the cell edge or may reflect a yet-to-be identified mechanism for increased formation of EGFR spots, or increased receptor concentration, at these sites. Clustering was apparent even at lower concentrations EGF (2 ng/ml; data not shown). However, we used the 100 ng/ml concentration of EGF because it has been previously suggested that at higher concentrations, the EGFR can be targeted to caveolae for internalization (Orlichenko et al., 2006; Sigismund et al., 2005).

To determine the route of endocytosis of activated EGFR we coexpressed either clathrin-dsRed or caveolin 1-mRFP along with EGFR-EGFP, and then imaged cells using simultaneous dual-color TIR-FM (Fig. 2). In order to evaluate whether activated EGFR enters the cell through clathrin and/or caveolae, clusters of EGFR were circled 10 minutes after addition of 100 ng/ml EGF. These regions were then transferred to either the corresponding clathrin or caveolin 1 images and the percentage of colocalizing spots was logged. Quantitative analysis of 250 EGFR spots per group from five cells revealed that there was ~90% colocalization with clathrin. Furthermore, as a control for random spot alignment 250 regions that did not contain EGFR clusters were analyzed; these were found to have only ~3% colocalization with clathrin. When the clusters of EGFR-EGFP that colocalized with clathrin-dsRed were subsequently followed, both fluorophores were observed to disappear from the cells surface at the same time and the same rate (supplementary material Fig. S2), consistent with previous reports that EGFR-EGFP can be visualized internalizing with clathrin tagged with a red fluorescent protein in TIR-FM studies (supplementary material Fig. S2) (Benesch et al., 2005).

Similar methods were applied to determine whether EGFR localized to caveolae after addition of EGF. When 250 EGFR spots were marked and these regions transferred to the caveolin 1 images, ~20% of these spots colocalized with caveolin 1. However, the proportion of the 250 regions that was lacking in EGFR clusters but which colocalized with caveolin 1-mRFP was also ~20% and statistically indistinguishable ($P$>0.05). This would suggest that the ~20% colocalization between EGFR and caveolin 1 represented a random alignment of spots, emphasizing the importance of controls in studies such as these. Thus our conclusion is that, even in the presence of 'high' EGF concentrations, activated EGFR internalizes solely through clathrin-mediated endocytosis.

The preceding results demonstrated that during incubation with EGF, the EGF receptor accumulates with clathrin, but not with caveolin 1. To resolve whether clathrin was being recruited to activated EGFR, or if activated EGFR was being recruited to pre-existing clathrin spots, the fluorescence of clathrin-dsRed and EGFR-EGFP was followed during the addition of EGF. After addition of EGF, it took 6-10 minutes for a maximal overlap of

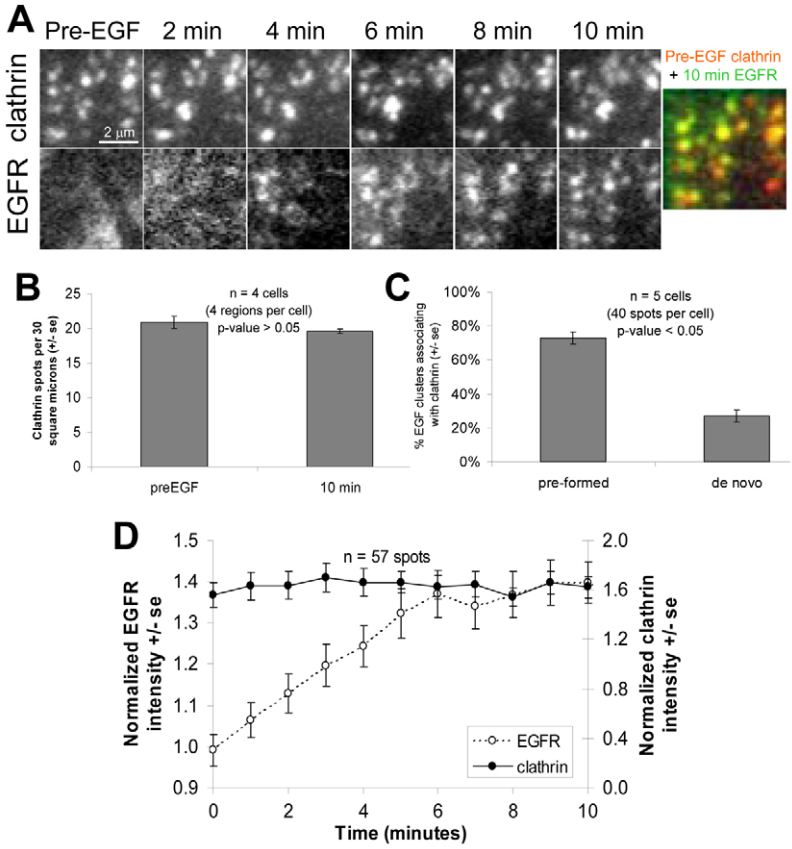


**Fig. 3.** Activated EGFR predominantly clusters at sites of preformed clathrin. (A) EGFR-EGFP and clathrin-dsRed were imaged by simultaneous two-color TIR-FM. Colocalization between clathrin imaged prior to addition of EGF (red), and EGFR observed at 10 minutes after stimulation (green) appears yellow in the overlay. (B) The number of clathrin spots on the cell surface counted before and 10 minutes after EGF addition is almost the same. (C) The percentage of EGFR clusters that formed at sites where clathrin was present prior to EGF addition was compared with the percentage that appeared where clathrin was not identified at time 0. (D) The clathrin and EGFR fluorescence intensities were logged every 2 minutes following EGF addition at the sites of EGFR clusters that colocalized with preformed clathrin.

EGFR and clathrin (Fig. 3). The first approach to analyzing the data was to identify clathrin spots just prior to EGF addition. The fluorescence of EGFR-EGFP and the fluorescence of clathrin-dsRed were quantified within these clathrin spots (Fig. 3D). These 'static clathrin spots' that existed prior to EGF, were the sites at which the EGFR clusters formed (yellow in overlay). A second analysis quantified the clathrin spots within 40 μm×40 μm square regions before, and 10 minutes after the addition of EGF (Fig. 3B). Four regions were analyzed in four cells per group to determine if the number of clathrin spots on the cell surface changed as a result of EGFR activation. Prior to addition of EGF 20.9±0.9 spots per unit area were counted, and 10 minutes after EGF addition 19.6±0.3, $P$=0.21. Thus, activation of EGFR signaling does not cause a detectable increase in the formation of new clathrin-coated pits in live cells at 37°C.

In a third approach to quantifying these results, 200 EGFR clusters (40 per cell from five cells) were identified 10 minutes after addition of EGF. These regions were then transferred to the corresponding clathrin images acquired before EGF stimulation. Then we determined the percentage of EGFR clusters that formed at preformed clathrin spots, as opposed to those that formed as clathrin was polymerizing de novo at the same site (Fig. 3C). There were 2.7-fold more EGFR clusters formed at pre-existing clathrin spots ('preformed'), than where clathrin was not present before EGF addition ('de novo'). Although 73% of EGFR clusters corresponded to sites where clathrin spots existed before EGF addition, only 27% of EGFR spots formed where clathrin was not present previously. It should be noted that lateral movement of clathrin spots in the plane of the plasma membrane could be a source of error in this analysis. This movement would lead to an over-estimation of the number of newly formed spots: any clathrin spots that moved even a short distance on the surface during the 10 minutes post-EGF treatment, would not be counted as pre-existing (Keyel et al., 2004; Rappoport et al., 2003b). Thus, the percentage of EGFR spots that are recruited to pre-existing sites of clathrin may be greater than 73%.

A fourth analysis involved imaging EGFR and clathrin for 10 minutes after the addition of EGF, then marking EGFR clusters which colocalized with preformed clathrin spots, and then running the images in reverse to quantify the fluorescence of EGFR and clathrin at these spots during the preceding 10 minutes. An average of the EGFR and clathrin fluorescence from 57 spots shows that although the intensity of clathrin is stable over time at these sites, EGFR signal gradually rose from background to a plateau ~6 minutes post-stimulation. Thus, activated EGFR predominantly clusters at sites of preformed clathrin, rather than inducing formation of new clathrin coats. These results demonstrate that although the EGFR signal increases over time, that of clathrin is constant. This demonstrates that there is neither an increase in the formation of new clathrin coats, nor an increase in clathrin signal at extant coats, as a result of activation of EGFR. Precedence for this type of behavior has been observed in other systems (e.g. G-protein-coupled receptors), which show that activated receptors cluster at preformed clathrin spots (Santini et al., 2002; Scott et al., 2002).

We next tested whether the hetero-tetrameric AP-2 adaptor complex is involved in the recruitment of EGFR to the clathrin spots or subsequent internalization of EGFR, by reducing the levels of α-adaptin with siRNA silencing (Fig. 4). The expression of α-adaptin was reduced ~85% by treatment with siRNA (Fig. 4B). This

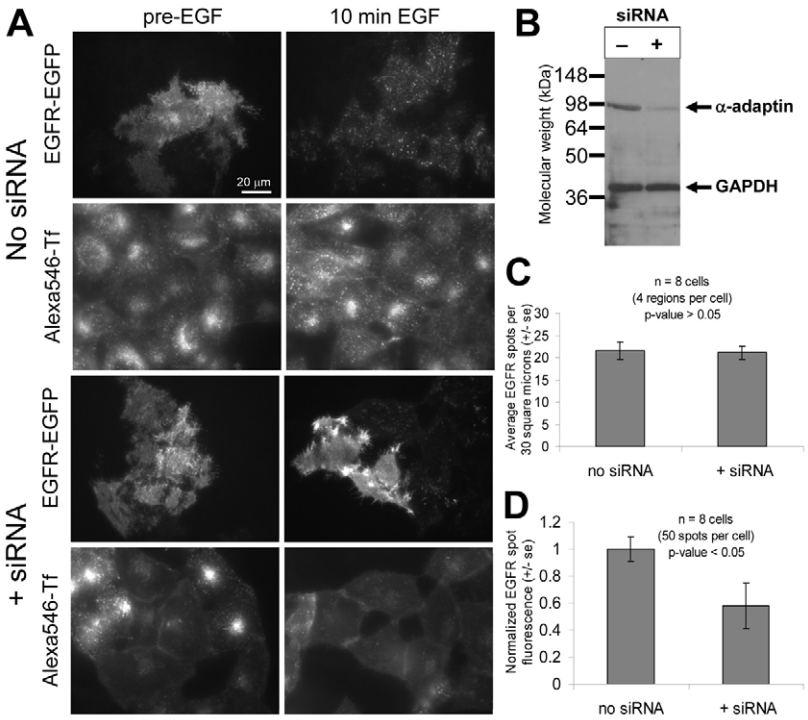


**Fig. 4.** The AP-2 complex is involved in the trafficking of activated EGFR. (A) TIR-FM comparing cells transfected with siRNA against α-adaptin demonstrates reduction of transferrin (Tf) uptake as well as apparent effects on EGFR distribution. Scale bars shown in image in upper left of (A) can be applied to all images in figure. (B) Western blot probed with anti-α-adaptin, as well as control anti-GAPDH, reveals potent and specific silencing. (C) Cells transfected with siRNA, demonstrating reductions in transferrin uptake, do not show alterations in the number of EGFR spots but do show (D) a ~40% reduction in EGF spot intensity relative to local background.

reduction of α-adaptin was sufficient to reduce the internalization of transferrin (in Fig. 4A, compare the second row with the fourth row). Use of transferrin as an independent marker for siRNA silencing is essential as not all cells appeared to be transfected. Treatment with the siRNA to α-adaptin also reduced internalization of the EGFR after treatment with EGF (compare Fig. 4A top row with the third row). By 10 minutes after EGF addition, the number of EGFR spots were the same with or without siRNA, but the fluorescence of EGFR spots relative to local background fluorescence was ~40% lower in the siRNA-treated group. This difference was due to an increase in EGFR background, rather than a decrease in gross intensity of the EGFR spots. Much of the receptor was fairly evenly spread all over the cell surface, except at the tips of the filopodia where it accumulated at fairly high concentrations (Fig. 4A, third row).

The observation of an ~40% decrease in EGFR entry with siRNA silencing of α-adaptin is consistent with the results from another study evaluating EGFR endocytosis by $^{125}$I-EGF uptake with and without siRNA treatment (Huang et al., 2004), although it is not consistent with two other studies which claimed that AP-2 siRNA has no effect on EGFR entry (Hinrichsen et al., 2003; Motley et al., 2003). One difference in how these studies were conducted is temperature: those that reported AP-2 siRNA to have an effect on EGFR entry were performed at 37°C; (Huang et al., 2004), whereas in the two claiming no role for AP-2 in EGFR endocytosis, EGF binding was performed at 4°C (Hinrichsen et al., 2003; Motley et al., 2003). Thus, it is our conclusion that AP-2 is indeed required for EGFR endocytosis, and our results confirm that this defect is not one of clustering per se, but instead one of entry.

These results demonstrate that when EGFR is examined at 37°C, upon activation, the EGFR accumulates at discrete spots of clathrin that existed prior to the addition of EGF. The EGFR then internalizes with the clathrin. At no point is the EGFR enriched at sites of caveolin 1. Efficient recruitment of the EGFR to clathrin requires the presence of AP-2, in that this process is blocked with expression of α-adaptin and is reduced by siRNA. Thus, these results demonstrate the benefits of live-cell imaging. However, it should be noted that TIR-FM is limited to observation of the adherent cell surface and it is possible that alternative pathways for EGFR entry exists on the opposite surface.

## Materials and Methods

### Plasmid constructs and siRNA

The construct encoding rat clathrin light chain A tagged with dsRed (clathrin-dsRed) was a gift from Tomas Kirchhausen (Harvard Medical School, Boston, MA). EGFP-tagged EGFR was a gift from Alexander Sorkin (University of Colorado Health Sciences Center, Aurora, CO). Caveolin 1-mRFP was a gift from Ari Helenius (ETH Institut of Biochemistry, Zurich, Switzerland). siRNA against α-adaptin was obtained from Dharmacon (Lafayette, CO) and matched that used in other studies (Huang et al., 2004; Motley et al., 2003).

### Cell culture

HeLa cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum in an incubator at 37°C in a humidified 5% $CO_2$ atmosphere. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the supplier's directions. Cells were imaged 48 hours post-transfection, except in siRNA studies in which cells were plated on day 1, transfected with siRNA on day 2 and then split 1:2 on day 3, transfected with siRNA + DNA on day 4 and then imaged on day 6. EGF was purchased from Sigma (St Louis, MO) and prepared according to the supplier's directions.

### Total internal reflection fluorescence microscopy (TIR-FM) image acquisition

TIR-FM was performed at 37°C using an Apo ×60 1.45 NA microscope objective (Olympus America, Melville, NY), as previously described (Rappoport and Simon, 2003). Clathrin-dsRed (or caveolin-1-mRFP) and EGFR-EGFP were excited with the 488 nm line of a tunable argon laser (Omnichrome, model 543-AP A01, Melles Griot, Carlsbad, CA) reflected off a 498 nm dichroic mirror. All mirrors and filters were obtained from Chroma Technologies (Brattleboro, VT). Green and red emissions were collected simultaneously using a dual-view splitter (Optical Insights, Santa Fe, NM) equipped with a 515/30 nm band-pass filter to collect green emission, a 550 nm dichroic mirror to split the emission, and a 580 nm long-pass filter to collect red emission. The camera used to acquire images was a ORCA-ER (Hamamatsu Photonics, Bridgewater, NJ). The camera and a mechanical shutter (Uniblitz, Vincent Associates, Rochester, NY) were controlled by MetaMorph software (Universal Imaging, Downingtown, PA).

### Dual-color processing

After subtraction of extracellular background, 12-bit dual-color TIR-FM image streams were aligned in MetaMorph. On the basis of single-fluorophore control experiments, green-to-red bleed-through corrections of 10% were employed; following background subtraction and alignment, 10% of the EGFP signal was subtracted from dsRed images.

### Data analysis

Paradigms determined from our previous studies in this area were applied to the current data sets (Rappoport and Simon, 2003). Spots of clathrin, caveolin 1 and EGFR were identified in single static images by circling 8 pixel × 8 pixel regions in MetaMorph. Only spots that appeared to overlap with neighbors were circled. Otherwise, identification was random. Spots were recorded as colocalizing if the majority of the spot intensity in the corresponding image from the other channel fitted within the confines of the drawn regions.

### Transferrin uptake studies

Cells were incubated at 37°C in serum-free DMEM for 15 minutes, and then for another 15 minutes at 37°C in Alexa-Fluor-546–transferrin (Molecular Probes, Invitrogen, Carlsbad, CA) diluted 1:500. After 5 minutes of incubation with transferrin, EGF was added at 100 ng/ml. After 10 minutes in transferrin plus EGF, cells were rinsed, fixed and analyzed as previously described (Rappoport et al., 2003a).

### Western blots

Cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer, clarified by centrifugation and then analyzed by 10% SDS-PAGE. After transfer and blocking in 5% milk, blots were probed with polyclonal rabbit anti-α-adaptin (M300) and polyclonal rabbit anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA). Bands were visualized with ECL+ (Amersham, Piscataway, NJ) following detection with HRP-conjugated goat anti-rabbit secondary antibody (Sigma).

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