

# A Novel Endocytic Mechanism of Epidermal Growth Factor Receptor Sequestration and Internalization

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## Abstract

**Cells form transient, circular dorsal ruffles or “waves” in response to stimulation of receptor tyrosine kinases, including epidermal growth factor receptor (EGFR) or platelet-derived growth factor receptor. These dynamic structures progress inward on the dorsal surface and disappear, occurring concomitantly with a marked reorganization of F-actin. The cellular function of these structures is largely unknown. Here we show that EGF-induced waves selectively sequester and internalize ~ 50% of ligand-bound EGFR from the cell surface. This process requires receptor phosphorylation, active phosphatidylinositol 3-kinase, and dynamin 2, although clathrin-coated pits or caveolae are not required. Epithelial and fibroblast cells stimulated with EGF sequestered EGFR rapidly into waves that subsequently generated numerous receptor-positive tubular-vesicular structures. Electron microscopy confirmed that waves formed along the dorsal membrane surface and extended numerous tubules into the cytoplasm. These findings characterize a structure that selectively sequesters large numbers of activated EGFR for their subsequent internalization, independent of traditional endocytic mechanisms such as clathrin pits or caveolae.** (Cancer Res 2006; 66(7): 3603-10)

## Introduction

Circular dorsal ruffles or “waves” are dynamic and transient actin-based protrusions on the surface of migratory epithelial and mesenchymal cells (1, 2) that form in response to receptor tyrosine kinase (RTK) stimulation with epidermal growth factor (EGF; ref. 3), platelet-derived growth factor (PDGF; ref. 4), or hepatocyte growth factor (1). These widely observed, yet enigmatic, structures extend in the *z* axis and first appear behind the leading edge, forming directly on the dorsal plasma membrane (5–7). While resembling traditional lamellipodial and peripheral ruffles, waves differ in their dynamics and composition. Mainly, these structures are transient, forming only once following growth factor stimulation into large rings that progress inward into a tight annulus before disappearing, usually within 10 to 20 minutes. Waves are rich in F-actin (8), cortactin, dynamin 2 (Dyn2), components of the Arp2/3 complex (2), and multiple kinases (9–11).

Currently, the function of waves is poorly understood. Recent studies indicate they facilitate a dramatic transformation of the actin cytoskeleton associated with lamellipodia of cells (2, 9). At nascent waves there is a reorganization of large actin stress fibers into highly branched F-actin filaments, as suggested by the presence of cortactin and Arp2/3 (2). This reorganized actin network may then support extension of the lamellipodial edge and directed cell motility (2, 7). Waves are also implicated in bulk fluid-phase endocytosis as these structures can terminate in macropinosomes (1). However, the role and requirement of waves during bulk macropinocytosis is not well understood (7). In this study, we report that waves act as endocytic structures that can specifically sequester and subsequently internalize up to ~ 50% of a specific RTK, such as the EGF receptor (EGFR). This sequestration process excludes other cell-surface receptors including the transferrin receptor and PDGF receptor (PDGFR) and transforms discrete plasma membrane regions into a complex tubular network used for ligand-receptor internalization. Whereas EGFR, phosphatidylinositol 3-kinase (PI3K; phosphatidylinositol-tri-3,4,5-phosphate, PIP<sub>3</sub>), Dyn2, and F-actin polymerization are required for wave function, traditional endocytic coat proteins such as clathrin, clathrin adaptors, and caveolin are not. These findings have broad implications toward our understanding of how receptor-mediated processes may affect receptor down-regulation, cell growth, motility, and metastasis.

## Materials and Methods

**Cell culture, transfection, and small interfering RNA.** Human pancreatic tumor cells (PANC-1), HeLa, COS7, and mouse NR6 fibroblasts expressing wild-type human EGFR (NR6WT, referred to here as NR6) were cultured in DMEM (with glucose and glutamine; CellGro, Herndon, VA) with 10% fetal bovine serum (FBS; Life Technologies, Inc., Carlsbad, CA). NR6 were maintained in G-418 (350 µg/mL; Life Technologies, Inc.). For NR6 in collagen, cells were either plated onto collagen-coated coverslips and covered with collagen (rat tail, type I, 1.5 mg/mL; BD Biosciences, San Jose, CA) or were suspended in collagen and plated onto coverslips. For all EGF stimulations, cells were washed in HBSS (CellGro) and maintained in 0.2% FBS medium for 16 to 24 hours before experimentation. Plasmids were prepared using Qiagen kits (Qiagen, Valencia, CA) and transfected via Lipofectamine Plus (Invitrogen, Carlsbad, CA). EGFR-green fluorescent protein (GFP), clathrin light chain- $\alpha$ -GFP (clathrin-GFP), AP180-C-FLAG, Dyn2K44A, and caveolin-1-GFP were expressed for 24 to 48 hours before experimentation.

Clathrin heavy chain (CHC) small interfering RNA (siRNA) duplex (human CLTC, sense GCAAUGAGCUGUUUGAAGAAU; Dharmacon, Lafayette, CO) was transfected with Oligofectamine (Invitrogen) at 200 nmol/L for HeLa and 400 nmol/L for NR6. HeLa were treated for 96 hours before experimentation. NR6 were treated a second time after 48 hours. For EGF stimulation, HeLa or NR6 were serum starved after 72 hours. Caveolin-1 siRNA duplex (human caveolin-1, sense AACCAGAAGGGACACACAGUUUU; Dharmacon) was used at 200 nmol/L for 96 hours. CHC reduction was confirmed by immunoblotting for CHC versus loading control ( $\beta$ -actin

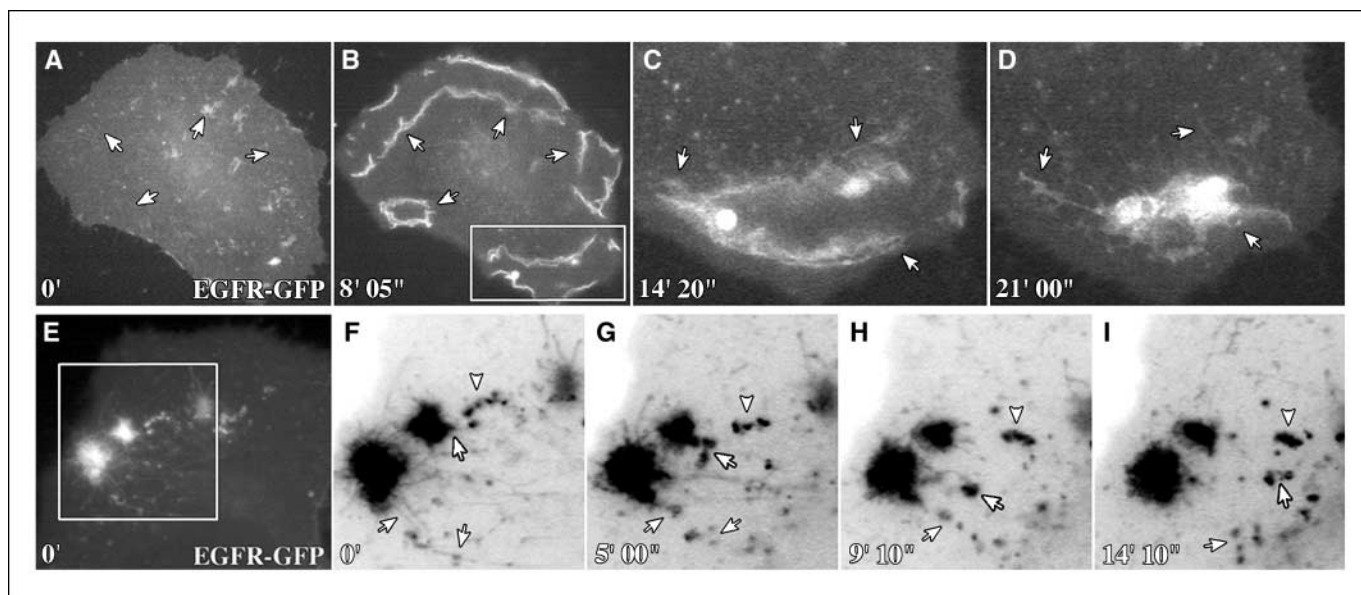
**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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**Figure 1.** EGFR is sequestered into forming waves and subsequently into dynamic tubular-vesicular structures that form at waves. Live PANC-1 cells expressing EGFR-GFP were stimulated with 30 ng/mL EGF and imaged. *A to D*, individual frames showed that EGFR-GFP was sequestered within newly forming waves in <5 minutes post-stimulation (*B*, arrows). By 15 minutes, the waves constrict and formed dynamic tubular-vesicular structures containing EGFR-GFP (*C* and *D*, arrows, and video 1). *E to I*, high magnification of a structure formed from a dorsal wave observed ~15 minutes post-EGF treatment. Close examination revealed that 2- to 5- $\mu$ m-long EGFR-GFP tubules (100-200 nm in diameter) extended toward the cell center (*F-I*, arrows, image inverted for clarity, video 2). *F to I*, nascent vesicles generated from the cluster (arrows, arrow with heavy border) trafficked toward the cell center. Associated vesicles were also found to coalesce as they moved toward the cell center (arrowhead). See videos 1 and 2.

and/or  $\gamma$ -tubulin) or fluorescence immunocytochemistry for CHC at the single cell level.

**Antibodies and phalloidin.** Dyn2 (2) and cortactin (10) polyclonal antibodies were as previously described. Eps15 polyclonal antibody was raised against the synthetic peptide EWAKRESEREEEQRLARLNQQEQED corresponding to amino acids 858 to 882 of rat Eps15L. Clathrin (X-22; American Type Culture Collection, Manassas, VA), anti-FLAG (Sigma, St. Louis, MO), EGFR (Santa Cruz Biotechnology, Santa Cruz, CA, extracellular domain), phospho-1173-EGFR (Upstate Biotech, Charlottesville, VA), PDGFR $\beta$  (R&D Systems, Minneapolis, MN), caveolin-1 (Zymed, Carlsbad, CA), and  $\gamma$ -tubulin monoclonal antibodies and EGFR (Santa Cruz Biotechnology, cytoplasmic tail), phospho-1068-EGFR (Cell Signaling Technology, Danvers, MA), and PDGFR $\beta$  (Santa Cruz Biotechnology) polyclonal antibodies were used. Fluorescent secondary antibodies were from Molecular Probes (Carlsbad, CA). Rhodamine-phalloidin (Sigma) was used at 80 to 160 nmol/L.

**Electron microscopy.** Scanning and transmission electron microscopy was done on EGF- or PDGF-stimulated cells using previously published techniques (11, 12). Serial transmission electron microscopic sections were registered using a computer and multiple location markers.

**Light microscopy.** Cells were fixed in 3.0% paraformaldehyde and processed (2). Images were acquired using a Carl Zeiss Axiovert 35 or 200M and Hamamatsu Orca II or Orca II ERG cameras (Hamamatsu Photonics, Hamamatsu City, Japan), or a Carl Zeiss LSM510 confocal microscope with heated stages for live-cell time-lapse microscopy. For time-lapse microscopy, cells were grown in glass-bottomed dishes. Cells were in 0.2% FBS, Ham's F-12K (Sigma) buffered with 15 mmol/L HEPES (pH 7.2). EGF (mouse submaxillary gland; Sigma) was used as indicated. For micropipette delivery, rhodamine-EGF (30 ng/mL in pipette; Molecular Probes) was in microinjection needles. Frames were collected at 1/5 seconds. Display rates are 10 frames/s. Lenses were Zeiss 63 $\times$  oil, plan apochromat, NA 1.4, 63 $\times$  oil, plan neofluar, NA 1.25, 40 $\times$  oil, achroastigmat, NA 1.3, and 40 $\times$  dry, plan neofluar, NA 0.75.

**EGF and transferrin internalization assays, EGFR kinase, and PI3K inhibitor assays.** Rhodamine-EGF (Molecular Probes) at 30 ng/mL and FITC-transferrin (Molecular Probes) at 5  $\mu$ g/mL were used. Rhodamine-EGF

and FITC-transferrin were together during stimulation/internalization to allow detection of both ligands. Acid stripping of rhodamine-EGF used ice-cold 0.2 mol/L acetic acid, 0.5 mol/L NaCl (pH 2.5) for 2  $\times$  2 minutes and 1  $\times$  1 minute, followed by neutralization in ice-cold HBSS (CellGro). EGF internalization was quantified by fluorimetric measurement of rhodamine-EGF signal in normal cells and cells expressing AP180-C-FLAG, Dyn2K44A, or with  $\geq$ 80% reduced CHC. The endocytic block was determined by comparison to normal cells. Transferrin internalization was as before (10) but in the presence of 30 ng/mL EGF. Inhibition of transferrin uptake by AP180-C-FLAG, Dyn2K44A, or siCHC was scored as blocked, no accumulation of internalized transferrin or not blocked, and normal clathrin-mediated endocytosis (CME); values are presented as percent cells with normal CME. The effect of AP180-C-FLAG, Dyn2K44A, or siCHC on wave formation was solved by scoring the percentage of nontransfected (control) versus transfected cells with waves, normalized to nontransfected. The EGFR kinase (PD168393; Calbiochem) and PI3K (wortmannin; Sigma) inhibitors were in DMSO. Inhibitors were diluted into 0.2% FBS-DMEM. Cells were treated with inhibitors for 1 hour before stimulating with 30 ng/mL EGF for 5 minutes (wave formation) or 15 minutes (transferrin or EGF internalization). Mock was 0.05% DMSO in 0.2% FBS-DMEM.

## Results

**Dorsal waves sequester EGFR.** Waves form within just minutes following RTK stimulation and use a variety of cytoskeletal and signaling proteins (13). Some of the cytoskeletal components include the large GTPase Dyn2 and the cortactin-Arp2/3-N-WASP complex. We confirmed wave formation in PANC-1, a metastatic human pancreatic adenocarcinoma cell line that expresses elevated levels of EGFR compared with normal pancreatic ductular cells and 2- to 3-fold more than other pancreatic adenocarcinoma cell lines (14, 15). At early time points, Dyn2-positive waves can be observed as large concentric ruffles along the cell perimeter (Supplementary Fig. S1A and A') that condense into tight, tubular-vesicular masses (Supplementary Fig. S1B and B'). Different cultured cells (COS7, NR6,

and PANC-1) were serum starved and then stimulated with concentrations of EGF ranging from 0.5 to 400 ng/mL (<1.0-65 nmol/L). COS7 were used as a reference as EGF-stimulated waves have been studied in these cells (3). The NR6 cells used here are a NIH 3T3 derivative that expresses physiologic levels of human EGFR (NR6; ref. 16). NR6 formed waves at a higher frequency than COS7 and plateaued at ~50% at 5 minutes of stimulation with 30 ng/mL (4.9 nmol/L) EGF, a physiologically relevant concentration (17). Occurrence of COS7 waves peaked at ~30% with 400 ng/mL (65 nmol/L). Even at low EGF concentrations (0.5 ng/mL), NR6 and COS7 cells formed waves (Supplementary Table S1). Wave formation is dose dependent but plateaus in all cell types tested (Supplementary Table S1). After 5 minutes, the frequency of waves declined steadily, with few present at 30 minutes post-stimulation, resulting in an average wave "life span" of 10 to 20 minutes (not shown).

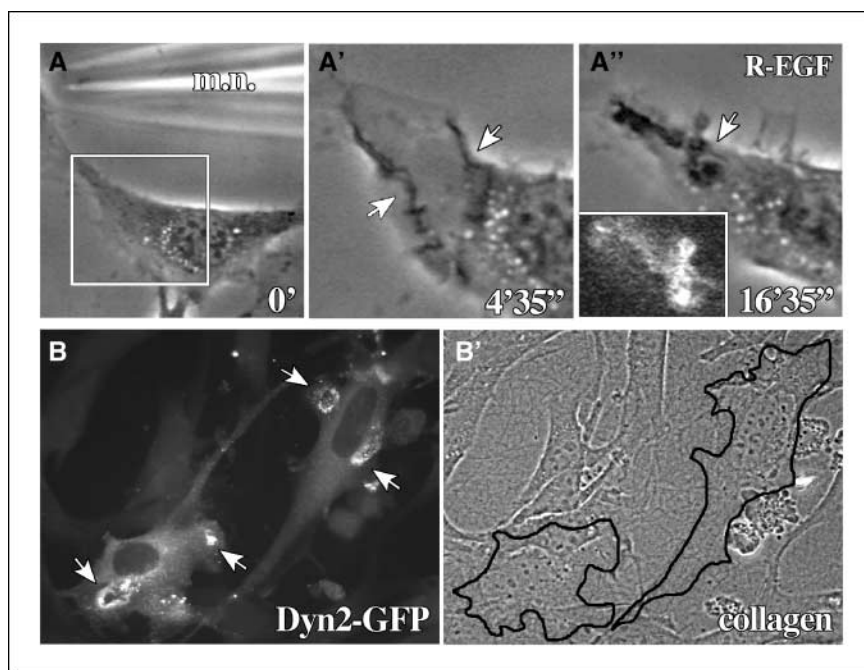
Because circular ruffles have only been implicated in bulk macropinocytosis, we first asked if these dynamic structures sequestered and trafficked EGFR when stimulated by EGF. Human EGFR-GFP (18) was expressed in PANC-1 or NR6 cells and monitored by live-cell time-lapse microscopy. Within only minutes post-EGF treatment, EGFR was sequestered within waves (Fig. 1, *A* versus *B*, *arrows*, and video 1, available online). As the waves closed, numerous EGFR-containing tubules and large puncta extended away from the main structure and often seemed to stream toward the cell center (Fig. 1*C, D, F-I, arrows* and videos 1 and 2, available online). Imaging of the EGFR containing waves late in formation (~15 minutes post-EGF; Fig. 1*F-I, video 2*) revealed large numbers of dynamic tubules of 100 to 200 nm in diameter and up to several microns long. Many vesicles were generated from these tubules that subsequently translocated toward the cell center (Fig. 1*F-I, arrows*, video 2). Vesicles associated with these structures also coalesced as they trafficked toward the cell center (Fig. 1*F-I, arrowhead*).

Many cells in culture form waves; however, it is unclear if this process occurs in a physiologic environment more representative of a cellular tissue. To address this, we stimulated cells via gradient (micropipette) delivery of EGF and also stimulated cells growing

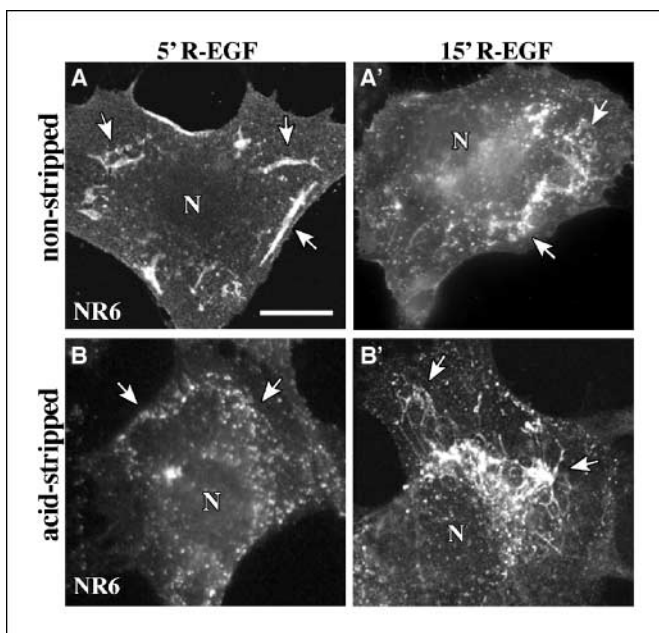
within a collagen matrix. Phase-contrast coupled with fluorescent time-lapse microscopy indicated that waves formed in a rhodamine-EGF gradient on the "side" of the cell closest to the micropipette tip (Fig. 2*A-A'*, video 3, available online) and that the Rhodamine-EGF (*A''*, *inset*, video 3) was sequestered in the waves and their associated tubular-vesicular structures, like those observed in the EGFR-GFP videos (video 2). Cells growing in a collagen matrix also formed waves that recruited Dyn2-GFP [Fig. 2*B* (*arrows*) and *B'*] and rhodamine-EGF (not shown). These data indicate that waves form and sequester ligand-bound EGFR within a gradient of EGF and that surrounding extracellular matrix does not act as a barrier to wave formation.

#### Dorsal waves internalize EGFR by a nontraditional pathway.

Live imaging (videos 1-3) of stimulated cells indicated that waves sequester and actually internalize activated EGFR. To confirm this, we tested directly if the tubular-vesicular-EGFR structures within waves (Fig. 1, videos 1 and 2) were "protected" from the plasma membrane surface. NR6 and PANC-1 (not shown) cells were treated with 30 ng/mL rhodamine-EGF for different times and the plasma membrane was thoroughly acid stripped and neutralized before fixation and staining. Waves in non-acid-stripped cells appeared as bright rings on a dim, diffuse plasma membrane stain (Fig. 3*A, arrows*). This indicated that at 5 minutes, ligand-bound EGFR was already sequestered. By 15 minutes with no stripping, tubular-vesicular structures were common and more near the cell periphery, with modest accumulation of the ligand-receptor complex in a late endosomal, perinuclear compartment (Fig. 3*A'*). As was observed by live-cell imaging (video 2), the proximity of the EGFR containing compartments to the nucleus was increased markedly by 30 minutes (not shown). In contrast to nonstripped controls, stripped cells displayed a near-complete loss of wave labeling at 5 minutes (Fig. 3*B, arrows*), indicating that at early time points, waves contained largely plasma membrane-associated, ligand-bound EGFR. Although some small ligand-containing endosomes were observed at the site of wave formation, indicating nascent endocytosis at the wave, the majority ( $\geq 90\%$ ) of



**Figure 2.** Dorsal waves form and sequester Dyn2 and ligand-bound EGFR in *in vivo*-like conditions. *A* to *A''*, NR6 cells exposed to a gradient of rhodamine-EGF (*R-EGF*; 30 ng/mL in needle) via a microneedle formed dorsal waves closest to the microneedle tip that actively sequester ligand-bound EGFR. By 4 minutes 35 seconds (*A*), a fully formed dorsal wave was visible that, by 16 minutes 35 seconds, closed in (*A''*) forming tubular-vesicular structures that contain rhodamine-EGF (*inset*). *B* and *B'*, NR6 expressing Dyn2-GFP and grown in a collagen "sandwich" formed dorsal waves (*arrows*) that sequestered Dyn2. *B'*, fibrils of collagen are visible by phase-contrast imaging. Black tracings in (*B'*) indicate cell perimeters. Stimulation was with 30 ng/mL EGF for 5 minutes.



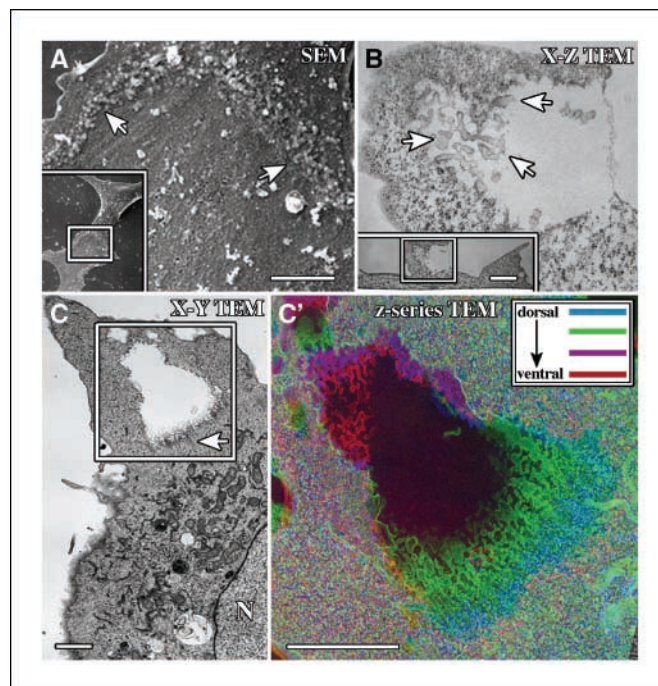
**Figure 3.** Waves internalize EGF-bound EGFR into tubular-vesicular structures. Rhodamine-EGF was used as a marker for EGFR localization and internalization in cells. *A* and *A'*, NR6 cells were stimulated with rhodamine-EGF (30 ng/mL) for 5 or 15 minutes, fixed, and imaged. *A*, at 5 minutes, large amounts of rhodamine-EGF localized to the waves and there were some associated vesicular puncta (arrows); there was also a diffuse plasma membrane association. Note the lack of accumulation in the late endosomal, perinuclear compartment at this early time point. *A'*, by 15 minutes, wave-based EGF-positive tubular-vesicular structures were observed (arrows). *B* and *B'*, NR6 cells stimulated as in (*A'*, *A''*) but acid stripped to remove surface-exposed ligand. The rhodamine-EGF signal in these cells indicates internalized EGF-bound EGFR. *B*, at 5 minutes, numerous small internalized puncta seem associated with waves (arrows). *B'*, similar to 15 minutes with no strip, there are numerous rhodamine-EGF, wave-based tubular-vesicular structures (arrows). Bar, 10  $\mu$ m for all cells. *N*, nucleus.

rhodamine-EGF was removed by acid stripping. Surface-stripped NR6 and PANC-1 (not shown) cells after 15 (Fig. 3*B'*) or 30 (not shown) minutes of stimulation showed internalized ligand-bound receptor similar to that seen by live-cell imaging, with much of it residing within tubular-vesicular (Fig. 3*B'*) and perinuclear endosomes, respectively. These data indicate that ligand-bound EGFR is sequestered at early time points (1-5 minutes) and then internalized into waves (5-30 minutes), where it is protected from acid stripping and internalized from the dorsal plasma membrane.

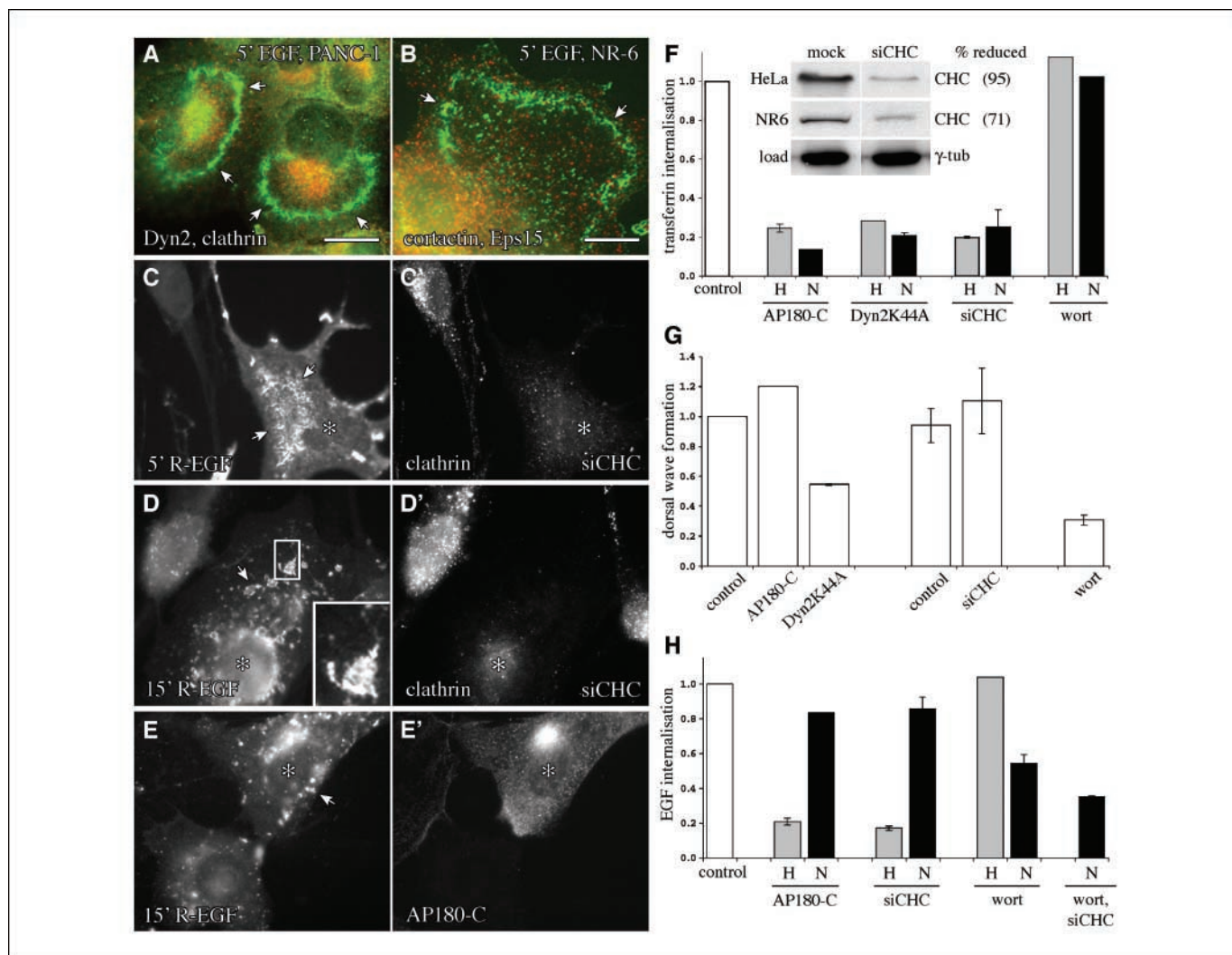
To gain insight into the endocytic processes of waves and to define the organization of waves at high resolution, we used both scanning and transmission electron microscopy. By scanning electron microscopy, waves appeared as ring-shaped arcs of uniformly sized bumps that deformed the dorsal plasma membrane (Fig. 4*A*, arrows). Vertical (*X-Z*) and serial-section transmission electron microscopy of EGF-stimulated cells showed that waves contained membranous tubules of a  $\sim$ 100 to 200 nm in diameter that extended both along and from the dorsal surface [Fig. 4*B* (arrows), *C*, and *C'*]. *En face* sections from the dorsal to the ventral surface revealed that waves are undulations of the dorsal plasma membrane, composed of numerous tubules extending along the wave perimeter (Fig. 4*C*). Four serial sections were superimposed (Fig. 4*C'*) to highlight the transformation of the dorsal plasma membrane into a complex, tubular network used to sequester EGFR. Numerous vesicle-buds emanated from the tubules that likely represented the biogenesis of the receptor-

containing motile vesicle carriers observed in living and fixed cells (Fig. 1, video 1). Notably, the membranes, tubules, and vesicle-buds at waves had no clathrin or caveolin-like coats.

Until recently (17, 19), it was accepted that EGFR sequestration and internalization relied exclusively on endocytic coat proteins such as clathrin and potentially caveolin. Therefore, we tested rigorously for the presence of multiple proteins involved in CME at the wave site. These included CHC using antibody staining and expression of clathrin light chain- $\alpha$  fused to GFP (20) and staining for Eps15, an EGFR substrate located at clathrin pits that internalize EGFR (21). These proteins did not localize to waves (Fig. 5*A* and *B*) or the tubular-vesicular structures formed from them (not shown). To provide a functional test for CME in wave formation and EGFR sequestration, we blocked clathrin pit formation and CME through the expression of two separate dominant inhibitory proteins, the CME scaffolding protein AP180-C (22) and GTPase-deficient Dyn2K44A (23), and by CHC knockdown using siRNA (24). The AP180-C protein lacks the plasma membrane targeting information of wild-type but does bind clathrin directly, subsequently reducing free cytosolic clathrin and inhibiting CME. GTPase-deficient Dyn2K44A has been used extensively to block CME and sufficient knockdown of CHC prevents clathrin pit formation directly. The sequence corresponding to the CHC siRNA oligo used is identical between mouse and human CHC. Following siRNA treatment of HeLa Cells, CHC



**Figure 4.** Waves represent a distended dorsal plasma membrane that is transformed into a vesicle-generating tubular network. Scanning electron microscopy (SEM), vertical section (*X-Z*) transmission electron microscopy (TEM), and serial section transmission electron microscopy identified the form of waves on the dorsal plasma membrane. *A*, scanning electron microscopy revealed that waves consist of a ridge of uniform width that is composed of numerous bumps (arrows). *B*, a vertical section through a wave identified numerous membrane tubules that extend within the ruffle and into the cytoplasm (arrows). *C*, transmission electron microscopy of an *en face* section through a wave region resolved numerous tubular invaginations along the plasma membrane. *C'*, four serial sections through the same region in (*C*) were pseudo-colored and aligned to provide greater visual depth. Large numbers of tubules are more easily resolved as an extension of the plasma membrane. Bar, 5  $\mu$ m (*A*), 1  $\mu$ m (*B*, inset, to *C'*).



**Figure 5.** Waves assemble and function independently of clathrin to internalize EGFR. PANC-1 and NR6 cells were stimulated with EGF for 5 minutes and stained for clathrin or Eps15. *A*, stimulated PANC-1 cells labeled for Dyn2 (green) and clathrin (red; X22 monoclonal antibody) show no accumulation of clathrin within waves (arrows; lack of yellow overlap). Clathrin-GFP also did not label waves (not shown). *B*, colabeling of cortactin (green) and the clathrin pit-associated protein Eps15 (red) in NR6 showed no enrichment of Eps15 at waves (arrows; lack of yellow overlap). *C* to *D'*, rhodamine-EGF is sequestered (*C*, arrows) and internalized significantly (*D*, arrow, inset) in cells devoid of CHC (*C'* and *D'*, asterisk). *E* and *E'*, cells expressing the CME inhibitor AP180-C (*E'*, asterisk) can also internalize EGFR significantly (*E*, arrow). *F*, inset, immunoblot analysis of total cell lysate from either mock treated cells or cells treated with siRNA to reduce CHC protein. Values adjacent to bands represent percent reduction of CHC levels as assessed by quantitative densitometry normalized to mock. CHC levels were reduced on average by 95% in HeLa cells (two experiments done) and 71% in NR6 (three experiments done).  $\gamma$ -Tubulin loading control is for NR6; HeLa loading control is not shown. Mock represents transfection reagent alone. AP180-C, Dyn2K44A, and CHC reduction ( $\geq 80\%$ ) potentially blocked CME of transferrin receptor in HeLa (*H*) and NR6 (*N*) and 100 nmol/L wortmannin (*wort*) had no effect. *G*, GTPase-deficient Dyn2K44A and wortmannin inhibited wave formation by  $\sim 50\%$  and  $\sim 70\%$ , respectively, whereas wave formation was modestly increased in AP180-C-expressing or CHC knockdown cells. *H*, waves internalize significant levels of EGFR. AP180-C blocked EGF internalization in HeLa (that do not make waves) by  $\sim 80\%$  but by only  $\sim 20\%$  in NR6. CHC knockdown in HeLa and NR6 yielded the same result as AP180-C, with an  $\sim 80\%$  block in EGFR internalization in HeLa and  $\sim 20\%$  in NR6. Wortmannin treatment had no effect on EGFR internalization in HeLa but did block  $\sim 50\%$  in NR6. The block in EGF internalization in NR6 was additive when cells were treated with both siCHC and wortmannin ( $\sim 65\%$ ). Bar, 10  $\mu$ m (*A* and *C-E'*), 5  $\mu$ m (*B*). *F* to *H*, control is nontransfected or mock treated (DMSO for wortmannin). Columns, mean ( $n = 3$ , with  $\geq 100$  cells in each condition); bars, SE.

was reduced  $\sim 90\%$  whereas the reduction in NR6 was  $\sim 70\%$  to  $80\%$ . This was assessed by immunoblotting of 20  $\mu$ g total cell lysate (Fig. 5*F*, inset blot). Loading controls were  $\beta$ -actin (not shown) and/or  $\gamma$ -tubulin to confirm the equal loading. This increased knockdown efficacy in HeLa cells, as assessed by blotting, was most likely due to transfection efficiency as 80% to 90% of these cells were transfected compared with NR6 (50-60%). Therefore, whereas more HeLa cells were reduced in CHC, the actual reduction of CHC in any individual NR6 cell appeared as low as was observed in HeLa. In addition to the biochemical quantitation, we did single-cell analysis using fluorescence

microscopy. This approach provided an advantage of being able to disregard cells that did not exhibit a marked protein reduction by siRNA treatment. We determined that a minimal cellular reduction of CHC by 80% was required to effectively block standard clathrin-mediated endocytosis of transferrin in HeLa and NR6 cells, as well as EGF in HeLa cells that do not form dorsal waves but do use clathrin for conventional receptor-mediated endocytosis. In contrast, knockdown of CHC had no significant effect on wave formation or EGFR sequestration into the waves (Fig. 5*G*). Expression of a truncated form of AP180, known to inhibit CME, also had no effects on wave formation or function. In contrast,

expression of Dyn2K44A blocked wave formation by 50% (Fig. 5G). These data corroborate our previous findings that expression of Dyn2 $\Delta$ PRD significantly inhibited wave formation (2). Importantly, we confirmed that rhodamine-EGF and EGFR were sequestered in waves that formed in cells depleted of CHC (Fig. 5C and C') or expressing AP180-C (not shown). Thus, consistent with the localization experiments (Fig. 5A and B) and the electron microscopic observations (Fig. 4), components of CME do not localize to, or participate in, dorsal wave formation or EGFR sequestration at the waves.

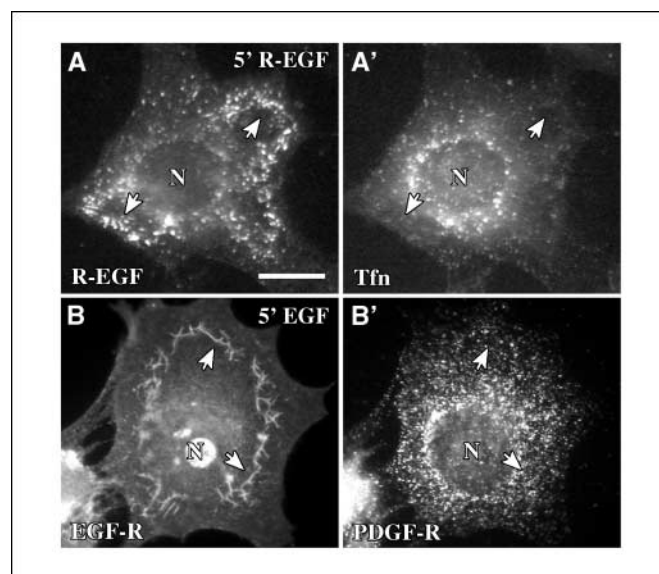
Several studies have implicated caveolae in the sequestration and internalization of EGFR (17, 25). Therefore, we used multiple reagents to caveolin-1 to test the role of caveolae in waves. Caveolin-1-GFP and caveolin-1 antibodies did not localize to waves (Supplementary Fig. S2A and B) nor did antibodies to caveolin-2 (not shown). Most often, the majority of caveolin-1 stain was completely absent from the wave area (Supplementary Fig. S2A and B). To examine the effects of a caveolin-1 knockdown on wave formation and EGFR sequestration, cells were treated with caveolin-1 siRNA, stimulated with EGF or rhodamine-EGF, and observed by fluorescence microscopy (Supplementary Fig. S2C and D'). Cells with 70% to 80% reduced caveolin-1 formed waves and sequestered ligand-bound EGFR into the waves normally. We conclude that whereas Dyn2 is required, conventional endocytic coat proteins are not involved in wave formation or EGFR sequestration and internalization by waves.

**Dorsal waves support the selective and large-scale internalization of EGFR.** The data described above suggest that waves internalize EGFR; therefore, it was important to define if this represented a minor or substantial portion of the endocytosed receptors. We differentially inhibited CME versus wave-based endocytosis and quantified the effects on total EGFR internalization. Clathrin-mediated endocytosis of transferrin was reduced by 80% to 90% in both HeLa and NR6 cells expressing AP180-C or with CHC reduction by siRNA (Fig. 5F). The PI3K inhibitor wortmannin (100 nmol/L) blocked waves by 70% (Fig. 5G and Supplementary Fig. S3D) but had no effect on transferrin uptake in HeLa or NR6 (Fig. 5F). Using these selective inhibitors, we observed that in HeLa cells that do not form waves, inhibiting traditional CME reduced EGFR internalization by >80% whereas wortmannin had no effect (Fig. 5H). Remarkably, the same treatments of NR6 cells that form waves reduced EGFR internalization modestly, by 15 to 20%, whereas wortmannin reduced uptake by an average of 50% (Fig. 5H). Importantly, the inhibitory effects of both clathrin disruption and wave inhibition by wortmannin were cumulative and reduced EGFR uptake by 65% to 70% in NR6 (Fig. 5H). This additive effect indicates that clathrin-dependent and wave-based internalization of EGFR occurs in parallel. These quantitative studies indicated that waves support the internalization of a substantial proportion (~50%) of the total endocytosed EGFR, making this pathway an important participant in receptor down-regulation in cells that form waves.

**EGF-stimulated waves selectively sequester and internalize EGFR and require EGFR and PI3K activity.** This study has focused on the formation of waves in response to EGF although other RTKs also stimulate wave formation (13). Is this process selective for only ligand-bound receptor? Does it exclude other nonactivated receptors resident in the dorsal plasma membrane? To address these questions, we stimulated cells with EGF and costained for EGFR and two additional receptors including transferrin receptor and PDGFR. Cells treated with EGF showed no significant sequestration of the transferrin receptor into the

waves (not shown). Cells were also stimulated with rhodamine-EGF medium containing FITC-transferrin (to mark the ligand-bound transferrin receptor). At 5 minutes post-stimulation, EGF-EGFR complexes were sequestered at early waves whereas transferrin-transferrin receptor complexes were in a separate population of small puncta resembling early endosomes of clathrin coated pits (Fig. 6A and A', arrows). These findings further indicate that traditional CME does not occur at waves, and receptors that do not possess tyrosine kinase activity, such as transferrin receptor, are not actively sequestered into waves when either ligand-bound or unbound. We also tested if another RTK, PDGFR, might localize to EGF-stimulated waves. EGF-stimulated waves contained EGFR but there was no significant accumulation of PDGFR (Fig. 6B and B', arrows). The selectivity for EGFR was confirmed using EGFR-GFP and two distinct PDGFR antibodies (not shown). We also found by antibody staining that EGFR-containing wave-based endosomes did not contain PDGFR and that PDGF-stimulated waves did not sequester EGFR (not shown), supporting the premise that EGF-stimulated waves mediate the selective internalization of EGFR. Therefore, waves selectively internalize significant levels of ligand-bound RTKs away from other receptors, including other nonactivated RTKs (PDGFR) or ligand-bound non-RTKs such as the transferrin receptor.

Because ligand-activated RTKs are sequestered into waves, we asked which specific signaling events are required for wave formation, including receptor phosphorylation and activation of immediate downstream effectors such as PI3K and accumulation of its major lipid product, PIP<sub>3</sub>. Antibodies against specific autophosphorylated tyrosines (1068 and 1173) of EGFR labeled waves heavily (Supplementary Fig. S3A and not shown). Importantly, the phospho-EGFR antibodies did not label cells



**Figure 6.** EGF-stimulated waves sequester and internalize EGFR specifically. To test if EGFR-containing waves specifically sequestered EGFR, we attempted to localize the transferrin receptor and PDGFR to waves. A and A', NR6 were stimulated for 5 minutes with medium containing both rhodamine-EGF (30 ng/mL) and FITC-transferrin (Tfn; 5  $\mu$ g/mL) to simultaneously mark the internalization of EGFR and transferrin receptor, then acid stripped. Only the internalized rhodamine-EGF is sequestered within the waves whereas the transferrin receptor distribution seemed normal. Little or no transferrin was observed at the wave site (arrows). B and B', PANC-1 stimulated with EGF for 5 minutes and colabeled for EGFR and PDGFR. EGF-waves contain EGFR, but not PDGFR (arrows). Bar, 10  $\mu$ m for all cells (A).

pretreated with the EGFR kinase inhibitor PD168393 (not shown), indicating the specificity of the antibodies for phosphorylated receptor. EGF-stimulated wave formation was exceptionally sensitive to PD168393 and was ~70% blocked at only 0.1  $\mu\text{mol/L}$  and >90% blocked at 2.0  $\mu\text{mol/L}$  of inhibitor (Supplementary Fig. S3B). PD168393-treated cells stimulated with PDGF showed normal wave formation and no labeling with phospho-EGFR antibodies (not shown). These data show that EGFR in waves is activated, and therefore waves may act to signal from the plasma membrane.

To explore the function of activated EGFR in waves, we tested the requirement of the immediate downstream effector PI3K, which, along with PIP<sub>3</sub>, regulates multiple actin-dependent processes, including cell motility and invasion (26). PI3K inhibition blocks PDGF-stimulated wave formation (3); however, the role of PI3K in EGF waves remains unclear. Kinase-active EGFR directly activates PI3K at the plasma membrane, resulting in the synthesis of PIP<sub>3</sub>. As waves contain activated EGFR (Supplementary Fig. S3A) and are dependent on actin-membrane remodeling, this effector lipid kinase could play an important early role in waves. To monitor PIP<sub>3</sub> synthesis (PI3K activity) in EGF-stimulated cells and determine if PIP<sub>3</sub> accumulated within waves, we expressed the PIP<sub>3</sub> biosensor PH-YFP, which consists of the pleckstrin homology domain of the serine/threonine kinase Akt fused to YFP (27). A prominent labeling of waves was observed using the PIP<sub>3</sub> probe (Supplementary Fig. S3C) with a distribution similar to that of the active EGFR (Supplementary Fig. S3C), suggesting that PI3K/PIP<sub>3</sub> function early at waves and may contribute to the sequestration of EGFR and/or actin remodeling at the wave site. Further, wave formation was blocked in a dose-dependent manner by wortmannin (Supplementary Fig. S3D), implying that PI3K/PIP<sub>3</sub> is a key early regulator of wave formation at the level of activated EGFR. Together with the phospho-EGFR results, we propose that activation of EGFR and its immediate effector kinase, PI3K, are required for wave formation and subsequent EGFR sequestration and internalization through waves.

## Discussion

In this study, we show for the first time that circular dorsal ruffles/waves are transient endocytic structures that selectively sequester and internalize activated EGFR from the dorsal plasma membrane, away from other surface receptors (Figs. 1, 2, 4, and 6). Because wave formation is actin (28), cortactin (2), Dyn2 (ref. 2 and this study), EGFR kinase (this study), and PI3K (3) dependent, subsequent internalization from these structures also requires these proteins. Importantly, wave-based internalization occurs independently of traditional endocytic coat proteins including clathrin and caveolin (Fig. 3). These findings provide new insights into the mechanisms by which cells can internalize a specific activated RTK from the plasma membrane. Importantly, waves form in a variety of different epithelial and mesenchymal cell types via stimulation of different RTKs (EGFR, PDGFR, and hepatocyte growth factor receptor/c-Met), suggesting that this endocytic process is seminal and widespread. Indeed, studies by others have suggested or observed non-clathrin-based internalization of EGF or EGFR in A431 (29, 30), E11 metastatic mammary tumor (31), or COS7 cells (19). Whether these internalization processes were by dorsal waves or a macropinocytotic mechanism is unclear as waves were not documented in the studies. Both macropinocytosis and wave-based internalization are actin and PI3K dependent. However, wave-based internalization does seem to differ from conventional macropinocytosis morpho-

logically, spatially, and temporally. Waves are invaginations of the dorsal plasma membrane that form a complex reticular network of interconnected tubules that persist on average for 10 to 20 minutes after their formation, compared with conventional macropinosomes that are spherical and form rapidly (<1 minute) and continuously from peripheral membrane ruffles. In addition, unlike waves, dynamin and cortactin do not seem to function during conventional macropinocytosis and it has not been shown that macropinosomes internalize sequestered EGFR. Further, Suetsugu et al. (7) have found that WAVE2 is required for circular ruffle formation, but not for bulk fluid uptake, indicating mechanistic differences exist between conventional macropinocytosis and wave-based endocytosis. Finally, Lanzetti et al. (32) have found that Rab5 and an associated GTPase-activating protein, termed RN-tre, are both important components of dorsal waves. Whereas overexpression of RN-tre reduces Rab5 activity and prevents dorsal ruffle formation, it seems to have little effect on the formation or activity of peripheral ruffles. In addition, whereas coexpression of Rab5 and active Ras (RasV12) stimulate dorsal ruffle formation in serum-starved cells, expression of either GTPase alone will activate peripheral cortical ruffles. Thus, although similar in many ways, there are clear morphologic, functional, and compositional differences between dorsal waves/ruffles and cortical macropinocytosis. Further study will likely define additional differences. Our study and others indicate that waves are defined by a specific collection of lipids (Supplementary Fig. S3C), cytoskeletal proteins (13), signaling kinases (13), and small GTPase/associated proteins (13) that together transform a localized region of the plasma membrane into tubulated invaginations used to sequester and internalize significant numbers of ligand-bound EGFR. We do not propose that this process is the sole mechanism for EGFR internalization in these cells, but rather it provides a significant parallel pathway to CME. A caveolin/lipid raft-based pathway for EGFR endocytosis has also been observed (17); however, our data indicate that caveolins do not function in waves, indicating that multiple pathways for EGFR internalization exist.

EGFR trafficking and down-regulation have been proposed to significantly influence downstream signaling. For example, the spatial and temporal activation and signaling amplitude of mitogen-activated protein kinase(s), PI3K, and phospholipase C $\gamma$  may be affected by the characteristics of the trafficking pathway, resulting in differential effects on cell growth and/or motility. How and when cells use a specific pathway is an important area for further investigation. It is interesting to note that fewer dorsal waves form in tumor cells than do in some normal nonneoplastic cells. For example, the pancreatic and prostate tumor cell lines BxPC3, PC3, HPAF, and PANC-1 form few waves (<5-10%) compared with mouse fibroblasts and primary human fibroblasts (>60%). The inability of tumor cells to rapidly and effectively endocytose large numbers of RTKs from the surface for degradation and subsequent signaling attenuation is likely to play a significant role in neoplastic transformation. Such tumor cell types often exhibit hallmarks of transformation and are generally more motile and invasive. Interestingly, it has also been proposed that waves may contribute to matrix degradation and cell migration three-dimensionally (7), an important process during tumor cell invasion in cells that, when occurring, may be cancer relevant.

How EGFR is selectively sequestered into waves without the use of a clathrin-adaptor complex is an important question. There are likely several features of waves that contribute to this nontraditional receptor sequestration and internalization. These features include a specific lipid environment rich in PIP<sub>3</sub> (Supplementary Fig. S3C),

a lipid that interacts with Dyn2 and a variety of actin regulatory proteins, additional cytoskeletal and scaffolding proteins (13), and, potentially, adaptor proteins including growth factor receptor binding protein 2 (Grb2) and Nck1/2. These adaptors contain SH2 domains that bind directly to phosphorylated RTKs such as EGF, PDGF, and hepatocyte growth factor/c-Met receptors. Notably, EGFR phosphorylated at tyrosine 1068, a site that binds Grb2 and Nck, is significantly accumulated in waves (Supplementary Fig. S3A). Further, Grb2 and Nck harbor SH3 domains that bind directly to Dyn2 (33), N-WASp (34), RN-tre (35), WIP (36), WAVE1/2 (37), PAK1 (38, 39), and c-Cbl (40), which are known protein components of waves (13).

Interestingly, Dyn2 and cortactin, both important in waves, are conserved among several membrane trafficking pathways. These pathways include CME (10), clathrin-independent endocytosis of the  $\gamma$ c cytokine receptor (41), anterograde trafficking from the Golgi (42), and, as presented here, wave-based endocytosis. This suggests that Dyn2 and cortactin are core components required for actin-dependent membrane trafficking of receptors in general. Furthermore, many of the key proteins in waves have characterized

roles in both membrane trafficking and cell motility (14), indicating a potential coupling of these cellular machineries at the interface of the plasma membrane and cytoskeleton.

Understanding the precise orchestration of events during dorsal wave formation, receptor sequestration, and endocytosis is a significant challenge as at least 30 proteins have been localized to these structures (13). Future studies should focus on the mechanism of EGFR sequestration into, and downstream signaling from, waves and on how these complex structures contribute to cell growth, motility, and metastasis.

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