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ERK-dependent threonine phosphorylation of EGF receptor modulates receptor downregulation and signaling

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SUMMARY

Epidermal growth factor (EGF) signaling is critical in normal and aberrant cellular behavior. Extracellular signal-regulated kinase (ERK) mediates important downstream aspects of EGF signaling. Additionally, EGFR undergoes MEK1-dependent ERK consensus site phosphorylation in response to EGF or cytokines such as growth hormone (GH) and prolactin (PRL). GH- or PRL-induced EGFR phosphorylation alters subsequent EGF-induced EGFR downregulation and signal characteristics in an ERK-dependent fashion. We now use reconstitution to study mutation of the sole EGFR ERK phosphorylation consensus residue, ⁶⁶⁹T. CHO-GHR cells, which lack EGFR and express GHR, were stably transfected to express human wild-type or T669A (⁶⁶⁹T changed to alanine) EGFRs at similar abundance. Treatment of cells with GH or EGF caused phosphorylation of WT, but not T669A EGFR, in an ERK activity-dependent fashion that was detected with an antibody that recognizes phosphorylation of ERK consensus sites, indicating that ⁶⁶⁹T is required for this phosphorylation. Notably, EGF-induced downregulation of EGFR abundance was much more rapid in cells expressing EGFR T669A vs. WT EGFR. Further, pretreatment with the MEK1/ERK inhibitor PD98059 enhanced EGF-induced EGFR loss in cells expressing WT EGFR, but not EGFR T669A, suggesting that the ERK-dependent effects on EGFR downregulation required phosphorylation of ⁶⁶⁹T. In signaling experiments, EGFR T669A displayed enhanced acute (15 min) EGFR tyrosine phosphorylation (reflecting EGFR kinase activity) compared to WT EGFR. Further, acute EGF-induced ubiquitination of WT EGFR was markedly enhanced by PD98059 pretreatment and was increased in EGFR T669A-expressing cells independent of PD98059. These signaling data suggest that ERK-mediated ⁶⁶⁹T phosphorylation negatively modulates EGF-induced EGFR kinase activity. We furthered these investigations using a human fibrosarcoma cell line that endogenously expresses EGFR and ErbB-2 and also harbors an activating Ras mutation. In these cells, EGFR was constitutively detected with the ERK consensus site phosphorylation-specific antibody and EGF-induced EGFR downregulation was modest, but was substantially enhanced by pretreatment with MEK1/ERK inhibitor. Collectively, these data indicate that ERK activity, by phosphorylation of a

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threonine residue in the EGFR juxtamembrane cytoplasmic domain, modulates EGFR trafficking and signaling.

1. Introduction

Epidermal growth factor (EGF) is a 53 amino acid peptide that has important roles in cell growth, differentiation, motility and adhesion [1]. These effects are mediated by binding and activating EGF receptor (EGFR). EGFR belongs to ErbB receptor superfamily, a group of transmembrane glycoprotein receptors, consisting of four members: EGFR (ErbB-1), ErbB-2 (c-neu, HER2), ErbB-3 (HER3), and ErbB-4 (HER 4) [2–5]. Except for ErbB-3, each has intrinsic tyrosine kinase activity in its cytoplasmic domain. EGF specifically binds to EGFR, promoting formation of either EGFR-EGFR homodimers or EGFR-ErbB-2 heterodimers and allowing the intracellular tyrosine kinase domains to approximate and undergo transautophosphorylation [6–10]. Consequent to kinase activation and autophosphorylation, C-terminal receptor phosphotyrosine residues (ten identified thus far) are engaged by signaling molecules including SHC, Grb-2, SHP2, phospholipase-C- γ , and others [11–16], leading to activation of downstream signaling by the ERK, PI3-kinase, and PLC- γ pathways. Dysregulated function of EGFR family proteins, including EGFR, is significant in onset and behavior of many human cancers [17–21] and measures targeting EGFR downregulation may alter cancer behavior [19,22]. Thus, it is important to understand mechanisms regulating EGFR signaling and trafficking.

The itinerary of EGF-induced EGFR trafficking has been intensely studied [23]. After cell surface EGF binding, EGFR undergoes clathrin coated pit-mediated endocytosis at a much enhanced rate compared to the constitutive (ligand-independent) rate. Thereafter, the activated receptor enters the endosomal pathway. If not recycled to the cell surface (as in the absence of EGF stimulation), EGFR progresses from early to late endosomes and then to the multivesicular bodies, ultimately undergoing degradation in lysosomes in a process termed receptor downregulation. Previous views held that signaling emanated only from activated cell surface EGFRs and that internalization terminated signaling [24]; more recent studies suggest that signaling in some measure emanates from EGFRs that are internalized, but not yet spatially reoriented in the MVB or degraded in the lysosome [25–30]. Thus, altered post-endocytic trafficking of activated EGFR may quantitatively and/or qualitatively impact EGF signaling.

We previously examined interplay between growth hormone [31–33] and prolactin [34] signaling and EGF signaling in murine preadipocytes and human T47D breast carcinoma cells, respectively. We observed that GH caused tyrosine phosphorylation of EGFR; this tyrosine phosphorylation has previously been shown to be catalyzed by JAK2 and unassociated with EGFR kinase activation [35]. In addition, we found that GH promoted serine/threonine phosphorylation of both EGFR and ErbB-2. Using a monoclonal antibody, PTP101, that detects (serine/threonine) phosphorylation at substrate consensus sites for extracellular signal-regulated kinase (ERK), we observed GH- and PRL-dependent PTP101-reactive EGFR and ErbB-2 phosphorylation that was prevented by pretreatment of the cells with inhibitors of MEK1, the upstream ERK-activating kinase [32–34]. For EGFR, this GH- or PRL-induced ERK-dependent phosphorylation retarded subsequent EGF-induced receptor downregulation and potentiated acute EGF-induced signaling [32–34]. Furthermore, in T47D cells, EGF itself caused EGFR PTP101-reactive phosphorylation and blockade of MEK1 resulted in enhanced EGF-induced EGFR downregulation, suggesting that EGF-induced ERK-mediated threonine phosphorylation may serve as a brake on receptor downregulation [34].

In the current work, we extend these observations by studying EGF-induced EGFR PTP101-reactive phosphorylation, EGFR downregulation, and signaling in a reconstitution system. We

map the EGFR threonine residue required for this ERK-dependent homologous phosphorylation and assess its impact on downregulation and signaling. Further, we investigate these issues using a human fibrosarcoma cell line that endogenously expresses EGFR and ErbB-2 and also harbors an activating Ras mutation. In these cells, constitutive PTP101-reactive phosphorylation of EGFR was detected and EGF-induced EGFR downregulation was modest, but was substantially enhanced by pretreatment with MEK1/ERK inhibitor.

2. Materials and methods

2.1. Materials

Recombinant human GH was kindly provided by Eli Lilly Co. (Indianapolis, IN). Recombinant human EGF was purchased from Upstate Biotechnology (Lake Placid, NY). The MEK inhibitors, PD98059 (New England Biolabs, Beverly, MA) and U0126 (Promega, Madison, WI), were purchased commercially.

2.2. Antibodies

Polyclonal anti-EGFR, monoclonal anti-EGFR, anti-ErbB-2, monoclonal anti-STAT5 (W-17) antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), polyclonal anti-EGFR used in co-immunoprecipitation with erbB2, monoclonal anti-phospho-threonine-proline antibody PTP101, polyclonal anti-phospho-EGFR antibodies anti-pTyr-992 and anti-pTyr-1068, polyclonal anti-phospho-AKT (recognizing phosphorylated serine 473), polyclonal anti-AKT antibodies (Cell Signaling Technology, Beverly, MA), anti-active mitogen-activated protein kinase affinity-purified rabbit antibody (anti-active ERK, recognizing the dually phosphorylated Thr-183 and Tyr-185 residues corresponding to the active forms of ERK1 and ERK2 (Promega, Madison, WI), monoclonal anti-phosphotyrosine antibody 4G10, anti-mitogen-activated protein kinase affinity-purified rabbit antibody (recognizing both ERK1 and ERK2) (Upstate Biotechnology, Lake Placid, NY), anti-ubiquitin monoclonal antibody (Stressgen Biotechnologies, Victoria, BC Canada) and anti-Flag antibody, anti-Flag affinity gel (Sigma, St Louis, MO) were all purchased commercially.

2.3. Cell culture and transfection

CHO-GHR cells [36] were cultured in Dulbecco's modified Eagle's medium containing 1.0 g/liter glucose (Cellgro, Inc. Hemdon, VA) supplemented with 5% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin (all from Biofluids, Rockville, MD), and 100 µg/ml hygromycin B (all from Biofluids, Rockville, MD). (Cell culture reagents were obtained via the UAB Comprehensive Cancer Center Media Core Facility.) For transient transfection, CHO-GHR cells were seeded in 10cm² dishes and used at 80% confluence. The plasmids pX-WT EGFR, pX-EGFR T669A, and pX-EGFR T654A, kindly provided by Dr. R. Davis (University of Massachusetts Medical School, Worcester, MA) [37] were transfected into CHO-GHR cells using lipofectAMINE Plus (Invitrogen) according to manufacturer's instruction. To generate stable CHO-GHR transfectants expressing the wild-type EGFR or T669A EGFR, cells were seeded in 10 cm² dishes and used at 50% confluence. The plasmids pX-WT EGFR and pX-EGFR T669A, from Dr. R. Davis were subcloned into the pcDNA (Invitrogen Corp, Carlsbad, CA) eukaryotic expression vector. pcDNA-WT EGFR or pcDNA-EGFR T669A was transfected into CHO-GHR cells using lipofectAMINE Plus (Invitrogen) according to the manufacturer's instructions. CHO-GHR cells expressing WT EGFR or EGFR T669A were selected in 1 mg/ml of G418 (Invitrogen) and cloned. Transfectants were maintained in culture medium containing 200 µg/ml of G418. For transient co-transfection with EGFR and ErbB-2, CHO-GHR cells were placed in 10cm² dishes and used at 80% confluence. The plasmids pCS105-FLAG-ErbB-2, kindly provided by Dr. Chenbei Chang (UAB), or pcDNA were transfected together with either pX-WT EGFR or pX-T669A EGFR

into CHO-GHR cells using methods as mentioned above. The plasmid ratio between pCS105-Flag-ErbB-2 or pcDNA and pX-WT EGFR or pX-T669A EGFR was 3:1.

2.4. Cell starvation, inhibitor pretreatment, cell stimulation, and protein extraction

Serum starvation of CHO-GHR cells was accomplished by substitution of 0.5% (w/v) bovine serum albumin (fraction V: Roche Molecular Biochemicals) for fetal bovine serum in the culture medium for 16–20h prior to experiments. Pretreatments and stimulations were carried out at 37°C in binding buffer (consisting of 25mM Tris-HCL (PH 7.4), 120mM NaCl, 5mM KCl, 1.2mM MgCl₂, 0.1% (w/v) bovine serum albumin, and 1mM dextrose). Serum-starved cells were pretreated with PD98059 (100 µM), U0126 (10 µM) or vehicle (as a control) for 60 min prior to treatment with EGF (concentrations indicated in figure legends), or vehicle as specified in each experiment. Stimulations were terminated by washing the cells once with ice-cold phosphate-buffered saline supplemented with 0.4 mM sodium orthovanadate (PBS-vanadate) and then harvested by scraping in PBS-vanadate. Cells were collected by brief centrifugation, and pelleted cells were solubilized for 30 min at 4°C in lysis buffer (1% (v/v) Triton X-100, 150 mM NaCl, 10% (v/v) glycerol, 50 mM Tris-HCl (pH 8.0), 100 mM NaF, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM benzamidine, 5 µg/ml aprotinin, and 5 µg/ml leupeptin). After centrifugation at 15,000×g for 15 min at 4°C, the detergent extracts (supernatant) were subjected to immunoprecipitation or were directly electrophoresed and immunoblotted, as indicated below.

2.5. Immunoprecipitation and immunoblotting

For immunoprecipitation, cell extracts (500–1000 µg) were mixed with 5 µl of monoclonal anti-EGFR antibody (1 µg) and incubated at 4°C overnight with continuous agitation. Protein A-sepharose beads (Amersham Biosciences, Piscataway, NJ) were added and incubated at 4°C for an additional hour. The beads were washed four times with lysis buffer adjusted to 0.5% (v/v) Triton X-100. Laemmli sample buffer eluates were resolved by SDS-PAGE and immunoblotted as indicated below.

Proteins resolved by SDS-PAGE were transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences). The membranes were blocked with TBST buffer (20mM Tris-HCl (pH 7.6), 150mM NaCl, and 0.1% (v/v) Tween 20) containing 2% (w/v) bovine serum albumin and incubated with primary antibodies (0.5–1 µg/ml) as specified in each experiment. After three washes with TBST, the membranes were incubated with appropriate secondary antibodies (1:10,000 dilution) and washed. The bound antibodies were detected with SuperSignal chemiluminescent substrate (Pierce Chemical Co., Rockford, IL). Membrane stripping was performed according to the manufacturer's suggestions (Amersham Biosciences).

2.6. Degradation of EGFR after blockade of protein synthesis

CHO-GHR-WT EGFR and CHO-GHR-EGFR T669A were grown to 80% confluence in six-well dishes and serum starved overnight. Cells were then incubated with cycloheximide (20 µg/ml) together with MEK inhibitor or vehicle for 1 h prior to stimulation with EGF (10nM) for the indicated durations. Cell lysates were resolved by SDS-PAGE and blotted with anti-EGFR.

2.7. Densitometric analysis

Densitometric quantitation of ECL immunoblots was performed using a high-resolution scanner and the ImageJ 1.30 program (developed by W.S. Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). Pooled data from several experiments are displayed as mean ± S.E. The significance of differences (*p* value) of pooled results was estimated using paired *t* tests.

3. Results

3.1 EGF causes PTP101-reactive phosphorylation of wild-type EGFR, but not T669A EGFR, in reconstituted CHO cells

EGFR can be phosphorylated at multiple sites in the intracellular domain, including serine, threonine, and tyrosine residues. Several reports have stressed the correlation between phosphorylation at certain serine or threonine residues and modulation of EGFR activity [37–40]. Our previous work indicated that GH, PRL, and EGF induce EGFR serine/threonine phosphorylation that requires ERK activity and is detected by PTP101, a monoclonal antibody that reacts with phosphorylated serine or threonine residues that reside within ERK substrate consensus sites [32–34]. To further pursue the mechanism and impact of such EGFR phosphorylation, we explored which EGFR residues are involved. Previous studies suggest that ⁶⁶⁹T is phosphorylated following EGF stimulation [41]. By using a synthetic peptide that encompasses ⁶⁶⁹T, it has been shown that this residue can be a substrate for MAP kinase (ERK) [42–44]. Indeed the EGFR intracellular domain encodes only one ERK consensus phosphorylation site (PX(S/T)P), which resides within the juxtamembrane region and includes PL ⁶⁶⁹TP (Figure 1A).

To understand whether ⁶⁶⁹T is required for PTP101 reactivity, we examined an EGFR mutant, EGFR-T669A, in which this residue is mutated to alanine. CHO-GHR cells, which express the rabbit GHR, but not EGFR [45], were used as a target for transfection with wild-type (WT) or mutant EGFR. Both WT EGFR and EGFR T669A were detected by immunoblotting at the expected M_r and acute stimulation of each transfectant with EGF resulted in tyrosine phosphorylation of EGFR (Figure 1B). These data indicate that each receptor was displayed at the cell surface and that EGF binds productively to each at the cell surface.

We next tested the ability of WT and EGFR T669A to undergo PTP101-reactive phosphorylation (Figure 2). Cells expressing vector control, WT EGFR, or EGFR T669A were treated with vehicle (–), GH 10min, or EGF for 15 minutes prior to detergent extraction. Each sample was immunoprecipitated with anti-EGFR and eluates were resolved by SDS-PAGE and immunoblotted with PTP101 (Figure 2A). As expected, both GH and EGF induced PTP101-reactive phosphorylation of WT EGFR. In contrast, neither GH nor EGF promoted such phosphorylation of EGFR T669A. Reprobing with anti-EGFR verified ample WT and EGFR T669A expression and the lack of endogenous EGFR in vector-transfected cells. Further, aliquots of extract from each sample were resolved and immunoblotted with anti-pERK, revealing that both GH and EGF promoted ERK activation in cells harboring either WT or EGFR T669A. Thus, the inability of EGFR T669A to undergo PTP101-reactive phosphorylation was not due to the inability of either GH or EGF to induce ERK activation in cells that express this mutant receptor.

ERK pathway involvement in EGF-induced PTP101-reactive EGFR phosphorylation was further examined in the experiment shown in Figure 2B. Cells expressing WT or EGFR T669A were pretreated with the MEK1 inhibitor, PD98059 (+), or vehicle (–) and then stimulated with EGF (+) or vehicle (–) for 15 min. As in Figure 2A, EGF promoted PTP101-reactive phosphorylation of WT, but not EGFR T669A. Additionally, EGF-induced WT EGFR PTP101 reactivity was blocked by PD98059, indicating that both ERK activation and an intact threonine-669 are required for this EGFR phosphorylation.

As a test of specificity, we also evaluated the consequences of mutation of threonine-654, another known phosphorylation site in the EGFR juxtamembrane intracellular domain; ⁶⁵⁴T phosphorylation is believed to be catalyzed by protein kinase C (PKC) [46,47]. As shown previously [37], we found that expression of EGFR T654A, in which ⁶⁵⁴T is mutated to alanine, yielded surface receptor expression and EGF-induced EGFR tyrosine phosphorylation (data

not shown). However, in distinction to EGFR T669A, PTP101-reactive phosphorylation of EGFR T654A was detected basally at a low level and further induced by EGF treatment; furthermore, this PTP101-reactive phosphorylation was inhibited by PD98059 pretreatment (Figure 2C). This indicates that ⁶⁶⁹T, but not ⁶⁵⁴T, is required for EGF-induced ERK-dependent EGFR PTP101- reactive phosphorylation.

3.2 EGF-induced EGFR downregulation is enhanced in CHO cells expressing EGFR T669A compared to WT EGFR

Our previous work indicated that PTP101-reactive EGFR phosphorylation correlated with diminished EGF-induced EGFR downregulation [33,34]. We thus examined the effect of the T669A mutation on EGF-induced EGFR loss in reconstituted CHO cells expressing either EGFR T669A or WT EGFR (Figure 3). In these stably transfected cells, EGFR expression is governed by a CMV promoter-driven eukaryotic expression vector. To nullify any effect of EGF signaling on the level of EGFR expression (via CMV promoter activation), we pretreated serum-starved cells for 1h with cycloheximide (CHX) to prevent new protein synthesis and then treated with EGF for 0.5–3h (Figure 3A). CHX itself had no substantial effect on EGFR abundance in either transfectant, even when present for 5h (data not shown). In WT EGFR-expressing cells, EGF induced detectable loss of receptor after 1h and further loss thereafter. Notably, this EGF-induced receptor loss was more profound in cells harboring EGFR T669A. Several such experiments were analyzed densitometrically and the degree of EGF-induced loss of WT EGFR vs. EGFR T669A was quantitatively compared (Figure 3B). This revealed that ligand-induced receptor loss was significantly greater for EGFR T669A than for WT EGFR at all EGF stimulation time points tested and that the degree of loss was progressively greater for EGFR T669A vs. WT EGFR with increasing duration of EGF treatment; 3h of EGF exposure caused nearly 50% loss of WT EGFR and >90% loss of EGFR T669A. (Regression analysis indicated receptor half-lives of 3.2 h for WT EGFR and 1.6 h for EGFR T669A in response to EGF.)

We further pursued EGF-induced EGFR downregulation by exploring the effect of pharmacological inhibition of the ERK pathway (Figure 3C). Cells expressing either WT EGFR or EGFR T669A were pretreated with the MEK1 inhibitor, U0126, prior to treatment for 2h with EGF (+) or vehicle (–). As expected, EGF treatment caused downregulation of EGFR T669A that was markedly enhanced compared to WT EGFR. However, when EGF-induced ERK activation was blocked, downregulation of WT EGFR was greatly increased, suggesting that ERK-mediated phosphorylation of the receptor lessens its downregulation. Indeed, pretreatment with U0126 did not affect EGF-induced downregulation of EGFR T669A. In concert with the results shown in Figure 2, these data indicate that EGF induces ERK-mediated phosphorylation of EGFR, likely at ⁶⁶⁹T, and this phosphorylation serves as a brake to receptor downregulation.

3.3 Effects of EGF-induced EGFR ⁶⁶⁹T phosphorylation on EGFR tyrosine phosphorylation and ubiquitination

Previous studies indicated that EGFR phosphorylation at sites other than tyrosine residues can affect receptor signaling. For example calmodulin-dependent protein kinase II (CaM kinase II) promotes phosphorylation at residues ¹⁰⁴⁶S and ¹⁰⁴⁷S; replacement of these residues by alanine results in decreased EGF-induced EGFR downregulation and enhanced EGF signaling [38,39]. Activation of protein kinase C promotes phosphorylation of EGFR at ⁶⁵⁴T; mutation of this residue results in enhanced EGF-induced signaling and inhibits phorbol ester-induced blunting of EGFR kinase activity [37,40]. In contrast to these EGFR phosphorylation sites, the impact of at ⁶⁶⁹T phosphorylation on EGF signaling in the context of intact cells has been relatively less clear. Hence, we sought to compare EGF-induced WT EGFR kinase activation with that of EGFR T669A (Figure 4).

Serum-starved cells harboring each of the receptors were treated with EGF (1 nM) for various durations from 15–180 min, after which proteins were extracted and immunoblotted with antibodies that detect phosphorylation of residues ⁹⁹²Y or ¹⁰⁶⁸Y (Figure 4A). Phosphorylation of these residues is correlated with EGFR kinase activation [48, 49]. As expected, EGF caused phosphorylation at WT EGFR at both ⁹⁹²Y or ¹⁰⁶⁸Y that peaked after roughly 30 min and persisted for up to 180 min. Likewise, EGF treatment induced phosphorylation of both residues in EGFR T669A; however, in this case the signals were maximal after 15 min and thereafter dissipated more rapidly than in WT EGFR. Notably, though, peak phosphorylation of EGFR T669A was substantially greater than that achieved for WT EGFR, such that even though it decayed more rapidly, the signals seen after 60 min for EGFR T669A were similar to those observed for WT EGFR at the same time point. Blotting for EGFR verified that these differences in maximal phosphorylation and kinetics could not be explained by differences in EGFR abundance between the two cells.

The effects of pharmacologic ERK inhibition on this EGF-induced EGFR tyrosine phosphorylation were tested in Figure 4B. Cells pretreated with vehicle (–) or PD98059 (+) were then exposed to vehicle (0 min) or EGF for 5 or 15 min. As seen in Figure 4A, acute (both 5 and 15 min) EGF-induced phosphorylation of ⁹⁹²Y and ¹⁰⁶⁸Y were enhanced in EGFR T669A compared to WT EGFR. Notably, pretreatment with the MEK1 inhibitor PD98059 led to marked enhancement of EGF-induced tyrosine phosphorylation of WT EGFR. In distinction, EGF-induced EGFR T669A tyrosine phosphorylation was not affected by PD98059 pretreatment. In concert with the findings in Figure 4A, these data strongly suggest that the ability of EGF to activate ERKs and thereby cause phosphorylation of EGFR ⁶⁶⁹T serves to dampen the degree of receptor kinase activation that ensues. EGF concentration dependence experiments (not shown) were consistent with these observations and indicated that EGFR tyrosine phosphorylation was easily detected with as little as 0.1 nM EGF (5 min) for EGFR T669A, but required 1 nM for WT EGFR.

EGFR is known to form dimers – homodimers (EGFR-EGFR) or heterodimers (EGFR-ErbB-2, for example; more below). We asked how coexpression of WT EGFR with EGFR T669A would compare with expression of each alone in terms of acute EGFR kinase activation in response to EGF (Figure 5). Cells were transiently transfected with expression plasmids encoding either WT EGFR, EGFR T669A, or an equal mixture of the two plasmids. After serum starvation, cells were treated with EGF for 0, 5, or 15 min, after which cell extracts were blotted with anti-pY-1068 and anti-EGFR (Figure 5A). EGF-induced EGFR tyrosine phosphorylation was enhanced in cells expressing EGFR T669A alone vs. WT EGFR, as expected. Notably, in cells harboring both wild-type and mutant receptors, the EGF-induced pattern of EGFR tyrosine phosphorylation was distinctly more similar to that seen in cells that express EGFR T669A alone than to those expressing WT EGFR alone. Densitometric quantitation with normalization for EGFR abundance of three similar experiments is displayed in Figure 5B and validates this observation. Thus, the propensity of EGFR T669A to enhance EGF sensitivity is not dampened by coexpression with WT EGFR, consistent with the notion that in WT-T669A heterodimers the mutant exerts dominance regarding this phenotype.

Consequent to EGF-induced EGFR kinase activation, the tyrosine phosphorylated receptor is rapidly ubiquitinated by c-Cbl, an SH2 domain-containing ubiquitin ligase that binds EGFR; this ubiquitination promotes post-internalization EGFR sorting to lysosomes [50–52]. Ubiquitinated EGFR can be detected after anti-EGFR immunoprecipitation, SDS-PAGE, and immunoblotting with anti-ubiquitin as a high-M_r smear. The smearing reflects multiple monoubiquitination, rather than polyubiquitination [53,54]. Polyubiquitination signals proteasomal degradation, while monoubiquitination is involved in endocytic/endosomal-lysosomal trafficking [55–59]. At each step of EGF-induced EGFR endocytosis and downregulation, multiply monoubiquitinated EGFR associates with ubiquitin interacting

motif-containing proteins [60]. As EGFR T669A underwent more robust acute tyrosine phosphorylation than did WT EGFR in response to EGF, we tested the effects of this mutation on EGF-induced EGFR ubiquitination (Figure 6).

Serum-starved CHO transfectants were pretreated with PD98059 (+) or vehicle (−) prior to stimulation with EGF (+) or vehicle (−) for 15 min (Figure 6A). Detergent extracts were precipitated with anti-EGFR and eluates were immunoblotted with anti-ubiquitin. Aliquots of extract were resolved without precipitation and immunoblotted with anti-pERK to verify the effectiveness of the PD98059. As expected, EGF acutely induced WT EGFR ubiquitination. Notably, inhibition of ERK activation with PD98059 markedly enhanced the effect of EGF on WT EGFR ubiquitination. In contrast, the degree of ubiquitination of EGFR T669A induced by EGF was substantially greater than that seen for WT EGFR and was not affected by PD98059 pretreatment. Three such experiments were analyzed densitometrically, estimating the relative abundance of EGF-induced EGFR ubiquitination normalized in each case by the EGFR abundance in the same immunoprecipitate. The graphical display of these data (Figure 6B) indicates that blockade of ERK-dependent phosphorylation of threonine-669 of EGFR (either pharmacologically or mutagenetically) in response to EGF leads to statistically-significantly increased EGF-induced EGFR ubiquitination.

3.4 Endogenous EGFR PTP101 phosphorylation and its effects in a human fibrosarcoma cell

The data outlined in Figure 1–Figure 6 derive from the study of EGFR (wild-type or mutant) expressed in the context of CHO cells, in which no ErbB family members are endogenously expressed. To further explore these issues in a more physiologically-relevant milieu, we used a human fibrosarcoma cell line that endogenously expresses EGFR and ErbB-2 and also harbors an activating Ras mutation. The C14 cell [61–63], is a derivative of the HT1080 human fibrosarcoma cell line, in which ERKs are constitutively active due to a Ras mutation [64,65] and in which EGFR is endogenously expressed. Serum-starved C14 cells exhibited activated ERK, which was blocked by PD98059; immunoprecipitated EGFR from the same extracts was recognized by PTP101, but not if the cells were treated with PD98059, indicating substantial basal ERK-dependent PTP101-reactive EGFR phosphorylation in these fibrosarcoma cells (Figure 7). EGF treatment for 15 min enhanced both ERK activity and PTP101-reactive EGFR phosphorylation somewhat, effects prevented by PD98059 pretreatment. Importantly, these findings extend the correlation between ERK activity and PTP101-reactive EGFR phosphorylation found in mouse preadipocytes [32,33], human T47D breast cancer cells [34], and CHO cells reconstituted with human EGFR (Figure 2 of this manuscript) to include another cell line, in this instance a human fibrosarcoma cell. Further, they indicate that constitutive Ras/ERK activation characteristic of many human cancers can be associated with such receptor phosphorylation even in the absence of growth factor or cytokine stimulation.

We examined the effect of EGF on EGFR abundance in C14 cells. Serum-starved cells were treated with EGF (1 nM) for 0–4 h, as indicated, and EGFR abundance in the detergent cell extracts was monitored by anti-EGFR immunoblotting (Figure 8A). Pretreatment with PD98059 to inhibit both basal and EGF-induced ERK activity resulted in significantly enhanced EGF-induced EGFR loss compared to cells not pretreated (Figures 8A and 8B). ErbB2 levels in the same extracts were determined by immunoblotting as a loading control; these levels did not vary with either EGF or PD98059 treatment. Notably, EGF-induced EGFR downregulation in C14 cells was relatively blunted. Densitometric quantitation of several such experiments (Figure 8B) indicated that EGF treatment for 4 h yielded only ~40% decrease in EGFR levels. (Although comparison between different cell systems may not be entirely valid, EGF treatment of CHO cells stably expressing WT EGFR for 3 h caused a slightly greater degree of receptor loss than that seen in C14 cells treated for 4 h.) Similarly enhanced EGF-

induced EGFR downregulation in C14 cells was observed when U0126, a distinct ERK pathway inhibitor, was used instead of PD98059 (Figure 8C).

To assess EGFR activation, serum-starved C14 cells were exposed to EGF for 0.5–2 h and extracts were immunoblotted with the antibody that detects phosphorylation of ^{1068}Y (Figure 9A, left side). As expected, EGF treatment caused EGFR tyrosine phosphorylation, although, interestingly, densitometric analysis of multiple experiments (Figure 9B) revealed that peak activation was observed in these cells 1 h after EGF exposure. (For comparison, peak EGF-induced ^{1068}Y phosphorylation was seen after 0.5 h in CHO cells that express WT EGFR (Figure 4A and data not shown).) Pretreatment of C14 cells with PD98059 significantly enhanced ^{1068}Y receptor phosphorylation detected after 0.5 h when compared to vehicle-pretreated cells (Figures 9A and 9B) and thereby shifted the peak for this EGF-induced signal from 1 h to 0.5 h in these cells. Thus, pharmacologic blunting of basal and EGF-induced ERK activity in C14 cells augmented early EGF-induced EGFR activation; however, the overall ^{1068}Y phosphorylation signal at later EGF stimulation time points in PD98059-pretreated cells was not enhanced, likely in part because EGFR abundance was lessened at these later points in the inhibitor-pretreated cells.

3.5 Coexpression of WT EGFR or EGFR T669A with ErbB-2

The data in Figure 7–Figure 9 indicate that endogenous (wild-type) EGFR present along with endogenous ErbB-2 in fibrosarcoma cells undergoes EGF-induced PTP-101-reactive phosphorylation and that in that setting pharmacologic prevention of ERK activity augments EGF-induced EGFR downregulation and EGF-induced EGFR tyrosine phosphorylation. In the experiments in Figure 10, we reverted to the CHO-GHR system (in which neither EGFR nor ErbB-2 is endogenously expressed) to probe the effects of coexpression or ErbB-2 on EGF-induced EGFR downregulation of EGFR T669A compared to WT EGFR.

WT EGFR- or EGFR T669A-expressing CHO-GHR cells were transfected with FLAG-tagged ErbB-2 or a vector control, as indicated (Figure 10A). After serum starvation and EGF treatment for 5 min, detergent extracts were immunoprecipitated with anti-FLAG and eluates were resolved and blotted sequentially for EGFR and ErbB-2. Both WT EGFR and EGFR T669A were specifically coprecipitated with ErbB-2 and EGF augmented this association. This indicates that the T669A mutation does not affect the ability of the EGFR to associate with ErbB-2. To examine EGF-induced EGFR downregulation (Figure 10B), CHO-GHR cells were cotransfected with FLAG-tagged ErbB2 or a vector control and either WT EGFR or EGFR T669A, as indicated, and treated with EGF for 3h in the presence of CHX, as in Figure 3. Notably, EGF-induced downregulation of EGFR T669A was substantially augmented in comparison to WT EGFR under these circumstances. This suggests that even though ErbB-2 is known to dampen EGF-induced EGFR downregulation (further discussed below), the difference between the WT EGFR and EGFR T669A is still maintained in the setting of ErbB-2 expression.

4. Discussion

Regulation of EGFR activity is critical for normal cellular function. Receptor phosphorylation on several cytoplasmic domain tyrosine residues (including ^{845}Y , ^{891}Y , ^{920}Y , ^{992}Y , ^{1045}Y , ^{1068}Y , ^{1086}Y , ^{1114}Y , ^{1148}Y , and ^{1173}Y) occurs in response to EGF binding and is catalyzed by the intrinsic enzyme activity embedded in the cytoplasmic tyrosine kinase domain (residues 688–957) [11–14]. Phosphorylation recruits a number of docking and signaling proteins, including Grb-2, SHC, PTP-1B, SHP-1, SHP-2, Cbl, and PLC- γ to these sites [15,16], and influences several downstream signaling pathways to ultimately govern gene expression and cell behavior. Thus, the strength (stoichiometry/abundance), timing (pace, duration), and location (cell surface vs. intracellular) of ligand-

induced EGFR tyrosine phosphorylation and the ligand-induced downregulation of receptor levels are believed to be key modulators of EGF action.

In addition to undergoing extensive cytoplasmic tail tyrosine phosphorylation, EGFR is also targeted for serine/threonine phosphorylation in response to several stimuli. Early studies indicated that four major non-tyrosine phosphorylation sites in the cytoplasmic domain include ⁶⁵⁴T, ⁶⁶⁹T, ¹⁰⁴⁶S, and ¹⁰⁴⁷S [37–39,41,43,44,46,47,66,67]. A more recent proteomic analysis [11] validated that EGF promotes phosphorylation of these four major sites and, in addition identified five other sites -- ⁶⁷¹S, ⁹⁶⁷S, ¹⁰⁰²S, ¹⁰⁵⁷S, and ¹¹⁴²S. While the significance of phosphorylation at these latter sites is yet unknown, phosphorylation of residues ⁶⁵⁴T, ⁶⁶⁹T, ¹⁰⁴⁶S, and ¹⁰⁴⁷S has been explored in some detail.

¹⁰⁴⁶S, and ¹⁰⁴⁷S, distal to the EGFR tyrosine kinase domain, were shown to be substrate phosphorylation sites for the serine/threonine kinase, calcium- and calmodulin-dependent protein kinase II (CaM kinase II) [38]. Replacement of ^{1046/1047}S with alanines in the context of the full-length EGFR and comparison with WT EGFR in a CHO cell reconstitution system revealed that EGFR S1046/1047A exhibited markedly decreased EGF-induced EGFR downregulation and enhanced EGF-induced signaling; moreover, a desensitizing effect of EGF on subsequent EGFR kinase activity found in WT EGFR was lost in EGFR S1046/1047A [38,39]. Thus, it was concluded that phosphorylation of ¹⁰⁴⁶S and ¹⁰⁴⁷S in the receptor tail facilitated several negative regulatory events in the itinerary of the EGF-stimulated EGFR, including receptor internalization/downregulation and desensitization of the kinase to further EGF stimulation [38,39].

In contrast to ¹⁰⁴⁶S and ¹⁰⁴⁷S, which reside in the distal tail, ⁶⁵⁴T and ⁶⁶⁹T are found in the juxtamembrane region of the EGFR cytoplasmic domain, proximal to the kinase domain. Initial studies of phosphorylation at ⁶⁵⁴T focused on understanding the effects of the phorbol ester, PMA, which was shown to diminish EGFR tyrosine kinase activity by activation of protein kinase C [68,69]. Mapping studies demonstrated that ⁶⁵⁴T phosphorylation was promoted by PMA-induced PKC activity [46,47]. Indeed, mutation of ⁶⁵⁴T to alanine (EGFR T654A) resulted in enhanced EGF-induced signaling and reduced the inhibitory effect of PMA on EGFR kinase activity [37]. Some studies with stimuli other than PMA have further linked such PKC-dependent EGFR phosphorylation to functional consequences. Sheffield and colleagues studied the effects of prolactin on EGFR function in normal murine mammary epithelial cells [70–73]. In those cells, prolactin caused substantial PKC-dependent EGFR threonine phosphorylation (although the residue was not mapped) and subsequent desensitization of EGF-induced EGFR tyrosine kinase activity. A more recent study indicated that in vitro and in vivo effects of leutinizing hormone releasing hormone (LHRH) on prostate cancer cell growth were mediated via PKC and were not observed when EGFR T654A was expressed in the cancer cells, further supporting the potential role of this site in regulating EGFR kinase activity [74]. A very recent study [75] may provide mechanistic insights into the role of the juxtamembrane domain and, in particular, phosphorylation of ⁶⁵⁴T in such EGFR kinase regulation. In that work [75], the juxtamembrane region was shown to be required for allosteric kinase activation and productive monomer interactions within the EGFR dimer that are believed important for EGF-induced kinase activation [10]. Expression of an intracellular domain with mutation of ⁶⁵⁴T to alanine (ICD T654A) augmented kinase activity of a coexpressed wild-type ICD, whereas introduction of a phosphomimetic mutation (ICD T654D) abrogated this activation and prevented allosteric interaction [75], thus implicating juxtamembrane domain phosphorylation in negative regulation.

⁶⁶⁹T is the only EGFR residue to reside in a consensus (PL⁶⁶⁹TP) for phosphorylation by ERKs [76] and it is well-conserved among species. Indeed, studies using synthetic peptides that encompass ⁶⁶⁹T showed that this residue is a substrate for MAP kinase [43,44]; our data,

derived using intact receptor in viable cells, support this finding by demonstrating PTP101 reactivity with WT EGFR, but not EGFR T669A. However, in contrast to ⁶⁵⁴T and ^{1046/1047}S, roles for ⁶⁶⁹T in EGFR signaling and/or downregulation are less clear [37,77,78]. We undertook the current studies because of our previous findings [32–34] that GH, PRL, and EGF each induced phosphorylation of EGFR that was detected with a monoclonal antibody (PTP101 [79,80]) that reacts with serine or threonine residues contained within ERK consensus sites. In those studies, PTP101-reactive EGFR phosphorylation was prevented by pretreatment with ERK inhibitors; however, a PKC inhibitor failed to prevent GH-induced PTP101 reactivity [32–34]. Furthermore, pretreatment of murine 3T3-F442A preadipocytes with GH [32,33] or human T47D breast cancer cells with PRL [34] blunted EGFR downregulation induced by subsequent EGF treatment and these effects of GH or PRL were also prevented by ERK inhibitors. Similarly, we observed synergism between GH and EGF (in 3T3-F442A cells) and PRL and EGF (in T47D cells) for downstream signaling that was dependent on the abilities of GH or PRL to activate ERKs [33,34]. Collectively, these findings suggested that (GH- or PRL-dependent) ERK-mediated phosphorylation of EGFR lessened the receptor's downregulation and thereby contributed to enhanced EGF-dependent signaling. Finally, we observed that ERK inhibitor pretreatment alone enhanced EGF-dependent EGFR downregulation [34], strongly suggesting that ERK-mediated EGFR threonine phosphorylation, whether accomplished by PRL or GH before EGF stimulation or resulting from EGF-induced ERK activation, can modulate EGF-induced EGFR downregulation.

In the current study, we used both mutagenetic and pharmacologic methods to better assess ERK-dependent PTP101-reactive EGFR phosphorylation and its impact on receptor trafficking and signaling. We found in a CHO reconstitution system in which no endogenous EGFR is expressed that WT EGFR and EGFR T654A, but not EGFR T669A, underwent GH- and EGF-dependent PTP101-reactive phosphorylation. This effect was blocked by an ERK inhibitor, indicating that ⁶⁶⁹T is indeed required for the ERK-dependent PTP101-reactive phosphorylation of the receptor. Both T669A mutation and pharmacologic ERK inhibition substantially enhanced EGF-induced EGFR downregulation in this system; the ERK inhibitor effect is consistent with our observations (mentioned above) in T47D cells [34] and the T669A mutation result furthers the argument that it is the EGF-induced ERK-dependent phosphorylation at ⁶⁶⁹T that tends to negatively regulate the receptor's own downregulation. Along these lines, our observations that both T669A mutation and pharmacologic ERK inhibition also allowed greater acute EGF-induced EGFR activation (as assessed by blotting of ⁹⁹²Y and ¹⁰⁶⁸Y phosphorylation) and ubiquitination are of substantial interest. They suggest that the enhanced downregulation seen when EGF-induced ERK-mediated phosphorylation of ⁶⁶⁹T is blocked may be related to a tendency of this threonine phosphorylation to negatively regulate kinase activation (more below).

Importantly, we extended our examination of these issues to a human fibrosarcoma cell line in which ErbB-2, as well as EGFR, is expressed and which is known to harbor a mutated Ras that displays constitutive activation of the Ras/ERK pathway. In these cells, ERK was basally active under serum-starved conditions and EGF only marginally enhanced this activity. Again, the ERK activation state correlated with PTP101-reactive EGFR phosphorylation and both basal and EGF-induced PTP101 reactivity were prevented by pretreatment with PD98059 for 75 min. This highlights the reversibility of ERK-dependent EGFR phosphorylation and emphasizes that this modification is apparently transient, consistent with our previous observation in 3T3-F442A cells that the time courses for GH-induced ERK activation / deactivation and PTP101-reactive EGFR phosphorylation / dephosphorylation were highly correlated, albeit shifted in that loss of PTP101 reactivity lagged behind decline in ERK activity by roughly 15–60 min [32]. Notably, PD98059 pretreatment of the fibrosarcoma cells also significantly hastened and enhanced EGF-induced EGFR downregulation and significantly augmented acute EGF-induced EGFR ¹⁰⁶⁸Y phosphorylation. These findings suggest that, as

we inferred for GH and PRL treatment in previous studies, “loading” of the EGFR with ERK-dependent phosphorylation (presumably at ⁶⁶⁹T) prior to EGF stimulation modulates the receptor’s responses to EGF in terms of its ability to be activated and/or its post-EGF binding itinerary. More detailed studies will be required to understand the potential significance of this regarding the threshold for and strength of EGFR triggering in cancers that are characterized by constitutive Ras/ERK activation.

EGFR is often found coexpressed in cells with the EGFR family member, ErbB-2, which itself has no ligand, but can heterodimerize with EGFR. In addition to potentiating effects on signaling offered by ErbB-2, heterodimerization of EGFR with ErbB-2 is known to dampen EGF-induced EGFR downregulation [81,82]. These two receptors are coexpressed in C14 cells. Thus, it is conceivable that the relatively decreased EGF-induced EGFR downregulation seen in these cells compared to the CHO cell EGFR transfectants could be accounted for in part by the presence in C14 cells of ErbB-2. However, it is notable that even in the context of ErbB-2 coexpression, blockade of ERK activity in C14 cells enhanced EGF-induced EGFR downregulation significantly. This finding suggests that ERK-mediated phosphorylation of EGFR has effects on EGFR downregulation that are independent of effects of heterodimerization with ErbB-2. These conclusions are further bolstered by the cotransfection experiments in the CHO cell background in which ErbB-2 was coexpressed with either WT EGFR or EGFR T669A. In that setting, both wild-type and mutant EGFRs coimmunoprecipitated with ErbB-2, but EGF-induced EGFR downregulation was augmented for EGFR T669A compared to WT EGFR.

Our current findings also highlight apparent differences in the roles of phosphorylation of ⁶⁶⁹T vs. those of ^{1046/1047}S and ⁶⁵⁴T described above. In particular, whereas EGFR S1046/1047A exhibited enhanced EGF-induced signaling and decreased EGF-induced EGFR downregulation and EGFR T654A manifested primarily enhanced EGF-induced signaling, EGFR T669A in our study displayed both enhanced acute EGF-induced EGFR activation and enhanced EGF-induced EGFR downregulation. In this respect, our findings may indicate two separate roles for ⁶⁶⁹T phosphorylation – negative regulation of kinase activity (perhaps mechanistically similar to that of ⁶⁵⁴T phosphorylation referred to above) and negative regulation of EGF-induced EGFR downregulation. However, it is also possible that the enhanced downregulation seen in the T669A results from the enhanced kinase activation of this mutant, in that EGFR kinase activity is generally taken to be a prerequisite for EGF-induced EGFR ubiquitination and downregulation.

Along these lines, it is important to note that our previous studies did not indicate that GH or PRL pretreatment resulted in obvious changes in subsequent EGF-induced EGFR tyrosine phosphorylation compared with EGF treatment alone [33,34]. Rather, the most prominent effect of GH- and PRL-induced ERK-mediated EGFR PTP101- reactive phosphorylation in those studies was to significantly diminish subsequent EGF-induced EGFR downregulation. Why would the two ERK-dependent effects (modulation of kinase activity and modulation of downregulation) be relatively uncoupled in our previous studies and so correlated in the current study? There could be many explanations, including that the effect of GH- and PRL-induced ERK activation on EGFR kinase activity (presumably a blunting effect) was present, but not detected, in our studies in 3T3-F442A and T47D cells.

In the aggregate, our current findings are important in that they implicate ERK-dependent phosphorylation of EGFR ⁶⁶⁹T as a regulator of EGF-induced receptor kinase activation and downregulation. Whether these are entirely separable effects remains a question worthy of further investigation. Further, these data highlight the likely importance of the juxtamembrane region in EGFR function and suggest that this region and ⁶⁶⁹T are important in both heterologous and homologous receptor regulation.

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Figure 1. Schematic structure of EGFR and expression of WT EGFR and EGFR T669A

A, Domain structure of EGFR. Highlighted are two cysteine-rich (CR) extracellular domain regions, the transmembrane (TM) domain, the juxtamembrane (Jx) cytoplasmic domain, and the kinase domain. The Jx domain region amino acid sequence including ⁶⁶⁹T that resides in an ERK phosphorylation consensus (PX(S/T)P) is indicated.

B, Expression of WT EGFR and EGFR T669A in CHO cells. Stably transfected cells expressing either WT EGFR or EGFR T669A were serum-starved and stimulated EGF (1nM) or vehicle for 15 min. Detergent extracts (800 µg) were immunoprecipitated with anti-EGFR and eluates separated by SDS-PAGE and sequentially immunoblotted with anti-phosphotyrosine (anti-pTyr) and anti-EGFR (upper panel). Detergent extracts (30 µg) were

resolved by SDS-PAGE and sequentially immunoblotted with anti-phospho Akt and anti-Akt (lower panel). The experiments shown are representative of three such experiments.

Figure 2. EGF causes PTP101-reactive phosphorylation of wild-type EGFR and EGFR T654A, but not EGFR T669A in reconstituted CHO cells

A, CHO cells stably expressing WT EGFR or EGFR T669A were serum-starved and stimulated with GH (500 ng/ml) for 10 min or EGF (1 nM) for 15 min or vehicle. Detergent extracts were immunoprecipitated with anti-EGFR and eluates were resolved by SDS-PAGE and immunoblotted sequentially with PTP101 and anti-EGFR (upper panel). Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-active ERK (anti-pERK) and anti-ERK (lower panel).

B, Serum starved cells were pretreated with PD98059 (100 μ M) or vehicle for one hour prior to stimulation with EGF (1 nM) for 15 min. Detergent extracts were immunoprecipitated with

anti-EGFR and eluates were resolved by SDS-PAGE and immunoblotted sequentially with PTP101 and anti-EGFR (upper panel). Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-pERK (lower panel). The experiments shown are representative of two such experiments.

C, CHO cells transiently expressing WT EGFR, EGFR T654A or EGFR T669A were serum-starved, pretreated with PD98059 or vehicle and then stimulated with EGF (1 nM) or vehicle for 15 min. Detergent extracts were immunoprecipitated with anti-EGFR and eluates were resolved by SDS-PAGE and immunoblotted sequentially with PTP101 and anti-EGFR. The experiments shown are representative of three such experiments.

Figure 3. EGF-induced EGFR downregulation is enhanced in CHO cells expressing EGFR T669A compared to WT EGFR

A, Serum starved cells were pretreated with cycloheximide (20 μ g/ml) for 1 h prior to stimulation with EGF (10 nM) for indicated durations. Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-EGFR and anti-STAT5.

B, Data in A along with those obtained from three other experiments, were subjected to densitometric analysis. EGFR mass at each time point was normalized to control (time 0; considered 100% in each experiment). Data are expressed as mean $\pm$ S.E. (n = 4). For comparison of WT EGFR vs. EGFR T669A, * - $p < 0.05$; ** - $p < 0.01$.

C, Serum starved cells were pretreated with cycloheximide and U0126 (10 μ M) or vehicle for 30 min prior to stimulation with EGF (10 nM) for 15 min. Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-EGFR antibody (upper panel) and anti-pERK and anti-ERK (lower panel).

Figure 4. EGF-induced EGFR tyrosine phosphorylation in CHO cells expressing EGFR T669A vs. WT EGFR

A,B, Serum starved cells were stimulated with EGF (1 nM) for the indicated durations. Detergent extracts were resolved in parallel by SDS-PAGE and immunoblotted with anti-pY-992, anti-pY-1068, or anti-EGFR. In *B*, cells were pretreated with PD98059 (100 μ M) or vehicle for 1 h prior to stimulation with EGF, as indicated. The experiments shown are representative of four such experiments.

Figure 5. EGF-induced EGFR tyrosine phosphorylation in CHO cells expressing EGFR T669A, WT EGFR, or cotransfected with both EGFR T669A and WT EGFR

A, CHO-GHR cells were transiently transfected with plasmids encoding either WT EGFR, EGFR T669A, or both receptors, as indicated. Serum starved cells were stimulated with EGF (1 nM) for the indicated durations. Detergent extracts were resolved in parallel by SDS-PAGE and immunoblotted with anti-pY-1068 or anti-EGFR.

B, Data in A, along with those obtained from two other experiments, were subjected to densitometric analysis. EGF-induced EGFR tyrosine phosphorylation was normalized by EGFR abundance and maximum signal achieved was considered 100%. Data are displayed as mean $\pm$ S.E. (n = 3). *p* values for indicated comparisons are listed.

Figure 6. EGF-induced EGFR ubiquitination in CHO cells expressing EGFR T669A vs. WT EGFR
A, Serum starved cells were pretreated with PD98059 or vehicle for 1 h prior to stimulation with EGF (10 nM) for 15 min. Detergent extracts were immunoprecipitated with anti-EGFR and eluates were resolved by SDS-PAGE and immunoblotted with anti-Ub and anti-EGFR. Detergent extracts were resolved by SDS-PAGE and immunoblotted with anti-pERK and anti-ERK.

B, Data in **A**, along with those obtained from two other experiments, were subjected to densitometric analysis. EGF-induced EGFR ubiquitination was normalized by EGFR abundance and maximum signal achieved was considered 100%. Data are displayed as mean $\pm$ S.E. ($n = 3$). p values for indicated comparisons are listed.

Figure 7. Constitutive Ras activation results in basal EGFR PTP101 phosphorylation

Serum-starved C14 cells were pretreated with PD98059 (100 μ M) or vehicle for 1 h prior to stimulation with EGF (1 nM) for 15 min. Detergent extracts were immunoprecipitated with anti-EGFR and eluates were resolved by SDS-PAGE and immunoblotted sequentially with PTP101 and anti-EGFR. Detergent extracts were resolved by SDS-PAGE immunoblotted sequentially with anti-pERK and anti-ERK. The experiment shown is representative of three such experiments.

Figure 8. EGF-induced EGFR downregulation in C14 cells

A, Serum starved C14 cells were pretreated with PD98059 (100 μ M) or vehicle for 1 h prior to stimulation with EGF (1 nM) for the indicated durations. Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-EGFR and anti-ErbB-2 (ErbB-2 is not downregulated in response to EGF and is thus used as a loading control.)

B, Data in A, along with those obtained from three other experiments, were subjected to densitometric analysis. EGFR mass at each time point was normalized to control (time 0; considered 100% in each experiment). Data are expressed as mean $\pm$ S.E. (n = 4). For comparison of PD98059 vs. DMSO pretreatment, * - $p < 0.05$; ** - $p < 0.01$.

C, Serum starved C14 cells were pretreated with UO126 (50 μ M) or vehicle for 30min prior to stimulation with EGF (1nM) for the indicated durations. Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-EGFR and anti-ErbB-2 antibodies.

Figure 9. EGF-induced EGFR signaling in C14 cells

A, Serum starved C14 cells were pretreated with PD98059 (100 μ M) or vehicle for 1 h prior to stimulation with EGF (1 nM) for the indicated durations. Detergent extracts were resolved by SDS-PAGE and immunoblotted sequentially with anti-pY-1068, anti-EGFR, anti-pERK, and anti-ERK.

B, Data in A, along with those obtained from three other experiments, were subjected to densitometric analysis. pTyr-1068 EGFR mass at each time point was normalized to EGFR abundance. Maximal signal achieved was considered 100%. Data are expressed as mean $\pm$ S.E. (n = 4). For comparison of PD98059 vs. DMSO pretreatment, * - $p < 0.05$.

Figure 10. Coexpression of WT EGFR or EGFR T669A with ErbB-2

A, WT EGFR- or EGFR T669A-expressing CHO-GHR cells were transiently transfected with either Flag-ErbB-2 or pcDNA, as indicated. Cells were serum starved overnight and then stimulated with EGF (1 nM) for 5min. Cell extracts were immunoprecipitated with anti-FLAG affinity gel and immunoblotted with anti-EGFR and anti-ErbB-2 antibodies sequentially.

B, CHO-GHR cells transiently cotransfected with Flag-ErbB-2 and either WT EGFR or EGFR T669A, as indicated, were serum starved overnight and pretreated with cycloheximide (20 µg/ml) for 1 h. Thereafter, cells were stimulated with EGF (10 nM) or vehicle for 3 h and detergent extracts were resolved by SDS-PAGE and sequentially immunoblotted with anti-EGFR and anti-ErbB2 antibodies.

The experiments in *A* and *B* are each representative of three independent experiments.