Combination antibody treatment down-regulates epidermal growth factor receptor by inhibiting endosomal recycling

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Due to its common dysregulation in epithelial-based cancers and extensive characterization of its role in tumor growth, epidermal growth factor receptor (EGFR) is a highly validated target for anticancer therapies. There has been particular interest in the development of monoclonal antibodies (mAbs) targeting EGFR, resulting in two approved mAb-based drugs and several others in clinical trials. It has recently been reported that treatment with combinations of noncompetitive mAbs can induce receptor clustering, leading to synergistic receptor down-regulation. We elucidate three key aspects of this phenomenon. First, we show that highly potent combinations consisting of two noncompetitive mAbs that target EGFR domain 3 reduce surface receptor levels by up to 80% with a half-time of 0.5–5 h in both normal and transformed human cell lines to an extent inversely proportional to receptor density. Second, we find the mechanism underlying down-regulation to be consistent with recycling inhibition. Third, in contrast to the agonism associated with ligand-induced down-regulation, we demonstrate that mAb-induced down-regulation does not activate EGFR or its downstream effectors and it leads to synergistic reduction in migration and proliferation of cells that secrete autocrine ligand. These new insights will aid in ongoing rational design of EGFR-targeted antibody therapeutics.

ErbB | monoclonal antibody | tyrosine kinase | trafficking

Epidermal growth factor receptor (EGFR) is a member of the ErbB family of single-pass transmembrane receptor tyrosine kinases (RTKs). Under normal conditions, EGFR activation is tightly regulated by its native ligands, epidermal growth factor (EGF) and transforming growth factor- α (TGF α), which bind to the receptor extracellular domain (1). Ligand binding induces conformational changes in EGFR that stabilize homo- or heterodimerization, leading to autophosphorylation of its intracellular domain. Phosphorylation of selected tyrosine residues activates signaling effectors in downstream pathways, including the mitogen-activated protein kinase (MAPK) and phosphoinositide kinase 3 (PI3K) pathways, eliciting responses such as growth, migration, differentiation, and apoptosis (2). The primary mechanisms of signal attenuation are receptor deactivation via phosphatase activity and receptor degradation following endocytosis (3). Ligand activation accelerates receptor endocytosis and simultaneously decreases the recycling to degradation ratio to terminate EGFR signaling (4).

Dysregulation of EGFR leading to unregulated growth has been observed in a variety of cancers. Means of dysregulation include receptor overexpression, which occurs in one-third of all epithelial-based tumors (5), mutation, aberrant localization, autocrine ligand secretion, and obstruction of endocytosis (3). Due to its prevalence and altered expression in cancer patients, several therapeutic strategies have been employed to target EGFR, one of which involves the use of monoclonal antibodies (mAbs) that bind the receptor ectodomain. mAbs act through

multiple mechanisms including immune cell recruitment, toxin delivery, and direct inhibition of receptor signaling via ligand competition, obstruction of dimerization, or modulation of trafficking (6). Both clinically approved mAbs targeting EGFR (cetuximab and panitumumab) bind domain 3 of the EGFR ectodomain, directly competing with ligand and thereby preventing dimerization and activation (7–9). Unfortunately, the monotherapy objective response rates of cetuximab and panitumumab are tepid: 11% (10) and 8% (9, 11), respectively, in the treatment of metastatic colorectal cancer. Although these rates improve when mAbs are used in combination with chemotherapy, poor tumor penetration, autocrine signaling, acquired resistance, and receptor mutation hinder drug performance (12). It is therefore of interest to develop complementary therapeutic strategies to enhance mAb efficacy.

It was recently established that particular combinations of noncompetitive anti-EGFR mAbs synergistically reduce surface receptor levels both in vitro and in vivo. This down-regulation of receptor leads to enhanced tumor cell killing and prolonged survival in mouse xenograft models of cancer (13–16). Consistency between down-regulation levels and combination efficacy in mouse models was also reported for ErbB2 (17). Friedman et al. proposed that synergism results from the formation of large clusters of cross-linked receptors on the cell surface following combination mAb treatment (13). Evidence for the formation of higher-order clusters of EGFR was established by Zhu et al. using the tyrosine kinase inhibitor decorin (18). In order to gain deeper mechanistic insights motivated by these exciting results and to inform the development of more potent antibody-based therapeutics, we investigated binding and trafficking processes underlying combination mAb-induced down-regulation. Our findings establish a connection between binding epitopes and down-regulatory potential of mAb pairs and show that synergistic down-regulation results from receptor recycling inhibition. We further demonstrate that unlike EGF-induced down-regulation, mAb-induced down-regulation is not agonistic, and it coincides with reduced migration and proliferation of autocrine ligand-secreting cells.

Results

Combinations of Anti-EGFR mAbs Reproducibly Down-Regulate Surface Receptor in Both Normal and Transformed Human Cell Lines.

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Using a panel of six anti-EGFR mouse mAbs, we compared the ability of pairwise combinations to down-regulate receptor on seven normal or transformed cell lines, whose origins and receptor densities are detailed in Table 1. The steady state (13 h) surface receptor levels for each combination are displayed in Fig. 1A and the observed reduction in surface EGFR directly corresponds to a reduction in total EGFR (Fig. S1). In general, mAb pairs down-regulate more effectively than single mAb treatment, and the relative performance of each combination is fairly consistent across cell lines. Also, down-regulation extent decreases as receptor density increases. This trend parallels that for ligand-induced down-regulation (Fig. 1B) and is suggestive of endocytic machinery saturability (19). To confirm that these results were a consequence of receptor abundance and not coincidental cell line variability, these trends were reproduced in U87 cell lines transfected with varying numbers of receptor (Table S1 and Fig. S2). Notably, one specific mAb combination, that of 225 (the murine version of cetuximab) and H11, down-regulates significantly better than other pairs, particularly in cell lines with high EGFR expression levels.

Combinations of Domain 3 Binders Down-Regulate Receptor Most Efficiently. To examine the correlation between down-regulation and epitope, we mapped the binding domains of the tested mAbs. By sorting a randomized EGFR ectodomain library displayed on the surface of yeast, we identified residues that were critical for the binding of each mAb (Table S2) with the exception of 225, for which a crystal structure was available (8) and which we had previously mapped by this method (20). Fig. 2 depicts the epitope locations on EGFR in its monomeric (1) and dimeric (21) forms. Consistent with the structural data, 111 and H11 cross-block, as do 199.12 and EGFR1. Also, 225, H11, 111, and 565 compete with EGF ligand (Fig. S3). A combination of two domain 3 binders (225 + H11) most consistently decreases surface receptor to the greatest extent. Among the antibodies tested, combinations consisting of two noncompetitive domain 3 binders down-regulate receptor more effectively than competitive combinations and combinations with one domain 3 binder and one domain 1/2 binder (Fig. 2C and Table S3), indicating stereospecific dependence of down-regulation.

mAb-Induced Down-Regulation Is Significantly Slower than EGF-Induced Down-Regulation. The kinetics of antibody-induced EGFR down-regulation were compared to those of EGF-induced down-regulation. Both combination mAb treatment and EGF treatment ultimately resulted in a maximum of 80% down-regulation, although EGF reduced surface receptor expression much more rapidly (Fig. 3). Down-regulation halftimes for the potent 225 + H11 pair, the moderately effective 111 + 565 pair, and EGF are provided in Table S4. Note that the mAb-induced down-regulation time scales are independent of receptor density and consistent across cell lines, typically ranging from 0.5–2.5 h.

Combination mAb Treatment Down-Regulates Surface Receptor by Abrogating Recycling. We next sought to elucidate the kinetic rate processes underlying EGFR down-regulation. To this end, we considered a simple material balance for receptor trafficking

Table 1. Cell line origins and surface EGFR densities

Cell line	Origin	EGFR per cell
HT-29	Colorectal adenocarcinoma	1.0×10^5
Hela	Cervical adenocarcinoma	1.7×10^5
U87-MG	Glioblastoma	1.9×10^5
HMEC	Human mammary epithelial cells	4.5×10^5
CHO-EG	Chinese hamster ovary (EGFR-GFP transfected)	1.6×10^6
U87-MGSH	Glioblastoma (mutant EGFRvIII transfected)	1.7×10^6
A431	Epidermoid carcinoma	2.8×10^6

Fig. 1. EGFR surface down-regulation following pairwise mAb treatment. (A) The seven cell lines shown were treated with the indicated antibody pairs for 13 h at 37 °C. Surface EGFR levels relative to those of untreated cells were analyzed via flow cytometry. (B) Comparison of the 13-h surface EGFR levels following treatment with 225 + H11 (circle), 111 + 565 (square), or EGF (triangle) plotted against receptor density ($\pm$ SD, $n = 3$).

(Fig. 4A). Solving for the steady state surface (R_S) and internal (R_I) receptor levels, we find that

$$R_S = \frac{P_{\text{syn}}}{k_e} \left(1 + \frac{k_{\text{rec}}}{k_{\text{deg}}} \right) \quad \text{and} \quad R_I = \frac{P_{\text{syn}}}{k_{\text{deg}}}.$$

Surface receptor levels can thus be lowered by (i) decreasing synthesis, (ii) increasing the endocytosis rate constant, or (iii) decreasing the recycling to degradation ratio, or by a combination of these factors. Cells treated with and without the protein synthesis inhibitor cycloheximide (22) exhibit similar down-regulation kinetics (Fig. S4A), implying that altered receptor synthesis is not responsible for the observed drop in surface EGFR. Furthermore, the relatively slow down-regulation rate (on the order of the constitutive endocytosis rate) indicates that first-pass endocytosis is certainly not accelerated in treated cells. We then considered the possibility that mAb treatment inhibits recycling. Assuming that synthesis and degradation rates are unchanged, the model predicts the fractional surface receptor remaining after treatment to be

$$\frac{R_{S,t}}{R_{S,u}} = \left(\frac{k_{e,u}}{k_{e,t}} \right) \left[\frac{1 + \frac{k_{\text{rec},t}}{k_{\text{deg}}}}{1 + \frac{k_{\text{rec},u}}{k_{\text{deg}}}} \right],$$

where “u” and “t” represent the untreated and treated cases, respectively. Thus, down-regulation extent depends on the ratio of the untreated and treated endocytic and recycling rates. Also, there is predicted to be a lower bound on EGFR levels, even if recycling is completely blocked by treatment (i.e., $R_{S,t} \neq 0$ even when $k_{\text{rec},t} = 0$). This is consistent with the 80% down-regulation limit observed and the fact that treatment with 225 + H11 plus additional monoclonal and polyclonal antibodies does not drive down-regulation further (Fig. S5).

To determine whether receptor recycling is hindered by mAb treatment, a pulse-chase quenching assay was performed. Briefly, A431 cells were pulsed with Alexa 488-labeled 225 in the presence or absence of H11. Surface 225 was then quenched by the addition of Alexa 488 quenching antibody. Cells were subse-

Fig. 2. Mapped epitopes of anti-EGFR mAbs. Using a yeast library of EGFR ectodomain mutants, the binding domains of five mAbs were determined. Domains 1 (orange), 2 (yellow), 3 (green), and 4 (cyan) are displayed in the monomeric (A) and dimeric (B) forms of the receptor. EGF ligand is shown in black in the dimeric structure. Epitopes for EGFR1 (dark green), 199.12 (magenta), H11 (red), 111 (pink), and 565 (blue) are shown as spheres. Starred residues are part of both the H11 and 111 epitopes. The 225 epitope identified in the published crystal structure is shown in gray (8). The monomeric and dimeric structures are adapted from 1nql (1) and 1ivo (21), respectively. (C) The 15 pairwise mAb combinations were ranked in order of down-regulation extent for each of the seven indicated cell lines. Pairs of two noncompetitive domain 3 binders (blue), pairs of one domain 1/2 and one domain 3 binder (green), and competitive pairs (yellow) are denoted.

quently chased in the continued presence of quenching antibody so that any incremental drop in signal during this period would result from internal 225 recycling to the cell surface, a surrogate for receptor recycling. As shown in Fig. 4B, 488 signal dropped during the chase period in the absence but not in the presence of H11, implying that combination treatment blocks the recycling that is observed following single mAb treatment. To independently confirm this result, cells were treated with or without the recycling inhibitor monensin (23) in the absence or presence of the 225 + H11 combination. As shown in Fig. 4C, treatment with monensin alone evoked essentially the same response as 225 + H11 treatment with or without monensin, in accord with the hypothesis that combination treatment substantively vitiates receptor recycling. Consistent with expectations, less potent mAb combinations induced greater receptor down-regulation in the presence than in the absence of monensin, suggesting that recycling was less dramatically impaired in these systems (Fig. S4B).

mAb Treatment Does not Agonize EGFR or Lead to Downstream Signaling. Since EGFR signals actively from endosomes following ligand stimulation (24), we examined whether or not mAb-induced internalization was similarly agonistic. We first evaluated EGFR activation in A431 cells at eight known phosphosites in the intracellular domain using in-cell Western assays (Fig. 5A). None of these phosphosites were stimulated by single or combination mAb treatment, including three major autophosphorylation sites (Y1068, Y1148, and Y1173) (25), one minor autophosphorylation site (Y1086) (26), and targets of Src tyrosine kinase (Y845) (27), Ca-calmodulin-dependent kinase II (S1046) (28), protein kinase C (T654) (29), and MAPK (T669) (30). In contrast, most sites were activated two- to threefold by EGF.

Luminex assays were also conducted on A431 cells to test for activation of downstream pathway effector MAPK. As Fig. 5B demonstrates, incubation with 225 and H11 singly and in combination failed to phosphorylate MAPK above control levels. To obtain a more global perspective of cell response, we used an iTRAQ-based mass spectrometry screen to assess phosphoryla-

tion following treatment with an isotype control mAb, 225, H11, 225 + H11, or EGF. Lists of all phosphoproteins identified and their relative signals compared to control treatment are provided (Fig. S6 and Table S5). Fig. 5C shows the relative phosphorylation of proteins associated with two critical pathways downstream of EGFR: MAPK and PI3K. Neither single nor combination mAb treatment activated any of these signaling effectors based on a cutoff of 1.7-fold background.

Down-Regulating mAb Combination Impedes Cell Migration and Proliferation. We further explored the therapeutic promise of the most potent down-regulating combination by examining its effects on migration and proliferation of cultured cells. An artificially constructed autocrine HMEC cell line (denoted ECT) that sheds surface-expressed chimeric EGF chimera at a fractional release rate of 0.3 h^{-1} (31) was tested. In ECT cells, the 225 + H11 combination significantly reduces the extent of cell migration compared to either mAb alone, whereas migration is not statistically significantly further inhibited by the combination compared to 225 treatment in normal HMEC cells (Fig. 6A).

Fig. 3. Surface EGFR down-regulation kinetics. HeLa (Left) and HMEC (Right) cells were incubated at 37°C in the presence of 225 + H11 (circle), 111 + 565 (square), or EGF (triangle). At the specified time points, surface receptor was quantified via flow cytometry. Surface EGFR levels relative to those of untreated cells are plotted as a function of time ($\pm\text{SD}$; $n = 3$).

Fig. 4. Inhibition of EGFR recycling via combination mAb treatment. (A) Basic model of EGFR trafficking following mAb treatment. (B) A431 cells were pulsed with Alexa 488-conjugated 225 for 2 h in the presence (square) or absence (diamond) of H11. Surface receptor signal was then quenched by adding $25 \mu\text{g/mL}$ Alexa 488 quenching antibody, and cells were chased at 37°C in the continued presence of quenching antibody. Total fluorescence was determined at each time point, and signal relative to the unchased control is plotted versus time ($\pm\text{SD}$; $n = 3$). (C) HT-29 (Left) and HMEC (Right) cells were incubated at 37°C with (circles) or without (squares) 225 and H11 in the absence (open symbols) or presence (closed symbols) of the recycling inhibitor monensin ($200 \mu\text{M}$). Surface EGFR levels compared to those of untreated cells are plotted against incubation time ($\pm\text{SD}$; $n = 3$).

Fig. 5. Downstream signaling implications of combination mAb treatment. (A) In-cell Western assays were performed on A431 cells for eight known EGFR phosphosites. Activation profiles are shown for EGF (closed square), 225 (open circle), H11 (open square), and 225 + H11 (closed circle). Phosphoprotein fluorescence was normalized by DNA fluorescence and signal relative to that of untreated cells is plotted versus time ($\pm$ SD; $n = 3$). (B) The time course of ERK 1/2 activation in A431 cells following mAb or EGF treatment was measured via bead-based immunoassay. Normalized phosphoprotein signal is plotted for cells treated with EGF (closed square), 225 (open circle), H11 (open square), 225 + H11 (closed circle), and an antibody-free control (open triangle) ($\pm$ SD; $n = 3$). (C) Serum-starved A431 cells were incubated with 225, H11, the 225 + H11 combination, and EGF at 37°C for 15 min (Top) or 60 min (Bottom). EGF stimulation was held constant at 15 min for both screens. Cells were then lysed and relative protein phosphorylation was measured using an iTRAQ-based mass spectrometry screen. Phosphorylation levels were normalized by total protein content, and signal strength relative to that in cells treated with an isotype control mAb is presented for MAPK and PI3K pathway components. Repetition of the 60-min screen yielded consistent results for proteins identified in both cohorts.

The same trend was observed for cell proliferation (Fig. 6B), demonstrating that combination treatment is more effective than monotherapy in a system that aberrantly expresses EGF.

Discussion

The use of noncompetitive antibody combinations to engage multiple therapeutic mechanisms is an increasingly popular and attractive strategy for enhancing drug efficacy (6). Understanding the biophysical and biochemical bases for antibody synergy will be helpful for designing the next generation of combination

Fig. 6. Cell migration and proliferation following mAb treatment. Migration (Left) and proliferation (Right) of HMEC (dark gray) and autocrine EGF-secreting ECT (light gray) cells were assessed using the scratch and MTT assays, respectively. For migration assays, monolayers were wounded and subsequently incubated with the indicated mAbs for 24 h at 37°C. Relative migration is shown as fractional wound replenishment compared to that of an untreated control ($\pm$ SD; $n = 6$). For proliferation assays, cells were treated with the specified mAbs for 72 h at 37°C. Relative proliferation is presented as viable cell abundance compared to that of untreated cells ($\pm$ SD; $n = 6$). Asterisks denote $P < 0.01$ for the 225 + H11 combination relative to either mAb alone.

and multispecific treatments. The present study offers insights into the structural dependence and mechanism of synergy.

Despite limited physical evidence for formation of receptor clusters, there has been speculation that combination mAb treatment induces clustering, leading to down-regulation (13, 15, 18). Our results support a mechanism that operates intracellularly to reduce endosomal recycling (Figs. 3 and 4). This finding resonates with previous experimental and theoretical analyses of endosomal sorting outcomes (32, 33) and agrees with our further observation that down-regulation is antibody-dependent and saturable (Figs. 1 and 2). All mAb pairs that were examined elicit some level of down-regulation, although the most effective combinations include two mAbs against EGFR domain 3, the ligand-binding domain (Fig. 2C). This finding is congruous with previous observations of domain 3-targeted mAb synergism (13, 16). In general, the extent of down-regulation induced by a mAb pair may depend on the structure of the bound receptor and the degree to which its orientation facilitates clustering. Saturability of down-regulation is consistent with previous findings in cell lines expressing elevated levels of EGFR (19) and results directly from a mechanism reliant on recycling inhibition, as surface levels are determined by the steady state balance between synthesis and constitutive endocytosis.

Combining our kinetic model with experimental findings (Figs. 3 and 4), we deduced that synergistic EGFR down-regulation is a consequence of a decrease in the ratio of the recycling to degradation rates. Treatment with the most effectively down-regulating combination completely blocked recycling in the three *in vitro* systems examined (Fig. 4), consistent with the formation of internalized clusters incapable of returning to the cell surface through recycling endosomes. Extending this result, less potent combinations fail to efficiently cluster receptors, thereby only partially obstructing recycling (Fig. S4B).

Encouragingly, contrary to ligand-induced internalization, mAb-induced internalization does not activate EGFR or its downstream effectors (Fig. 5). Lysosomal targeting of the receptor is expected to require ubiquitinylation (34), but if so we find it to be independent of kinase activity. This is consistent with previous work showing that EGFR and its effector pathways are differentially activated by distinct ligands and mAbs (16, 35). Our results support a therapeutic strategy that utilizes endogenous trafficking machinery to reduce surface EGFR expression without activating downstream signaling pathways. Encouragingly, while combination therapy did not affect migration and proliferation relative to monotherapy in normal cells, it inhibited both processes in a cell line that exhibits autocrine ligand secretion, a common means of EGFR dysregulation in cancer (Fig. 6).

In vivo, other studies have shown strong agreement between receptor down-regulation and drug efficacy in mouse xenograft models (13, 14, 17), offering promise for the therapeutic potential of synergistically down-regulating mAb pairs identified in our screen. Also, the observation of ErbB2 down-regulation by mAb combinations (17) suggests that clustering receptor may be an effective approach for targeting other ErbB family receptors and RTKs in general.

Overall, our work demonstrates that combinations of anti-EGFR mAbs reproducibly reduce surface receptor levels in a structurally dependent fashion by inhibiting receptor recycling. These mAb combinations show therapeutic promise in that they do not agonize signaling, and they hamper the migration and proliferation of cells that secrete autocrine EGF.

Materials and Methods

Cell Lines and Antibodies. The transfected CHO-EG (36), U87-MGSH (37), and ECT (31) cell lines were established as described previously, and all other lines were obtained from ATCC. Cells were maintained in their respective growth media (from ATCC unless otherwise indicated): DMEM for A431, U87-MG, U87-MGSH, and CHO-EG cells, McCoy's Modified 5A media for HT-29 cells, EMEM for HeLa cells, and HuMEC Ready Medium (Invitrogen) for HMEC and ECT cells. U87-MG, U87-MGSH, and CHO-EG media were supplemented with 1 mM sodium pyruvate (Invitrogen) and 0.1 mM nonessential amino acids (Invitrogen), and transfected lines U87-MGSH and CHO-EG were selected with 0.3 mM Geneticin (Invitrogen). ATCC media were supplemented with 10% FBS. mAb 225 was secreted from the hybridoma cell line (ATCC). EGFR1 (AbCam) and H11 and 199.12 (LabVision) were purchased. mAbs 111 and 565 were prepared as described previously (13). Unless otherwise noted, all washes were conducted in PBS containing 0.1% BSA and all mAbs were used at a concentration of 40 nM for single treatment and 20 nM each for combination treatment. EGF (Sigma) was dosed at 20 nM. Trypsin-EDTA (Invitrogen) contains 0.05% trypsin and 0.5 mM EDTA.

Receptor Quantification. Cells were serum starved for 12–16 h, washed, digested in trypsin-EDTA (20 min at 37 °C), neutralized with complete medium, and labeled with 20 nM 225 for 1 h on ice. They were then washed, labeled with 66 nM phycoerythrin (PE)-conjugated goat anti-mouse antibody (Invitrogen) for 20 min on ice, washed again, and subjected to quantitative flow cytometry on an EPICS XL cytometer (Beckman Coulter). Receptor density was calculated based on a curve of identically labeled anti-mouse IgG-coated beads (Bangs Laboratories).

Receptor Down-Regulation Assays. Cells were seeded at 5×10^4 per well in 96-well plates, serum starved for 12–16 h, treated with the indicated mAbs in serum-free medium, and incubated at 37 °C. At each time point, cells were washed and treated with trypsin-EDTA for 20 min at 37 °C. Trypsin was neutralized with medium (10% FBS) and cells were transferred to v-bottom plates on ice. They were then washed, acid stripped (0.2 M acetic acid, 0.5 M NaCl, pH 2.5), and washed again prior to incubation with 20 nM 225 for 1 h on ice to label surface EGFR. Cells were then washed and labeled with 66 nM PE-conjugated goat anti-mouse antibody (Invitrogen) for 20 min on ice. After a final wash, plates were analyzed on a FACS Calibur cytometer (BD Biosciences). Cell pelleting was conducted at $1000 \times g$.

Fine Epitope Mapping of Anti-EGFR mAbs. A mutagenized library (average of one mutation per clone) of yeast expressing the EGFR ectodomain followed by a c-myc tag was generated, induced, and sorted as previously described (20). The library was subjected to two rounds of selection: one against the mapped mAb and for c-myc binding and one for simultaneous 225 and c-myc binding to select for properly folded, full-length clones. Selected clones were sequenced to identify residues that interact with the mAb.

Alexa 488 Quenching Pulse-Chase Assays. mAb 225 was labeled using an Alexa 488 microscale labeling kit (Invitrogen). A431 cells were seeded at 5×10^4 per well in 96-well plates and serum starved for 12–16 h. They were then pulsed with Alexa 488-labeled 225 with or without H11 in serum-free medium and incubated at 37 °C for 2 h. Cells were then treated with 25 μ g/mL anti-Alexa-488 quenching antibody (Invitrogen) for 30 min on ice and chased at 37 °C for the indicated length of time in the presence of quenching antibody. Cells were returned to ice, washed, incubated with trypsin-EDTA for 30 min, and neutralized (DMEM, 10% FBS). They were then transferred to v-bottom plates, washed, and analyzed on a FACS Calibur cytometer (BD Biosciences).

Percent unchased signal was calculated relative to cells that were not returned to 37 °C after quenching (38, 39).

Monensin Recycling Assays. Cells were seeded at 5×10^4 per well in 96-well plates and serum starved for 12–16 h. They were then treated with or without 200 μ M monensin (Sigma) in serum-free medium and incubated at 37 °C for 20 min. The indicated mAbs were then added and incubation proceeded at 37 °C. At each time point, surface EGFR was analyzed as in receptor down-regulation assays.

In-Cell Western Assays. A431 cells were seeded at 4×10^4 per well in 96-well plates and allowed to adhere for 24 h. Following 12–16 h of serum starvation, cells were treated with the designated mAbs in serum-free medium at 37 °C for the specified time length. All subsequent incubations were performed at room temperature. Cells were fixed for 20 min (PBS, 4% formaldehyde), permeabilized via four 5-min incubations (PBS, 0.1% triton), blocked for 1 h in Odyssey blocking buffer (Licor Biosciences), and labeled for 1 h with 15 nM antiphosphosite antibodies (Genscript) in blocking buffer. Cells were then washed three times with PBST (PBS, 0.1% Tween-20) and labeled with 66-nM 800-conjugated goat anti-rabbit antibody (Rockland Immunochemicals) and 400 nM TO-PRO-3 DNA stain (Invitrogen) in blocking buffer for 30 min. After three final PBST washes, wells were aspirated dry for analysis on a Licor Odyssey Scanner (Licor Biosciences). Signal was normalized to cell abundance by dividing 800 (phosphoprotein) by 700 (TO-PRO-3) channel fluorescence.

Luminex Phosphoprotein Quantification Assays. A431 cells seeded in 96-well plates at 3×10^4 per well were allowed to settle for 24 h prior to 12- to 16-h serum starvation. Cells were then incubated with the specified mAbs in serum-free medium at 37 °C. At the indicated times, cells were lysed using the Bio-Plex cell lysis kit (Bio-Rad). Phosphorylated ERK1/2 abundance was quantified using the Luminex bead-based immunoassay, performed with the Bio-Plex Phospho-ERK1/2 (T202/Y204, T185/Y187) bead kit and the Bio-Plex Phosphoprotein Detection Reagent kit on the Bio-Plex 200 platform (Bio-Rad).

Global Phospho-Mass Spectrometry Screens. A431 cells were seeded at 1×10^6 per well in six-well plates, grown to confluence, and incubated with the appropriate mAbs in serum-free medium at 37 °C for 15 or 60 min. Cells were washed once with chilled PBS and lysed at 4 °C (8 M urea, 1 mM Na_3VO_4). Protein concentration was measured via bicinchoninic acid assay (Pierce). Lysate reduction, alkylation, trypsin digestion, and peptide fractionation were performed as previously described (40). Samples were labeled separately with eight isotopic iTRAQ reagents (Applied Biosystems) for 2 h at room temperature, combined, and concentrated. Immunoprecipitation (IP) with pooled antiphosphotyrosine antibodies [4G10 (Millipore), pTyr100 (Cell Signaling), and PT-66 (Sigma)] proceeded for 16 h at 4 °C using protein G agarose beads (Calbiochem) in IP buffer (100 mM Tris, 100 mM NaCl, 1% Nonidet P-40, pH 7.4). Phosphopeptide enrichment by immobilized metal affinity chromatography and analysis and quantification of eluted peptides were conducted via ESI LC/MS/MS on an LTQ-Orbitrap (Thermo Fisher Scientific). Phosphopeptides were identified using Mascot analysis software (41), and spectra were manually validated (42). Signal intensities were normalized by total protein levels and compared to isotype control treatment.

Migration Assays. HMEC and ECT cells were seeded at 5×10^4 per well in 96-well plates and grown to confluence. Monolayers were wounded with a pipet tip, washed with PBS, and placed in complete medium with the indicated mAbs. Scratch area was measured immediately and after 24 h incubation at 37 °C using Image J software analysis of images from a Nikon confocal microscope (Nikon Instruments). Percent migration was calculated as the fractional reduction in scratch area in the treated wells divided by that of the untreated wells.

Cell Proliferation Assays. HMEC and ECT cells were seeded at 5×10^3 per well in 96-well plates and allowed to adhere for 24 h. They were then treated with the indicated mAbs in complete medium and incubated at 37 °C for 72 h. Cell viability (relative to an untreated control) was assessed using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay (Invitrogen) (43).

Statistical Analysis. Heteroscedastic two-tailed student's *t* tests were performed on migration and proliferation assay results to compare combination and single mAb treatment.

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