Supplementary Figure Legends

Supplementary Figure 1. AC480 blocks EGF-induced responses *in vitro. A*, Western blot quantification (the plot shows the mean and SEM of 4 blots) of phospho-ERBB1 (residue 845) in MTLn3E cells treated with varying concentrations of AC480 (AC480). Cells were stimulated with or without 5 nmol/L EGF for 60 seconds, lysed and analyzed by western blotting. Phospho-ERBB2 (residue 1248) levels were also assayed and followed the trend of EGF-induced phospho-ERBB1. β-actin loading control for the pERBB1 blot is shown; the same amounts were loaded for the pERBB2 blot. *B*, Lamellipod extension in MTLn3E cells is blocked by AC480 in a dose-dependent manner, with concentrations of 1 umol/L or higher resulting in a complete blockade of extension. After 10 minute pre-treatment, cells were followed in time lapse and cell area measured pre and 3 minutes post stimulation with 5 nmol/L EGF. Data are mean and SEM of at least 10 cells for each experiment, done in triplicate. *C*, Images from time lapse sequences showing representative lamellipod extension for cells stimulated by EGF (5 nmol/L) in the presence of carrier (DMSO, Control) or 1 umol/L AC480 (AC480) before and 3 minutes post stimulation. *D*, Chemotaxis of MTLn3E cells to EGF in the presence of AC480(AC480). Chemotaxis to EGF in presence of the inhibitor was measured using a 48-well microchemotaxis chamber as described in Methods. Data are mean and SEM of 11-12 measurements from 3 separate experiments. *E*, *In vitro* invasion of MTLn3E cells in response to 10 nmole/L EGF in the presence or absence of 1 umole/L AC480. *F*, Proliferation of MTLn3E cells in the presence of varying concentrations of AC480. For E and F, data are mean and SEM of 3 experiments.

Supplementary Figure 2. Gefitinib blocks EGF-induced responses *in vitro*. *A*, Western blot quantification (mean and SEM of 4 blots) of phospho-ErbB1 (residue 845) in MTLn3E cells. Cells were treated with gefitinib or DMSO and stimulated with 5 nmol/L EGF for 60 seconds,

lysed and resolved using western blotting. Phospho-ErbB2 (residue 1248) levels were also assayed and followed the trend of EGF-induced phospho-ErbB1, consistent with the stimulation of ErbB1/ErbB2 heterodimers by EGF. β-actin loading control for the pErbB1 blot is shown; the same amounts were loaded for the pErbB2 blot. *B*, Lamellipod extension is blocked by gefitinib in a dose-dependent manner, with concentrations higher than 1umol/L having a complete blockade of extension. Cells were treated with gefitinib or DMSO control for 10 minutes prior to stimulation with EGF. Cells were followed in time lapse and cell area measured pre- and 5 minutes post stimulation with 5 nmol/L EGF. Data are means and SEM of least 10 cells for each experiment, done in triplicate. *C*, Images from time lapse sequences showing representative lamellipod extension for cells stimulated by EGF (5 nmol/L) in the presence of DMSO (Control) or 1umol/L gefitinib (Gefitinib) 5 minutes post stimulation. *D*, Chemotaxis to EGF is inhibited by gefitinib. Chemotaxis to EGF in the presence of varying concentrations of inhibitor was measured as described in Materials and Methods. Data are means and SEM of at least 10 measurements from 3 experiments. *E*, *In vitro* invasion of MTLn3E cells in response to 10 nmole/L EGF in the presence or absence of 1 um/L gefitinib. *F*, Proliferation of MTLn3E cells in the presence of varying concentrations of gefitinib. For E and F, data are mean and SEM of 3 experiments.

Supplementary Movie 1. Mice bearing MTLn3E tumors were treated with carrier (top image) or AC480 (bottom image) by oral gavage, and tumor cell motility *in vivo* imaged as described in Materials and Methods. Frames are taken at 1 minute intervals with a scale of 1 um/pixel.

Supplementary Movie 2. Mice bearing MDA-MB-231 pBabe or 1R tumors were imaged as described in Materials and Methods. Frames are taken at 1 minute intervals with a scale of 1 um/pixel.

Supplementary Materials and Methods

Immunoblotting. MTLn3E cells were grown to 70% confluency and incubated with serum-free starvation medium for 4 hours. The medium was changed to fresh serum-free medium with or without 5 nmol/L EGF, and cells were incubated for 60 seconds. Cells were then washed with cold PBS and lysed in 2X sample buffer (100mM NaF, 50mM Navanadate, 130mM Tris-HCl, 20% glycerol (w/v), 4.6% SDS, 0.02% Bromphenol Blue, 4% beta-mercaptoethanol and Complete Protease Inhibitor Cocktail (Roche Diagnostics Corp., Indianapolis, IN)). The plates were scraped, sonicated, and lysates boiled for 5 minutes. Protein samples were separated by SDS-PAGE, blotted to nitrocellulose membranes, and probed with anti-ß-actin (#A 5441 Sigma, St. Louis, MO), anti-phospho-ErbB1 (#2231 Tyr_{845}, Cell Signaling Technology, Beverly, MA), or anti-phospho-ErbB2 (#sc-12352-R pNeu, Santa Cruz). The primary antibodies were detected by using IRDye anti-Mouse and Anti-Rabbit secondaries on a Licor quantitative infrared imaging scanner (Li-COR Biosciences).

Microchemotaxis chamber assay. A 48-well microchemotaxis chamber, manufactured by Neuroprobe, Inc.(Gaithersburg, MD) was used (1). Briefly, filters were coated with 27 μg/mL rat tail collagen (BD Biosciences, Palo Alto, CA). Cells were starved in L15 containing 0.35% BSA, detached with 2mM EDTA in PBS and 20,000 cells were plated

into the wells of the upper chamber. 5nM EGF (Life Technologies Inc., Gaithersburg, MD) was placed in the lower chamber. Inhibitors or DMSO controls were placed in both top and bottom chambers during the assays. The chambers were incubated for 3 hours at 37°C before fixing, scraping the upper surface free of nonmigrating cells, staining and counting the number of cells crossing the filter.

Lamellipod extension assay. MTLn3E cells were grown on glass-bottom tissue culture plates overnight. Cells were placed in serum-free media for 4 hours, then treated with inhibitors or carrier control for 10 minutes prior to stimulation with 5nM EGF. Stimulation of cells was recorded on a Nikon 20X Diaphot with JVC 3-CCD camera. Images were analyzed using ImageJ. Maximal cell area extension was measured and compared to the pre-stimulatory cell area and compared for different drug concentrations.

In vitro **invasion assay.** MTLn3-ErbB1 cells were plated on top of Matrigel coated transwells (BD Biosciences, 354480) at a density of $1X10^5$ in serum free media containing 0.3% BSA. Transwells were placed in wells containing buffer or 10 nmole/L EGF and incubated at 37 degrees overnight in the absence (DMSO, f.c. 0.1%) or presence of Iressa, AG825 or BMS (1 umole/L each). Transwell filters were then fixed in 3.7% paraformaldehyde for 15 minutes followed by staining with 0.2% crystal violet for 10 minutes. The number of cells on the bottom of the filter was ascertained by counting the number of cells per field. Results shown are mean -/+ SEM of at least three independent chambers.

Proliferation studies. Cells were plated on 35mm culture dishes at densities of 3,100 cells per plate for MTLn3 cells in triplicate for each inhibitor concentration tested and the DMSO control (0.1%, equivalent to the concentration of DMSO added to the culture media used in all the drug treatments). After 24 hours, cells were counted using a hemocytometer, and parallel cultures treated with 10, 3.16, 1, or 0.316 umole/L drug in culture media in 0.1% DMSO. Following incubation, cells were counted in triplicate for each drug concentration and the DMSO control at 24 hours for MTLn3 cells and 48 hours for the MDA-MB-231 cells. Cell number was normalized to the DMSO control for each cell line. Results shown are mean +/- SEM of 3 separate measurements.

1. Xue C, Wyckoff J, Liang F, *et al.* Epidermal growth factor receptor overexpression results in increased tumor cell motility in vivo coordinately with enhanced intravasation and metastasis. Cancer Res 2006;66: 192-7.