A431, H1703, HCC4006, and HCC827 cells were obtained from the American Type Culture Collection. EBC1 cells were obtained from the National Cancer Institute. MKN45 cells were kindly provided by Matthew Meyerson (Dana Farber Cancer Institute). PTENloxPloxMEF were provided by Hong Wu (UCLA). All cells except for H1703 and PTENloxPloxMEFs were grown in DMEM supplemented with 10% FBS and antibiotics. H1703 cells were cultured in RPMI supplemented with 10% FBS and antibiotics. MEFs were cultured in DMEM supplemented with 5% FBS. TS-543 is a cell line derived from a human glioblastoma tumor with foci of amplification of the PDGFRA gene. PC3 cells expressing tetracycline-inducible PTEN were obtained from NeoMarkers.

**Plasmids.** To generate a human-specific PTEN hairpin with a hygromycin resistance marker, the puromycin-resistance cassette in pSIREN-retroQ-PTEN was swapped with the hygromycin-resistance cassette from pMSCV-Hygro (Clontech) by restriction digest. To generate constitutively active drug-resistant ErbB2, wild-type ErbB2 was first shuttled from pLXSN-ErbB2 (kindly provided by David Riese, Purdue University) into pMSCV-Neo. pMSCV-ErbB2 was then subjected to two rounds of site-directed mutagenesis using the QuikChange XL II kit (Stratagene) to generate pMSCV-ErbB2-YVMA. pMSCV-ErbB2-YVMA was then subjected to an additional round of site-directed mutagenesis to introduce the C805S mutation and generate pMSCV-ErbB2-YVMA-C805S. pCMSV-Cbl-70Z (DN Cbl) was kindly provided by Nader Rahimi (Boston University). The E17K mutation was engineered into human Akt1 by site-directed mutagenesis with the QuikChange XL II kit using the pLNX-HA-Akt1 plasmid (originally generated by William Sellers and obtained from Addgene (plasmid 903)) as a template. Wild-type and lipid-phosphatase-dead (G129E) mouse PTEN cDNAs (resistant to the G129E mutation) were introduced into human cells by transfection. Excess biotin was quenched with 3% hydrogen peroxide in water. Primary antibody treatments, cells were placed on ice. One plate from each duplicate was washed twice with ice-cold PBS and lysed, while the other was washed three times with glutathione cleavage solution (50 mM glutathione, 75 mM NaCl, 1 mM EDTA, 1% albumin, and 0.75% 10N NaOH) for 8 min, twice with ice-cold PBS, and then lysed. Lysates were subjected to immunoprecipitation using 10 μg of cetuximab as described in Materials and Methods.

**Cleavable Biotin Internalization Assay.** The assay has been described elsewhere (3) and was carried out here with a few minor modifications. Briefly, cells were serum starved for 6 h and washed once with ice-cold PBS. After the wash, cells were incubated with Sulfo-NHS-S-S-Biotin (0.25 mg/mL) for 15 min at 4 °C. This step was repeated once for an additional 15 min. Excess biotin was quenched with 250 μL quenching solution (Thermo Scientific cat. no. 89881). Cells were then incubated with 50 ng/mL EGF at 37 °C where indicated. For each treatment group (including the “no EGF” control), cells were plated in duplicates. Following treatments, cells were placed on ice. One plate from each duplicate was washed twice with ice-cold PBS and lysed, while the other was washed three times with glutathione cleavage solution (50 mM glutathione, 75 mM NaCl, 1 mM EDTA, 1% albumin, and 0.75% 10N NaOH) for 8 min, twice with ice-cold PBS, and then lysed. Lysates were subjected to immunoprecipitation using 10 μg of cetuximab as described in Materials and Methods.

**Determining Genetic Associations Between Gene Copy Number Alterations.** Data files for 272 individual tumor samples were downloaded from the TCGA public-access data portal, and by the institutional review board of the University of California, Los Angeles. Pretreatment tumor samples from patients with recurrent glioblastoma who received EGFR kinase inhibitor therapy in the context of a clinical trial (2) were examined. These include five patients who responded to EGFR kinase inhibitors and five patients that failed EGFR kinase inhibitor therapy (Fig. S1B). Sections were stained with polyclonal antibody to phospho-EGFR (Tyr-1086) (Invitrogen). Antigen retrieval was performed using 0.01 M citrate buffer, pH 6.0 for 30 min in an oven. Peroxidase activity was quenched with 3% hydrogen peroxide in water. Primary antibody for rabbit phospho-EGFR (Tyr-1086) was diluted in 1% normal goat serum in TBS 0.1% Tween and applied for 16 h at 4 °C, followed by biotinylated secondary antibodies (Vector Laboratories) at 1:200 dilution for 30 min, and avidin-biotin complex (Elite ABC, Vector Laboratories) for 40 min. Negative control slides received blocking serum (10% normal goat serum in TBS 0.1% Tween). Vector NovaRed was used as the enzyme substrate to visualize specific antibody localization. Slides were counterstained with Harris hematoxylin. Representative images from phospho-EGFR immunostained sections were photographed using a ColorView II camera mounted on an Olympus BX41 microscope at 20× magnification. Three images were captured from representative regions of the tumor (and adjacent normal brain if present). Borders between individual cells were approximated using a separator function of the Soft Imaging software (with the parameter of smooth and fine/coarse, 2 and 10, respectively). Quantitative analysis was done using HSI color algorithm based on hue, saturation, and intensity. Saturations of the separated cell in the images were quantified in the red-brown hue range to exclude the negative staining area with hematoxylin nuclear staining. To compare the staining intensity of all slides, mean saturation of total cells on each image was quantified and calculated. A total of 1,500–2,000 cells per case (on average) were measured. As an internal control, mean saturation was measured in adjacent normal brain tissue. For samples in which no adjacent normal brain was present on the slide, a normal reference standard was established by analyzing 3,560 cells from five normal brain sections. Ratios of mean phospho-EGFR staining per tumor cell/mean phospho-EGFR staining per normal brain were determined. False color images representing the distribution of such cells were generated.
samples were cross-referenced by tumor IDs. A description of data types, platforms, and analyses are as previously described (4). Specific data sources were as follows: (i) For aCGH data, we used “level 3” normalized and segmented copy number data from the Cancer Genome Characterization Center at Memorial Sloan-Kettering Cancer Center (Agilent 244K CGH array). One unique profile was selected for each tumor based on the highest signal to noise. For the purpose of tabulation, copy number aberration calls for genes were derived from the mean log2 ratios of spanning segments as follows: “deletion”: log2R < −1.0; “loss”: −1.0 < log2R < −0.2; “amplified”: log2R > 2.0. Thresholds were derived from the distribution of segment mean log2 ratios. (ii) For sequencing data, we used all sequencing data summaries available as of May 2009 in “multiple alignment format” (“MAF”): http://www.broad.mit.edu/GBM.ABI.1.maf, genome.wustl.edu_GBM.ABI.53.maf, hgsc.bcm.edu_GBM.ABI.1.maf, hgsc.bcm.edu_GBM.ABI2.maf. Mutations were further filtered by excluding events which were classified as “somatic,” “synonymous,” or “silent,” or “unvalidated.” Sequencing information was not available for all 272 tumors. Overall, 106/272 (39%) of GBMs harbored focal amplification of the EGFR gene, consistent with the reported frequency of ~40% in the literature (5, 6). Loss of at least one PTEN allele was found in all but one tumor with EGFR amplification/mutation (105/106 = 99%), but in a substantially lower fraction of GBMs without EGFR amplification/mutation (115/166 = 69.3%). From the full set of 272, we considered the subset of GBMs for which there was evidence of one-and-only-one mutation or copy number aberration among EGFR, PDGFRα, MET, and NF1 (n = 103, 14, 6, 30, respectively).

**Immunofluorescence.** Cells were cultured on glass coverslips for 18 h, followed by serum starvation in binding buffer (DMEM, 0.2% BSA, 1 mM hepes) for 4 h. Starved cells were then placed on ice and allowed to bind EGF for 1 h with ice-cold binding buffer containing 50 ng/mL of EGF. To induce EGFR internalization, cells were shifted to 37 °C for 15 min in medium without EGF. Cells were then washed with ice-cold PBS and fixed in 4% paraformaldehyde. Fixed cells were permeabilized with 0.1% Triton X-100 and blocked with 10% normal donkey serum in 2% BSA for 1 h, followed by a 1-h incubation with anti-EGFR-Alexa-488 and anti-EEA1 antibodies. Cells were then incubated for 1 h with secondary antibodies conjugated to Alexa-488 (for EGFR) or Alexa-555 (for EEA1) (Invitrogen). For nuclear staining, cells were incubated with DAPI and then mounted onto microscope slides using Fluromount G (Electron Microscopy Sciences). Laser scanning confocal microscopy analysis was performed on a Leica TCS AOBS SP2 (upright stand) using a 63×, 1.2-numerical aperture water immersion lens for imaging.

Colocalization was quantified using MetaMorph software (Universal Imaging). Images were analyzed by applying the color threshold tool to differentiate positively stained cells from unstained cells. The extent of EGFR/EEA1 colocalization was measured using the colocalization tool. For each cell type, 15 images were analyzed and the results were presented as averages ± SE.

**Generation of 293T-EGFRvIII Cells and Transfection with Cbl-b shRNA.** 293T cells were retrovirally infected with pLHCX-EGFRvIII (7). Infected cells were selected using 300 μg/mL hygromycin. To validate EGFRvIII as a target of Cbl proteins (as has been shown by others in a different cell system) (8), stable 293T-EGFRvIII cells were transiently transfected with 10 μg vector (pSirenRetroQ), 10 μg pCMV6-XL4-Cbl-b (OriGene, cat. no. SC107022), or 10 μg pLKO.1-Puro-Cbl-b (Sigma-Aldrich, cat. no. TRCN0000007749) using the calcium phosphate method.

Fig. S1. PTEN inactivation impairs EGFR-inhibitor-induced cell death. (A) A431 and A431-PTEN-shRNA cells were treated with the indicated doses of EKB-569 for 6 h. After treatment, lysates were generated and apoptotic cell death was assessed by immunoblot using antibodies against the cleaved form of the Caspase-3 substrate PARP (PARP*). Antibodies against p85 were used to control for loading. (B) A431 cells were transduced with shRNA-resistant variants of PTEN and subsequently transduced with a PTEN shRNA that targets the endogenous message. The resulting cells were seeded at equal densities and allowed to grow in the presence or absence of increasing EKB-569 doses for 6 days. After 6 days, cell death was assessed by trypan blue exclusion (Left). A replicate set of samples was treated for 6 h and lysed for Western blot analysis with the indicated antibodies (Right).

Fig. S2. PTEN-associated EGFR inhibitor response correlates with the level of EGFR phosphorylation in GBM patient samples. (A) Immunohistochemical quantification of EGFR phosphorylation in glioblastoma tumor samples. The panel shows pEGFR stains (original image) and corresponding false-color conversion from a normal brain section (Top row) and one glioblastoma sample (GBM 1, area 3). (B) Table of pEGFR staining results in pretreatment samples from glioblastoma patients that subsequently responded (n = 5) or failed to respond (n = 5) to single agent EGFR kinase inhibitor therapy. Patient numbers and results for PTEN IHC, EGFR copy number, and EGFRvIII status in tumor tissue are according to previously published results (9) (C) Table of pEGFR staining results in 10 untreated human gliomas tumors that lack the EGFRvIII mutant, harbor amplification of EGFR gene locus, and either do (Top five patients) or do not (Bottom five patients) express the PTEN tumor suppressor protein by IHC.
Fig. S3. Determination of EGFR kinase activity using a microfluidic-based phosphopeptide array. (A) Schematic of phosphopeptide array assay. Whole cell lysates are applied to a chip spotted with peptides and a kinase reaction is then carried out on-chip after addition of ATP. Peptide phosphorylation is quantified every 5 min using FITC-labeled antiphosphotyrosine antibodies (PY20-FITC) and imaging with a CCD camera (see Materials and Methods for details). (B) Increased phosphorylation of 10 peptides in astrocyte sublines with increased EGFR expression. The graph shows phosphorylation rates of 10 matrix-spotted peptides after arrays were incubated with whole cell lysates from two human astrocyte sublines expressing different EGFR levels (EGFR low and EGFR high). The Inset shows immunoblots for total EGFR and p85 (loading) from the same lysates.
PTEN knockout does not impair EGFR endocytosis. (A) EGF-induced degradation of biotinylated EGFR in PTEN+/+, but not PTEN−/− mouse embryonic fibroblasts (MEFs). MEFs carrying a "floxed" PTEN allele were engineered to overexpress wild-type EGFR. EGFR-expressing sublines were subsequently transduced with Cre recombinase through retroviral infection. MEFs of the indicated genotype were serum starved, cell-surface biotin-labeled at 4 °C with a cell impermeable biotinylation reagent (Thermo Scientific), and challenged with EGF at 37 °C for the indicated times. Each treatment group was plated in duplicates. The first sample from each treatment group was used to assess total biotinylated EGFR and was therefore lysed immediately. The second sample was used to measure endocytosed EGFR and was therefore treated with glutathione to cleave off biotin from cell surface (but not internalized) EGFR before lysis. All lysates were subjected to immunoprecipitation using the EGFR antibody cetuximab. The amount of biotinylated EGFR was determined by streptavidin-HRP detection. (B) EGFR routing to EEA1-positive early endosome is not impaired in PTEN−/− MEFs. PTENlox/lox and PTEN−/− MEFs were serum starved and challenged with 50 ng/mL EGF for 15 min. Cells were fixed and analyzed by immunofluorescence (Left) using antibodies for EGFR (green) or for the early endosomal marker EEA1 (red). Shown are representative images for each immunostain as well as an overlay of both stains. The bar graph on the Right is the quantification of EGFR/EEA1 colocalization for each genotype.
**Fig. S5.** PTEN regulates ligand-induced EGFR ubiquitylation. (**A**) PTEN reconstitution restores ligand-induced EGFR ubiquitylation. PC3 cells stably expressing a tetracycline-inducible PTEN allele (1) and engineered to overexpress wild-type EGFR were cultured in the presence or absence of 500 ng/mL doxycycline for 24 h. Doxycycline treatment was continued for an additional 12 h, but under serum-free conditions. Following serum starvation, cells were stimulated with EGF as indicated, lysed, and subjected to immunoprecipitation with the EGFR antibody cetuximab. Immunoprecipitates were analyzed by Western blot as shown. (**B**) PTEN reexpression in PTEN-deficient U87 glioblastoma cells accelerates EGFR protein down-regulation and enhances EGFR ubiquitylation in response to EGF. U87 cells expressing an ecdyson-inducible allele of PTEN (U87-ind-PTEN) were treated with the ecdyson analog muristerone A (MurA) for 18 h. MurA treatment was continued for another 24 h under serum-free conditions, followed by EGF stimulation for the indicated times. Lysates were collected and analyzed by immunoblot as indicated (Left) or subjected to immunoprecipitation with cetuximab before immunoblot (Right). (**C**) PTEN knockdown impairs ubiquitylation of mutant EGFR. Parental and HCC827-PTEN-shRNA cells were serum starved and challenged with EGF for the indicated times. Cells were lysed and subjected to immunoprecipitation with the EGFR antibody cetuximab. Shown are Western blots of the immunoprecipitates probed with the indicated antibodies. The brace indicates more extensively ubiquitylated EGFR of higher molecular weight, whereas the arrow points toward less extensively ubiquitylated receptors.

**Fig. S6.** Mutational activation of Akt impairs EGFR ubiquitylation and confers EGFR inhibitor resistance. (**A**) Akt activation is sufficient to blunt ligand-induced EGFR ubiquitylation. A431 cells were stably transduced with constitutively active HA-tagged Akt1 (Akt1-E17K). A431 and Akt1-E17K cells were serum starved, challenged with EGF (100 ng/mL) for the indicated times, and lysed. EGFR immunoprecipitates from these lysates (Top) and whole cell lysates (WCL, Bottom), were analyzed by Western blot with the indicated antibodies. Expression of mutant Akt was confirmed by Western blot using an antibody to the HA epitope. The Left bracket indicates more extensively ubiquitylated EGFR of higher molecular weight. (**B**) Expression of a constitutively active Akt1 allele confers EGFR-inhibitor resistance. Parental A431 cells and a subline stably transduced with a constitutively active Akt1 (Akt1-E17K) mutant were grown in the presence or absence of increasing concentrations of the EGFR kinase inhibitor EKB-569. Cell death was assessed by trypan blue exclusion after 5 days of treatment.
Inactivation of Cbl confers EGFR kinase inhibitor resistance. A431 and HCC827 cells were stably transduced with a dominant negative allele of Cbl (Cbl-70Z) and cultured in the presence or absence of EKB-569 for 5 days. Cell death was evaluated using the trypan blue exclusion method and plotted as the fraction of blue cells over the total number of cells.

Fig. S7.

EGFR gene amplification is associated with PTEN loss in human glioblastoma. The bars indicate the relative frequency of PTEN loss (in %) for primary glioblastomas with either EGFR amplification/mutation (n = 106), PDGFRA amplification/mutation (n = 14), MET amplification/mutation (n = 6), NF1 deletion/mutation (n = 30), or all other examined GBMs (n = 119). The EGFR-amplified group was the only one that showed statistically significant enrichment for PTEN loss (P = 5.2 × 10^{-10}; one-sided Fisher’s exact test).

Fig. S8.

Cbl-b regulates EGFRvIII protein levels. 293T cells were stably transduced with an EGFRvIII cDNA. After antibiotic selection, cells were transiently transfected with either a Cbl-b cDNA (Left) or a Cbl-b shRNA (Right). Forty-eight hours after transfection, cells were harvested and lysates analyzed by immunoblot with the indicated antibodies.

Fig. S9.

Other Supporting Information Files

Table S1 (DOC)