Supporting Information

Rothenberg *et al.* 10.1073/pnas.0803217105

SI Methods

DNA and shRNA Constructs. The putative target sequences for the shRNA vectors are:

shCTRL: 5′-GCAAGCTGACCCTGAAGTTCAT-3′
shEGFR 1: 5′-GCTGGATGATAGACGCAGATA-3′
shEGFR 2: 5′-GCTGAGAATGTGGAATACCTA-3′
shEGFR 3: 5′-AGAATGTGGAATACCTAAGG-3′
shEGFR 4: 5′-GCTGCTCTGAAATCTCCTTTA-3′
shErbB-3: 5′-AATTCTCTACTCTACCATTG-3′

The shCTRL vector encodes an shRNA that minimally affects the levels of Green Fluorescent Protein (GFP). The shEGFR1 vector targets a sequence within the EGFR coding region. The shEGFR vectors 2–4 target sequences within the 3′-untranslated region (UTR) of the primary EGFR mRNA. All shRNAs were expressed in pLKO, a self-inactivating lentiviral vector (William Hahn), from the U6 snRNP promoter. This vector also contains a puromycin resistance cassette expressed from the SV40 promoter. shRNA inserts were subcloned into the EcoRI-AgeI sites of pLKO, and the correct orientation and sequence were confirmed by dideoxynucleotide sequencing. cDNA expression constructs were generated in the lentiviral vector pLenti 6V5 DEST, which also contains a blasticidin resistance cassette expressed from an SV40 promoter, using the Gateway cloning system (Invitrogen), or in pWPI, a lentiviral cDNA expression vector containing a co-cistronic green fluorescent protein reporter (Didier Trono). The entire EGFR coding sequence and mutations therein for each construct was verified by dideoxynucleotide sequencing. The HIV-1 packaging construct pCMV-deltaR8.91 encodes the viral GAG, PRO, POL, TAT and REV proteins. pMD.G expresses the VSV-G envelope protein from a CMV promoter.

Lentivirus Production and Transduction. shRNA or cDNA lentiviral expression constructs were co-transfected into 293T cells with pCMVdeltaR8.91 and pMD.G using TransIT-LT-1 transfection reagent (Fisher Scientific). Viral supernatants were collected at 48 and 72 h posttransfection in DMEM with 30% FCS, filtered through 0.45 μm syringe filters to remove cell debris and stored in aliquots at −80 °C. Transductions were carried out in the presence of 8 μg/ml Polybrene (Sigma) by spinoculation at 1200 × *g* and at 32 °C for 60 min in a Sorvall Legend RT table-top centrifuge. Viral supernatant was exchanged for fresh media 30–36 h after spinoculation. To generate stable EGFR variant-expressing cell lines, cells were transduced by serial spinoculation. 24 h after the second round of transduction, blasticidin was added (where appropriate) to 1 μg/ml and the cells were cultured for an additional two passages. Individual shRNA vector supernatants were titered using the A549 human lung cancer cell line, which is relatively insensitive to the toxic effects of shRNA vectors targeting EGFR, by comparing cell survival in the absence or presence of puromycin. Vector titers on each PC9-derived cell population for each experiment was compared in the same manner using the lentiviral shCTRL construct at the same time as the cells were transduced with the lentviral shEGFR vectors. Viral supernatant was stable to multiple freeze-thaw cycles.

Cell Lines and Reagents. NSCLC cell line PC-9 (derived from a patient with adenocarcinoma previously untreated) was provided by Prof. Hayata (Tokyo Medical University Tokyo, Japan). NSCLC cell line A549 was obtained from the American Type Culture Collection. Cell lines were cultured with RPMI-1640 (GIBCO-BRL/Invitrogen) supplemented with 10% FBS (GIBCO-BRL/Invitrogen), penicillin and streptomycin (100 U/ml and 100 μg/ml, respectively; GIBCO-BRL). 293-T cells were cultured in DMEM (GIBCO-BRL/Invitrogen) supplemented with 10% FBS, penicillin, and streptomycin. All cell lines were cultured in a humidified atmosphere of 5% CO_2 at 37 °C. Erlotinib was obtained from the Massachusetts General Hospital Pharmacy. PHA-665752 was obtained from Pfizer. PI-103 was obtained from Cayman Chemical.

Immunoblotting. Cell lysis buffer consisted of 1% Nonidet P-40, 20 mM Tris, 150 mM NaCl, 10 mM $MgCl_2$, 0.25% sodium deoxycholate, 10% glycerol, 50 mM sodium fluoride, 1 mM sodium vanadate, 4 μg/ml leupeptin, 4 μg/ml aprotinin, and 1 mM PMSF. Protein levels were determined by immunoblot of cell lysates after SDS/PAGE and transfer to PVDF membranes. Reactive bands were visualized using the SuperSignal West Pico Peroxide reagent (Pierce) after incubating membranes with anti-rabbit or anti-mouse HRP-linked IgG (Cell Signaling). Levels of EGFR, Akt, ERK1/2, and ErbB-3 were determined using rabbit polyclonal anti-EGFR (Santa Cruz Biotechnology), anti-Akt (Cell Signaling), anti-ERK 1/2 (Cell Signaling), or anti-ErbB-3 (Santa Cruz Biotechnology) antibodies. The phosphorylated forms of EGFR, ERK1/2, and ErbB-3 were detected using rabbit polyclonal anti-EGFR [pY1068] (Abcam), anti-ERK1/2 [pT202/S204] (Cell Signaling), or anti-ErbB-3 [pY1289] (Cell Signaling) phosphospecific antibodies. Phosphorylated Akt was detected using rabbit monoclonal anti-Akt [pS473] phosphospecific antibody (Biosource). Levels of polyADP ribose polymerase (PARP) and GAPDH were detected with mouse monoclonal anti-PARP (BD PharMingen) or anti-GAPDH (Chemicon) antibodies. Anti-p85 antibody was from Upstate Biotechnology. Phospho-Tyr antibody was from Cell Signaling.

Flow Cytometry. Adherent cells were gently detached with trypsin, collected by centrifugation, resuspended in PBS containing 3% BSA and incubated at 37 °C for 30 min before addition of 0.5 μg mouse monoclonal antibody 528 (Santa Cruz) for 30 min. Cells were washed 3 times with PBS and then incubated with 1 μg Alexa Fluor 647 goat anti-mouse IgG (H+L) (Molecular Probes/Invitrogen) for 30 min. Cells were washed 3 times with PBS, resuspended in 1% paraformaldehyde, and analyzed using an LSRII flow cytometer (Becton-Dickinson). For fluorescence-activated cell sorting, GFP-expressing cells were isolated using a FACSAria cytometer (Becton-Dickinson).

1. Rokudai S, Fujita N, Hashimoto Y, Tsuruo T (2000) Cleavage and inactivation of antiapoptotic Akt/PKB by caspases during apoptosis. *J Cell Physiol* 182(2):290–296.
2. Fan QW, *et al.* (2006) A dual PI3 kinase/mTOR inhibitor reveals emergent efficacy in glioma. *Cancer Cell* 9(5):341–349.
3. Fan QW, *et al.* (2007) A dual phosphoinositide-3-kinase alpha/mTOR inhibitor cooperates with blockade of epidermal growth factor receptor in PTEN-mutant glioma. *Cancer Res* 67(17):7960–7965.

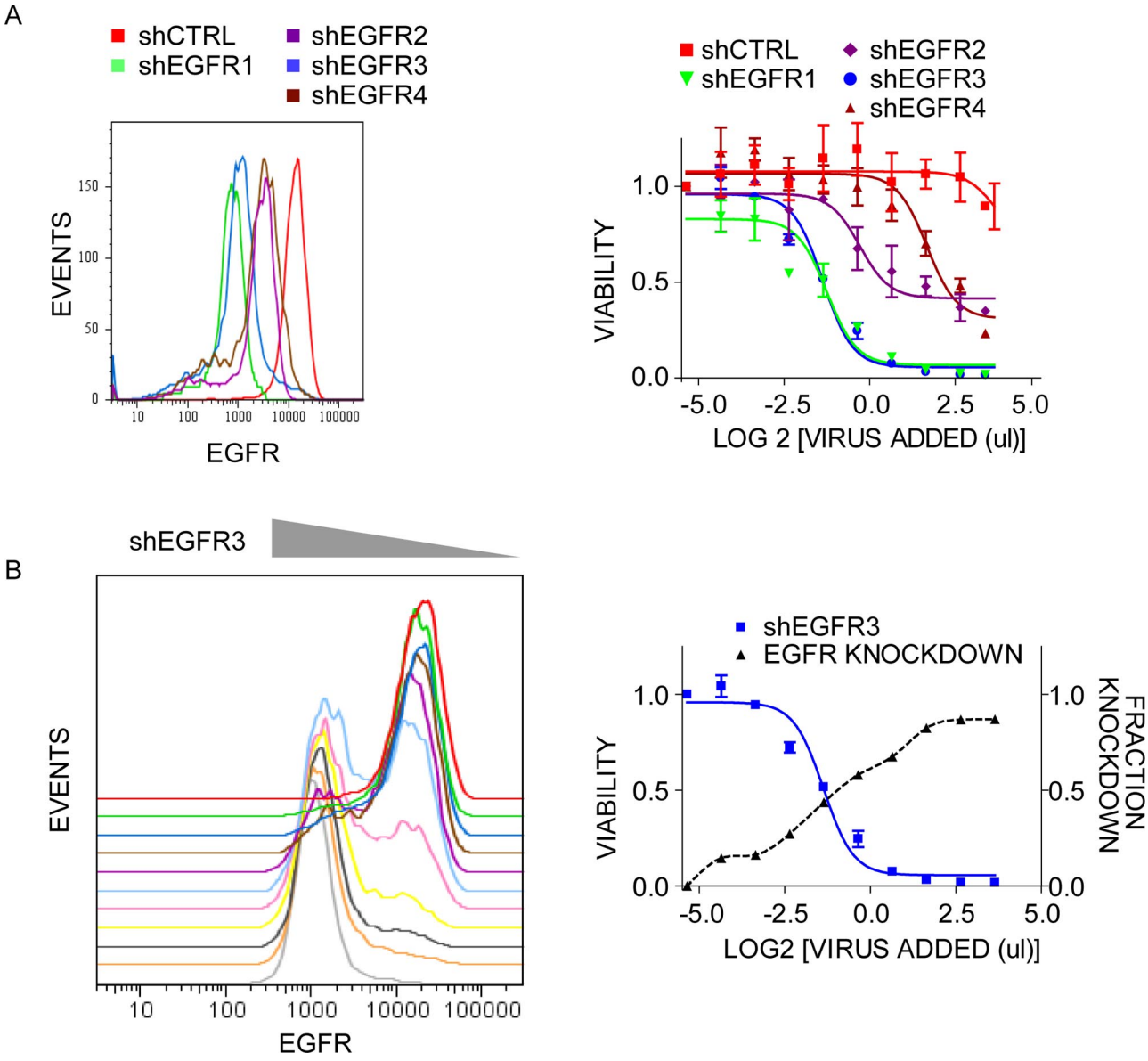


Fig. S1. Lentiviral shRNA vectors targeting EGFR demonstrate different efficiencies of knockdown and resulting toxicity. (*A*) PC9 cells transduced with the indicated lentiviral shRNA vectors were examined for EGFR by flow cytometry (*Left*). Cellular toxicity correlates with knockdown by each vector (*Right*). (*B*) Increasing the titer of the shEGFR3 vector causes progressively increased loss of EGFR protein (*Left*) and increased toxicity (*Right*).

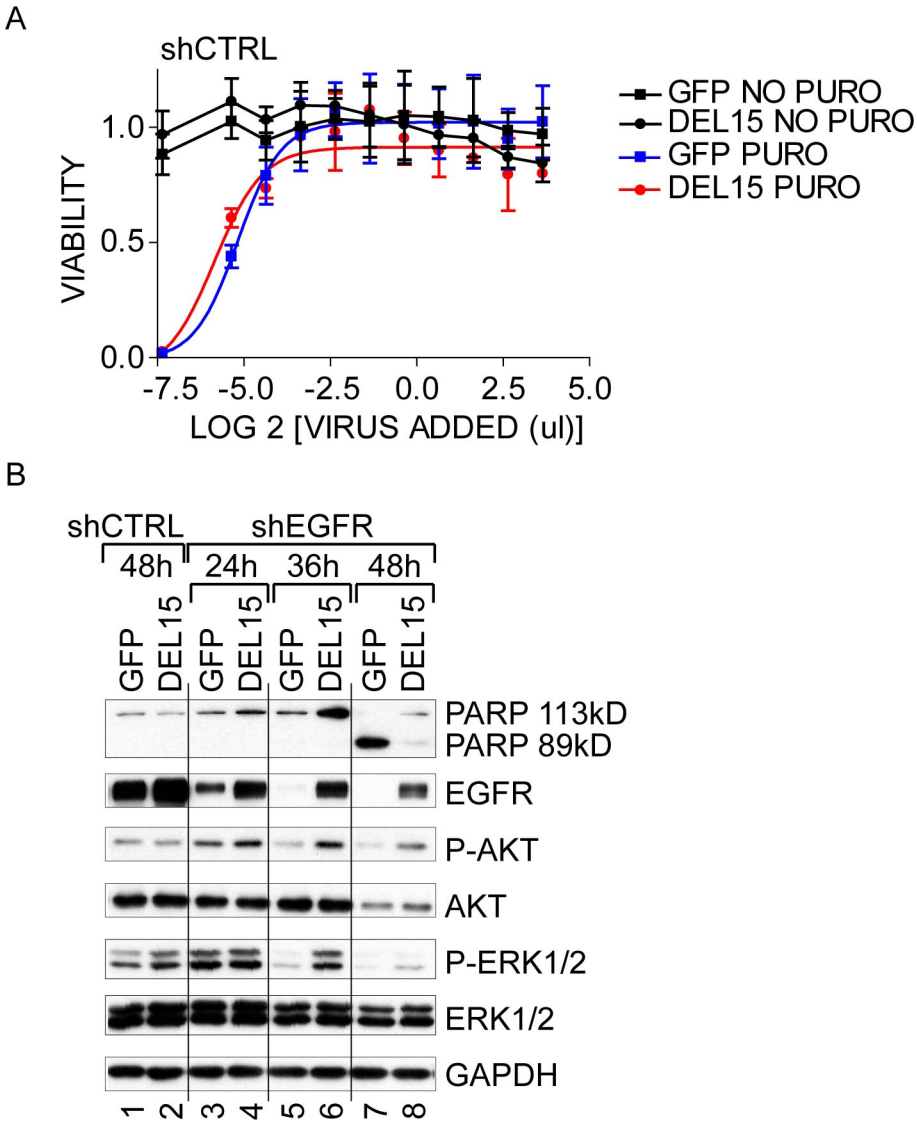


Fig. S2. Sources of off-target toxicity for shEGFR vectors. (*A*) PC9 cells expressing GFP or DEL15-delUTR EGFR are resistant to the shCTRL vector across the entire range of viral titers (black curves). Therefore, selection with puromycin gives a measure of the relative fraction of transduced cells, which is similar for each cell type (blue and red curves). (*B*) Following infection with the shCTRL or shEGFR vectors, levels of the indicated proteins were monitored by immunoblot every 12 h. The shCTRL vector does not significantly affect signaling output from EGFR or cause apoptosis (lanes 1–2). In control (GFP) cells treated with the shEGFR vector, the levels of phosphorylated Akt and ERK begin to decrease at 36 h posttransduction (lanes 3 vs. 5) but are preserved in DEL15-delUTR cells (lanes 4 vs. 6). The decrease in phosphorylated Akt clearly precedes the decrease in total Akt that is evident at 48 h posttransduction (lanes 7–8) as a result of caspase-mediated cleavage (1). The loss of phosphorylated and total Akt even in the DEL15-delUTR cells is due to incomplete reconstitution of EGFR activity or an off-target effect of the shRNA vector. Despite this, the appearance of the cleaved 85kD PARP fragment indicative of apoptosis in control cells is significantly delayed in cells expressing DEL15-delUTR (lanes 7 vs. 8).

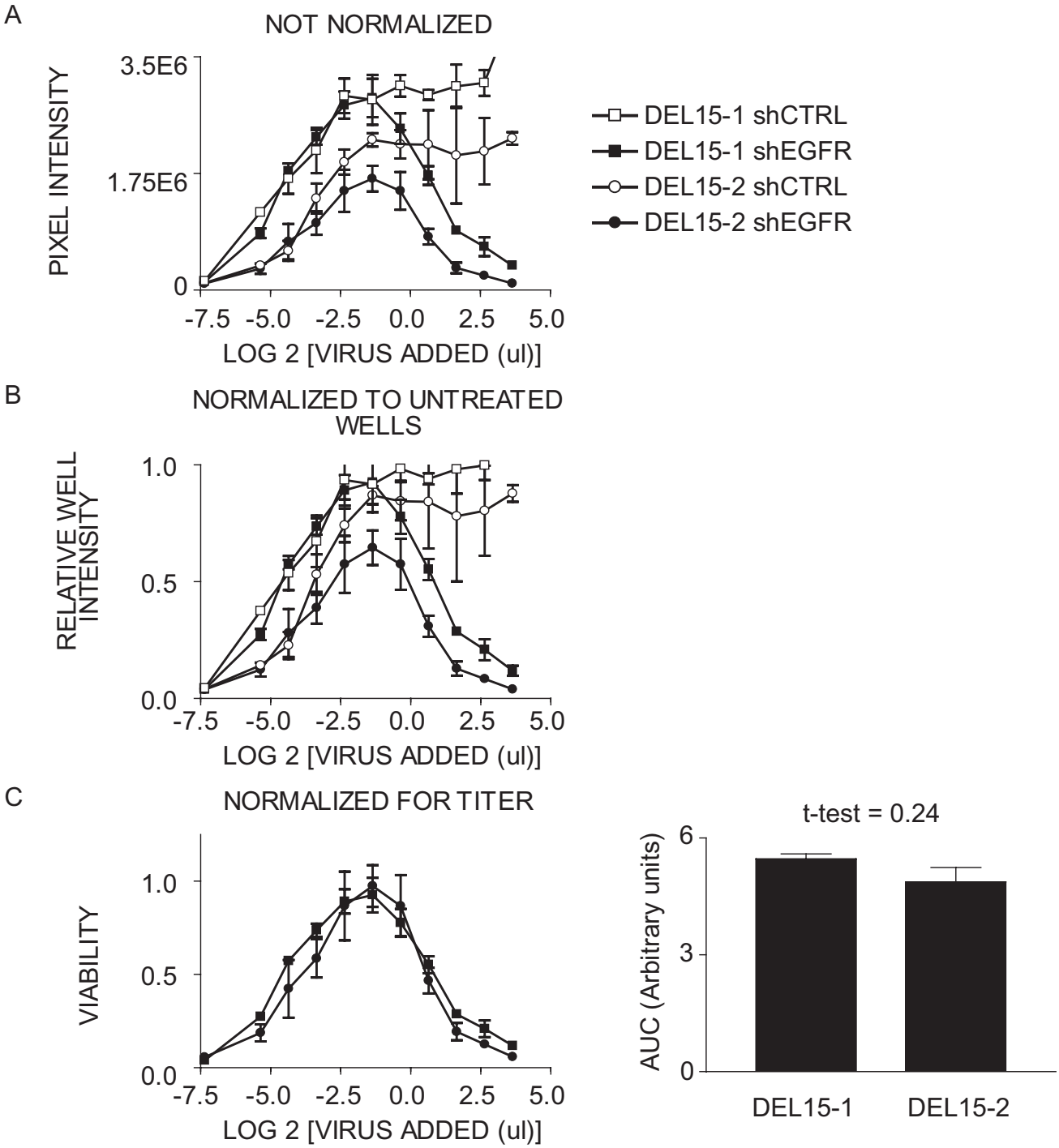


Fig. S3. Reproducibility of AUC analysis. Two PC9 cell populations (DEL15–1 and DEL15–2), each expressing exogenous DEL15-delUTR, were generated independently on different days, and AUC analysis for each population was carried out, also on different days for each cell line. (*A*) Raw well intensity data (from puromycin-treated wells) before any normalization. (*B*) Intensity data for puromycin-treated wells normalized to untreated wells. (*C*) Intensity data (e.g., viability) normalized for variations in shRNA viral titer as determined with the shCTRL virus. After normalization, the AUCs for each cell population are not statistically different (bar graph). Data are presented as the mean of four replicates ±1 STDEV.

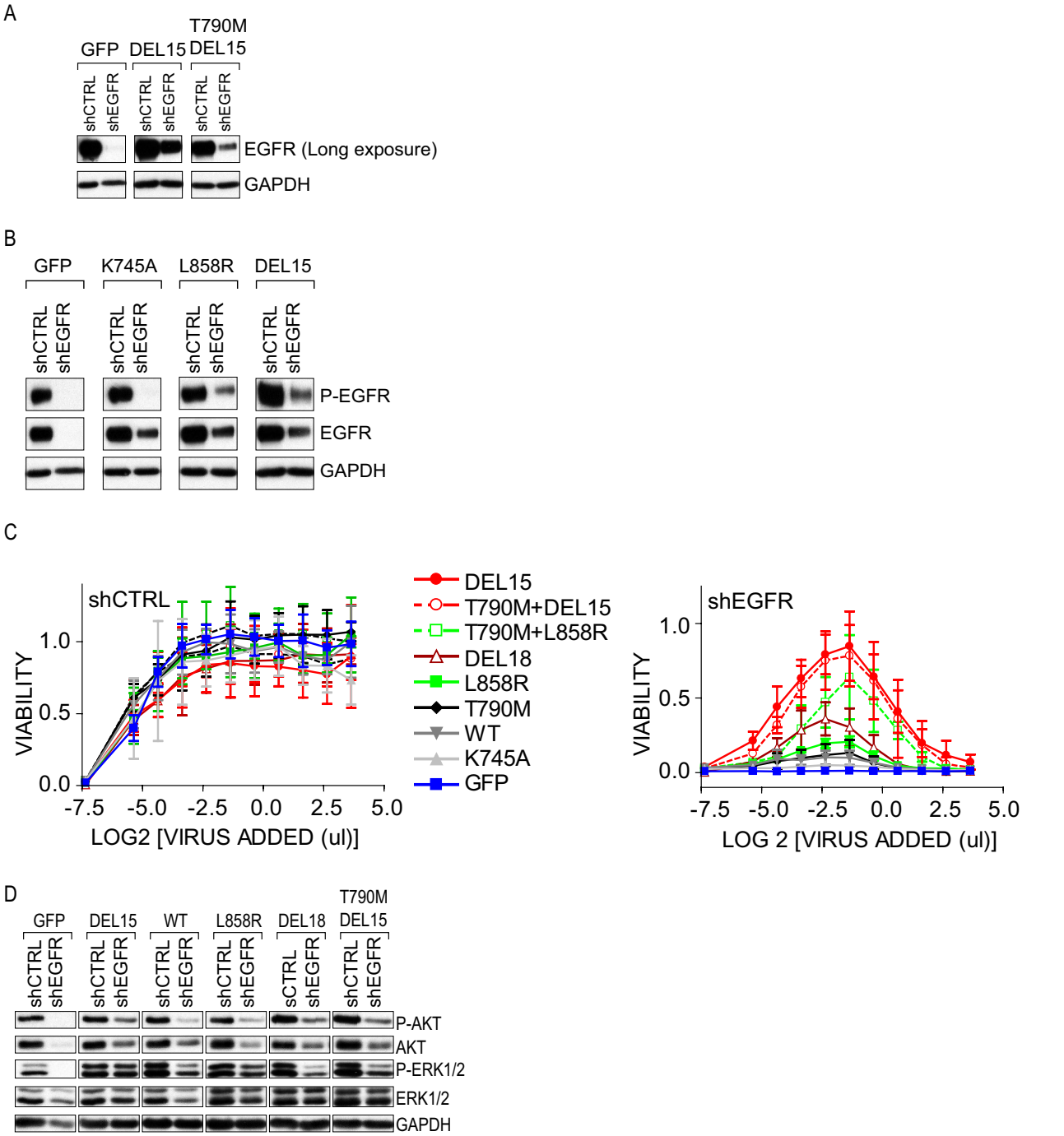
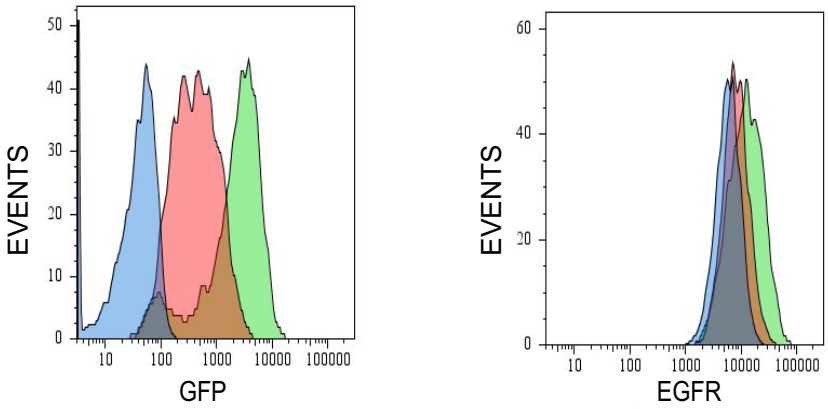


Fig. S4. Relative activity of EGFR variants. (*A*) Levels of the dual EGFR mutants achieved through heterologous expression are less than the single mutants (but are still detectable compared to control cells). (*B*) EGFR levels were analyzed in PC9 cells engineered to stably express the indicated EGFR variants (minus the endogenous 3′-UTR) 2 days after transduction with the control shRNA vector or the shEGFR vector targeting the EGFR 3′-UTR. 20 μg of each extract was probed with the indicated antibodies. All endogenous EGFR protein is eliminated in control GFP-expressing cells (lanes 1 vs. 2). Therefore, the levels of phosphorylated EGFR remaining after knockdown reflect the relative kinase activity of the exogenous EGFR variants. As expected for the kinase-inactive K745A variant, although it is detectable after knockdown of endogenous EGFR, it is completely unphosphorylated (lanes 3 vs.4). (*C*) AUC curves for the complete panel of EGFR variants. (*D*) Lysates were examined by immunoblotting with the indicated antibodies after transduction of cells as in Fig. 2*B*.

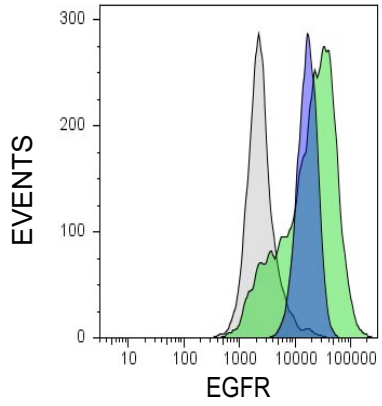
A

B



	GFP	EGFR
PARENTAL	1.00	1.00
UNSORTED	8.97	1.43
SORTED	45.05	2.33

C



	EGFR
PARENTAL	1.00
PAR. KO	0.13
SORTED KO	1.27

Fig. S5. Methodology for increasing exogenous EGFR levels. (*A*) EGFR variants were subcloned into the pWPI vector, which encodes a co-cistronic GFP reporter. (*B*) After transduction of PC9 cells, highly GFP-positive cells were collected by FACS (*left panel*), and levels of EGFR were determined by flow cytometry as described in SI Methods (*right panel*). The table shows mean EGFR levels for the indicated cell populations. (*C*) The sorted cells retain as much cell-surface EGFR, after knockdown of the endogenous protein (green histogram), as parental PC9 cells before knockdown (blue histogram). The decreased EGFR in a small fraction of the sorted cells is likely due to the approximately 10% GFP ''low'' cells that remain after sorting (Fig. S5*B* green histogram). The table shows the median EGFR levels for the indicated cell populations.

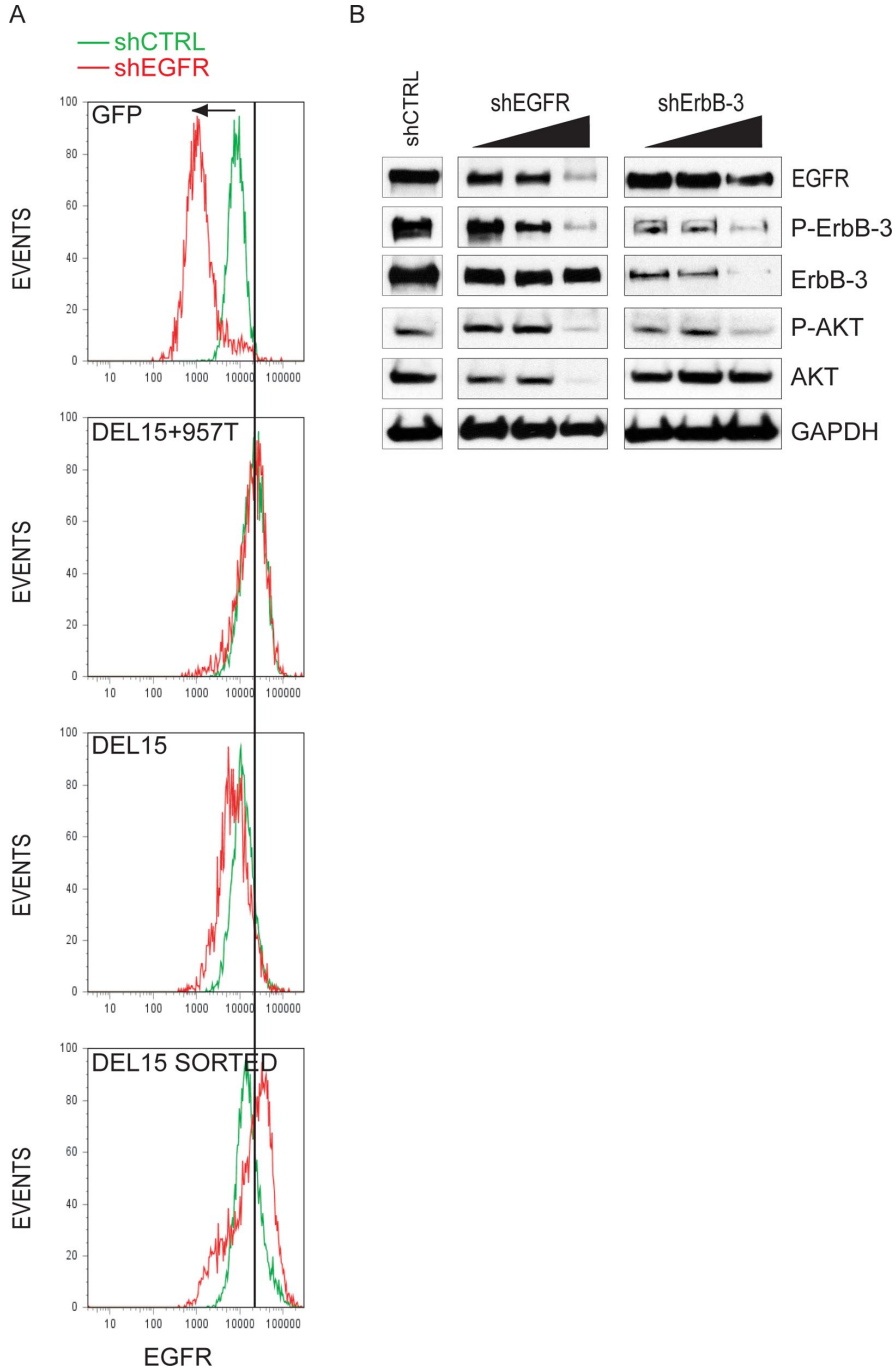


Fig. S6. Truncated DEL15 mutants retain the ability to activate ErbB-3. (*A*) Deletion of the C-terminal portion of EGFR removes the epitope for the antibody used for immunoblot. Therefore, the exogenous truncation mutants were detected by flow cytometry using an antibody against the EGFR ectodomain. In GFP-expressing control cells, the shEGFR vector results in significant loss of cell-surface EGFR (histogram of EGFR expression in shCTRL-treated cells [green] shifts to the left in shEGFR-treated cells [red]). However, cells expressing either full-length or truncated DEL15 EGFR retain detectable EGFR after knockdown of the endogenous protein (green and red curves overlap). The levels of the truncated EGFR at the cell-surface are higher than full-length (compare the peak of the red histograms for ''DEL15 957T'' and ''DEL 15'' plots); however, the sorting approach described in Fig. S5 leads to equivalent levels of each protein (compare red histograms for ''DEL15 957T'' and ''DEL 15 SORTED'' plots). (*B*) Knockdown of EGFR decreases both phosphorylated ErbB-3 and phosphorylated Akt (lanes 2–4), while knockdown of ErbB-3 (lanes 5–7) decreases phosphorylated Akt.

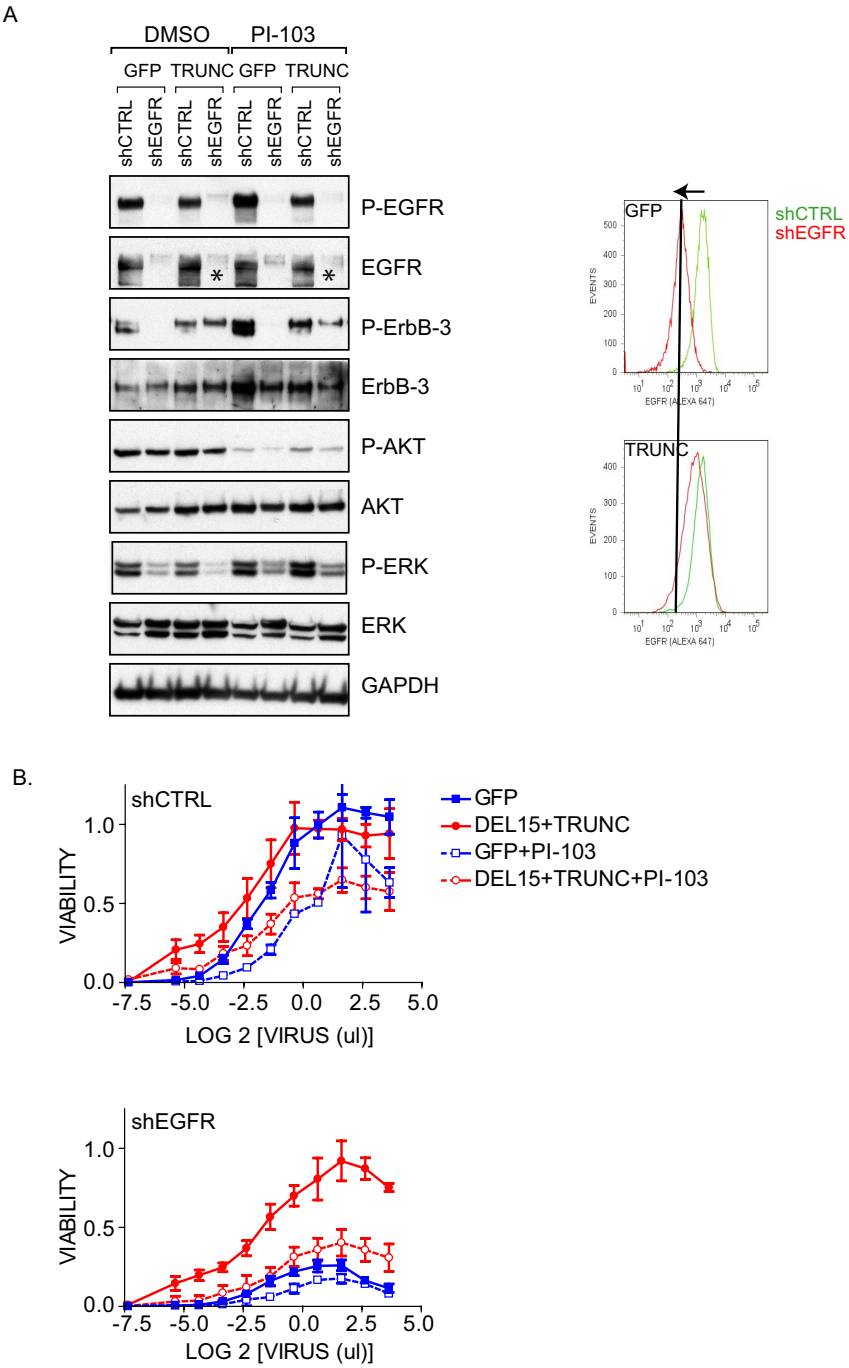


Fig. S7. EGFR C-terminal deletion mutants are sensitive to PI3K inhibition. (*A*) PC9 cells stably expressing expressing GFP or the shRNA-resistant DEL15 C-terminal truncation were transduced with control or EGFR shRNA vectors. 24 h after transduction, 1 μM PI-103, a highly specific and potent class IA PI3K inhibitor (2, 3), or DMSO was added. 48 h after transduction, extracts were prepared and immunoblot analysis was performed with the indicated antibodies (*Left*). The levels of phosphorylated Akt (compare lanes 5–8 to 1–4) are significantly reduced as a result of PI-103 treatment. The expression of the C-terminal truncation EGFR was verified by flow cytometry after knockdown of endogenous EGFR as in Fig. S6 (*Right*). (*B*) PC9 cells were plated at 1e4 per well in 96-well format and transduced the following day with a dilutions series (0.12–12.5 μl) of the shCTRL or shEGFR vectors. 30 h after transduction, puromycin and 1 μM PI-103 or DMSO were added. Cell viability was determined as in Fig. 2 *C*.