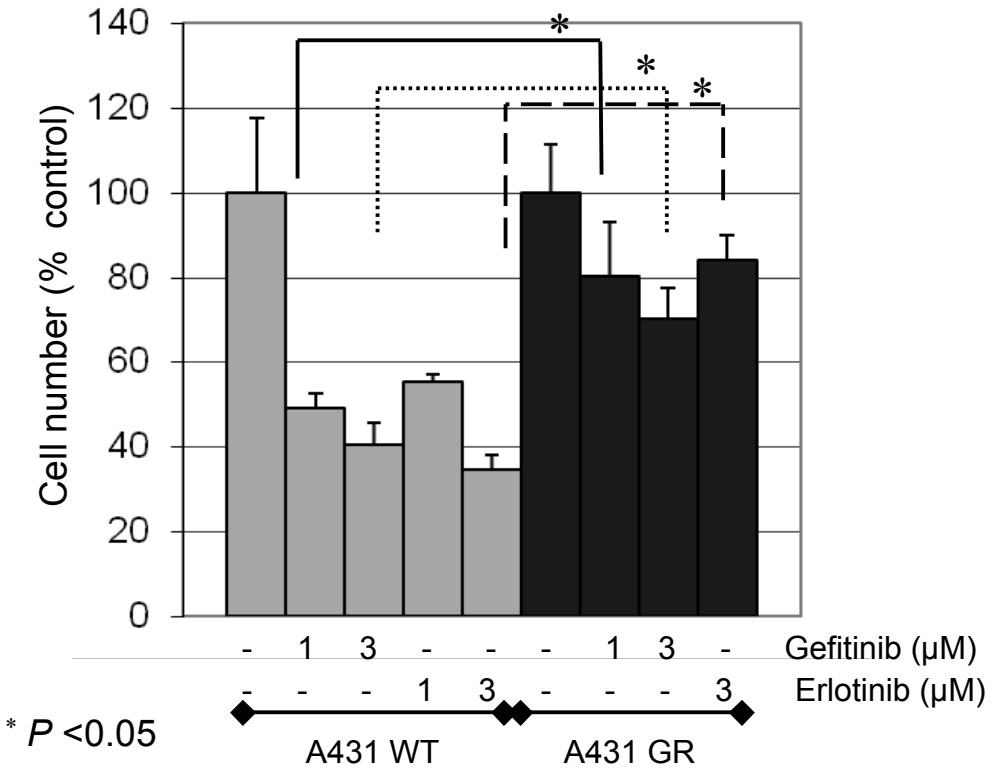
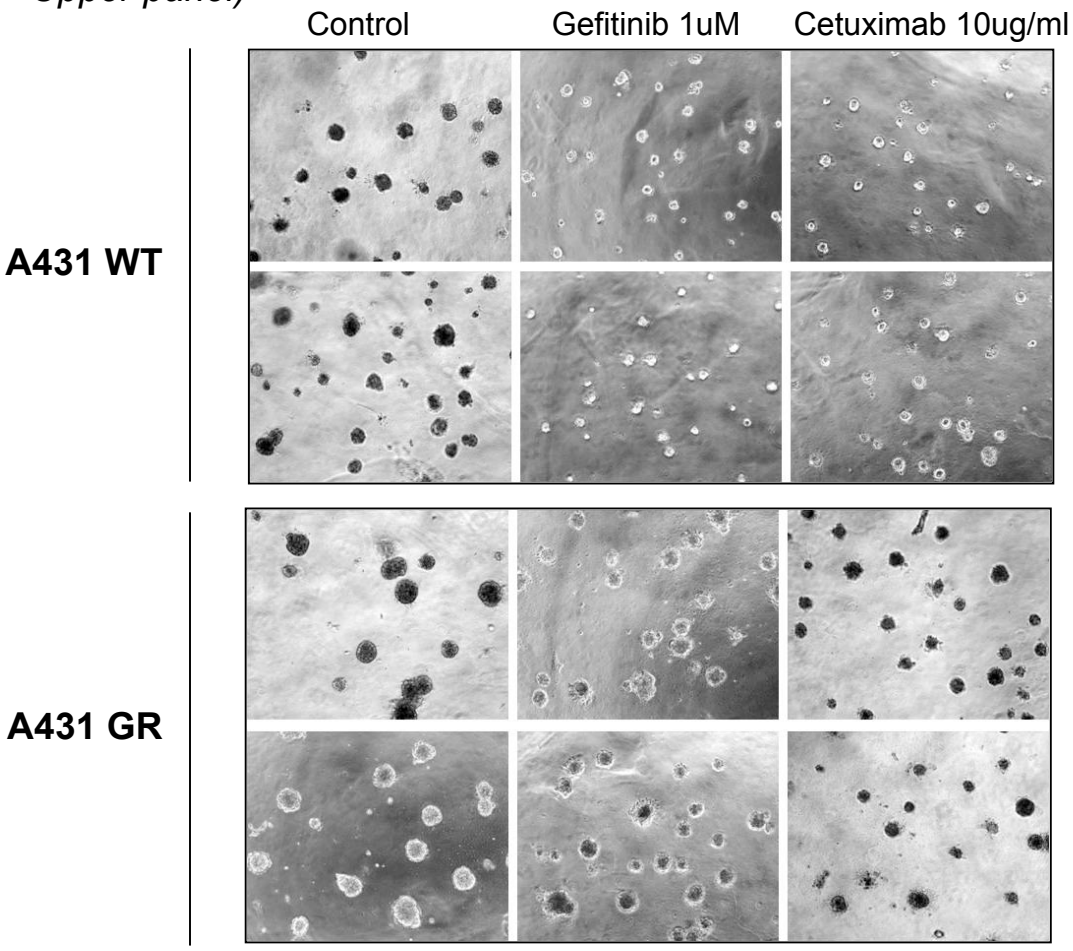
A



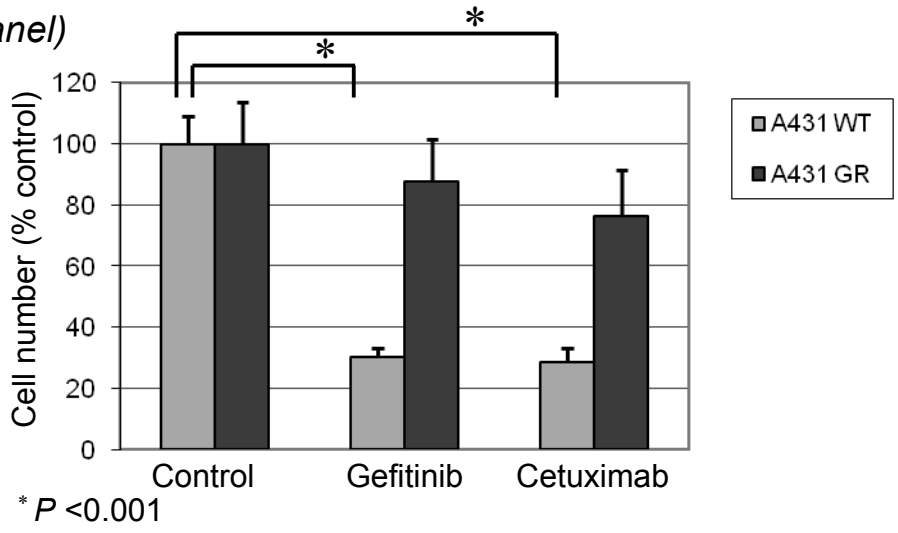
Guix et al., Supplemental Figure 1A

B

Upper panel)

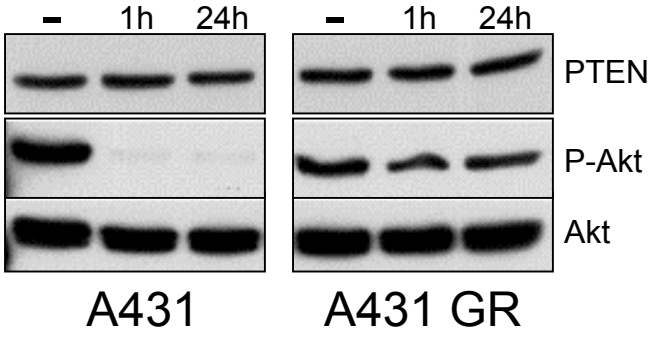


Lower panel)

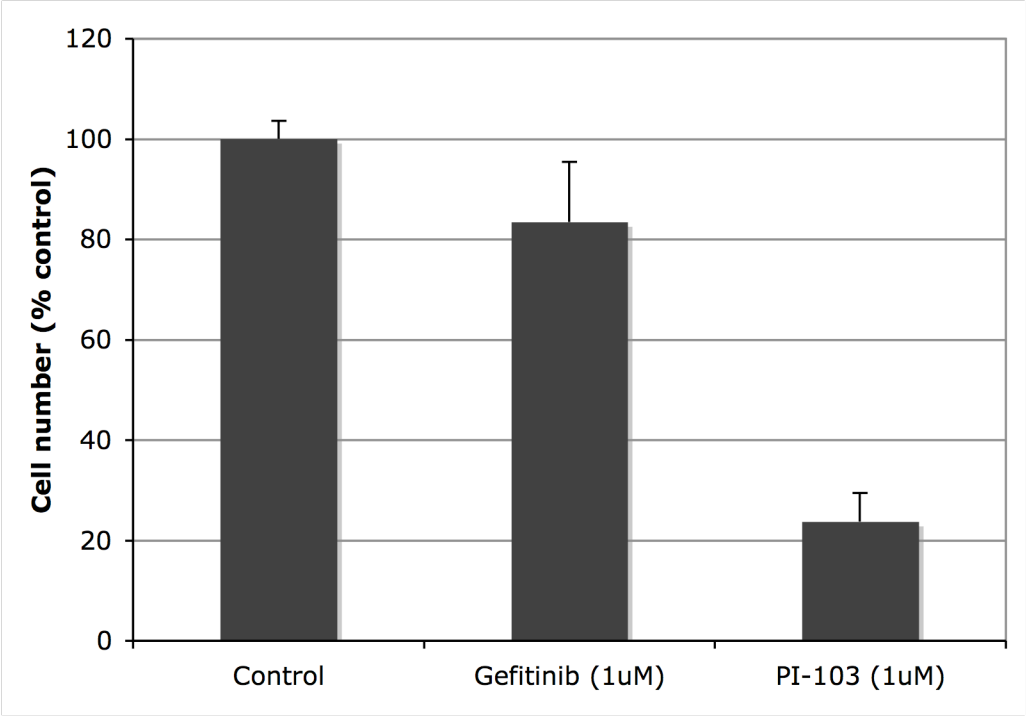


* *P* <0.001

Guix et al., Supplemental Figure 1B

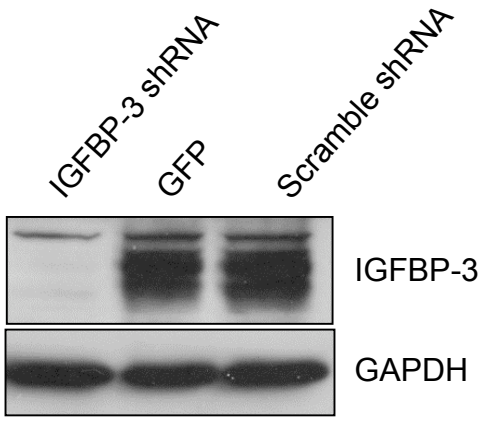


Guix et al., Supplemental Figure 2



Guix et al., Supplemental Figure 3

A



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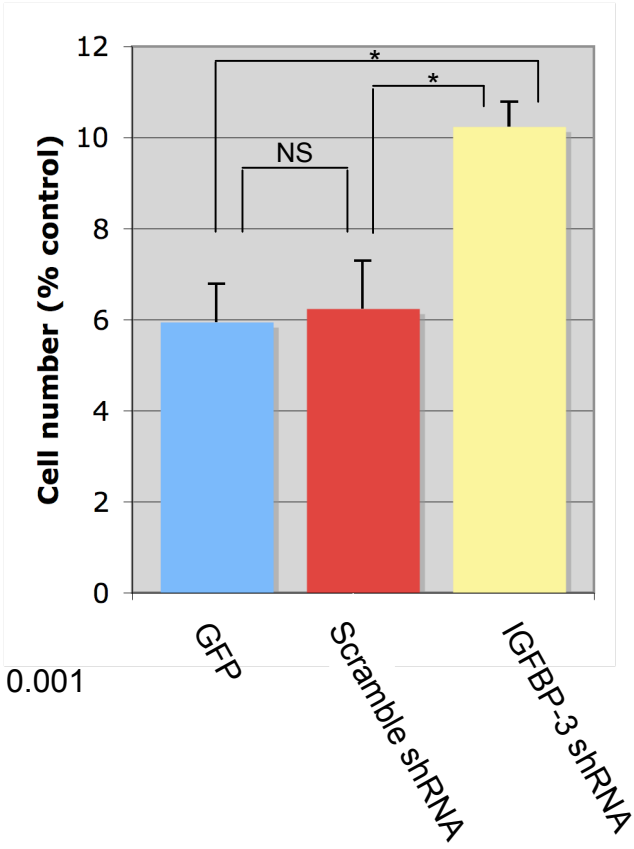
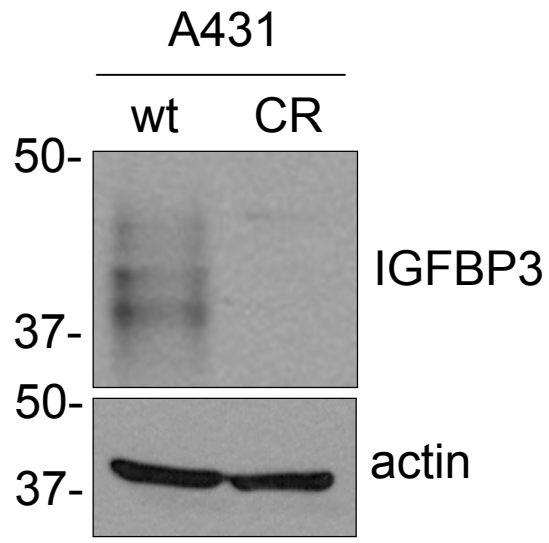


* p < 0.001

Guix et al., Supplemental Figure 4

A



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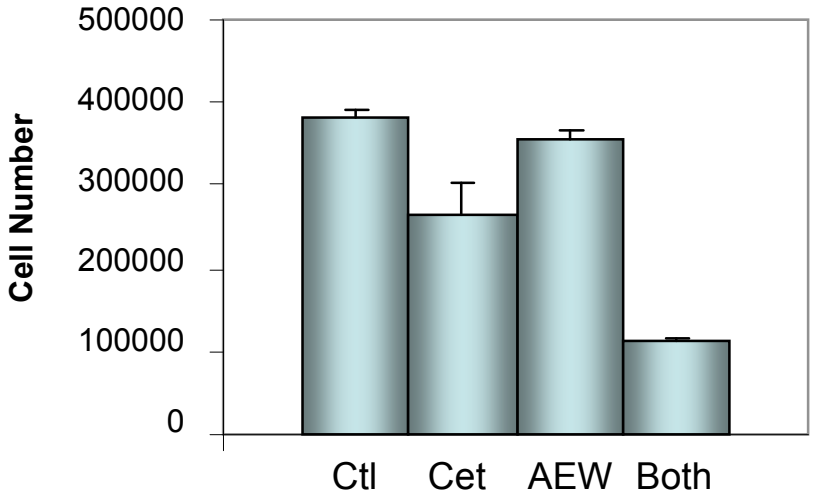
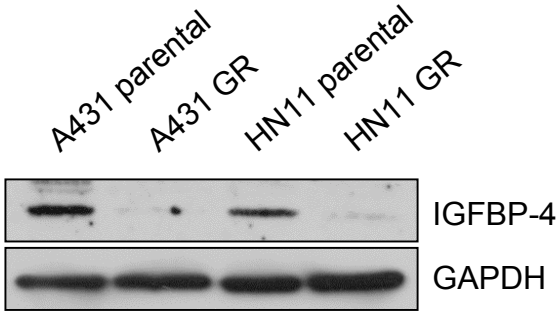
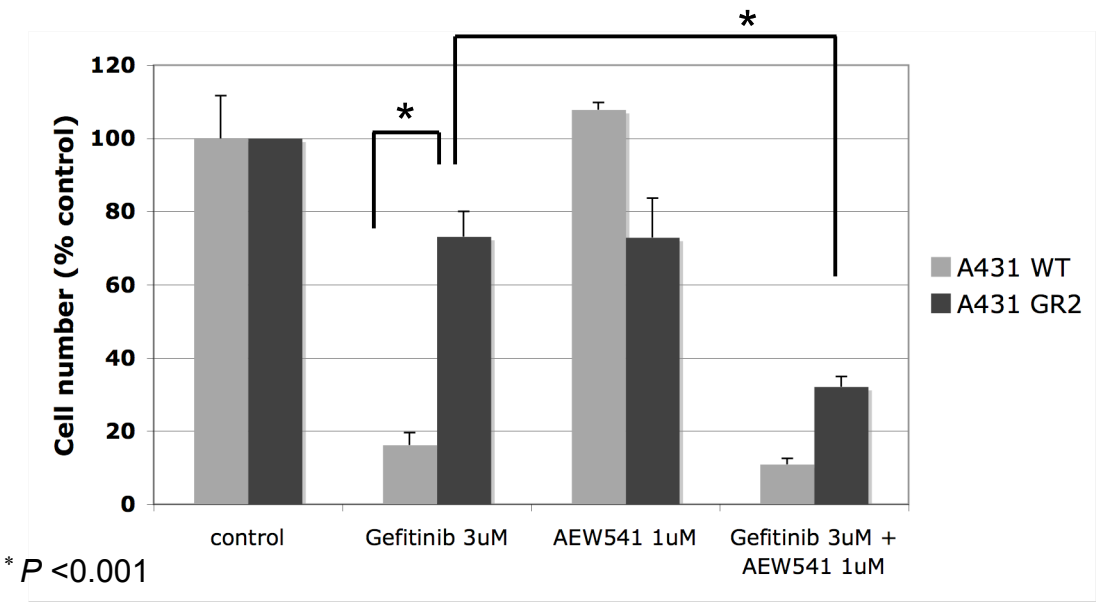


Guix et al., Supplemental Figure 5



Guix et al., Supplemental Figure 6

A



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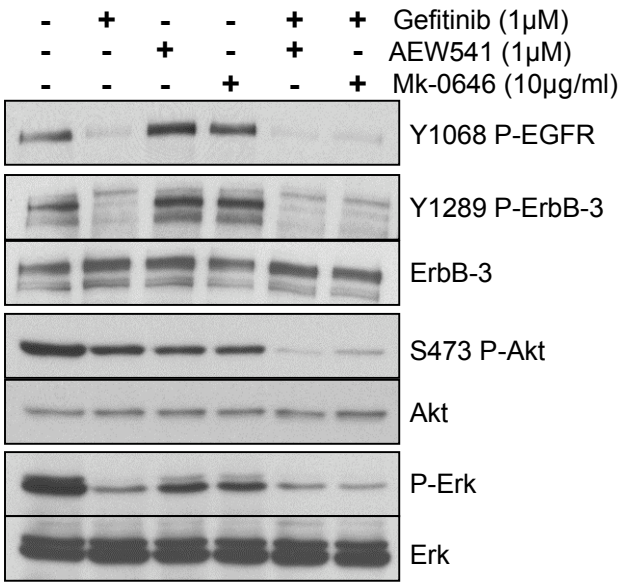
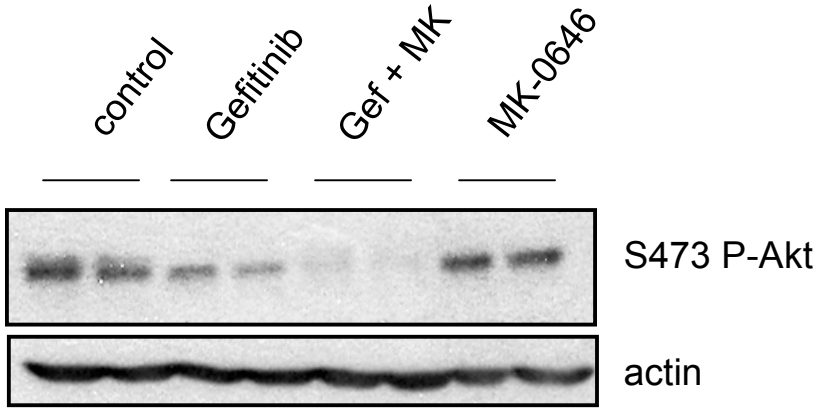


Guix et al., Supplemental Figure 7

A



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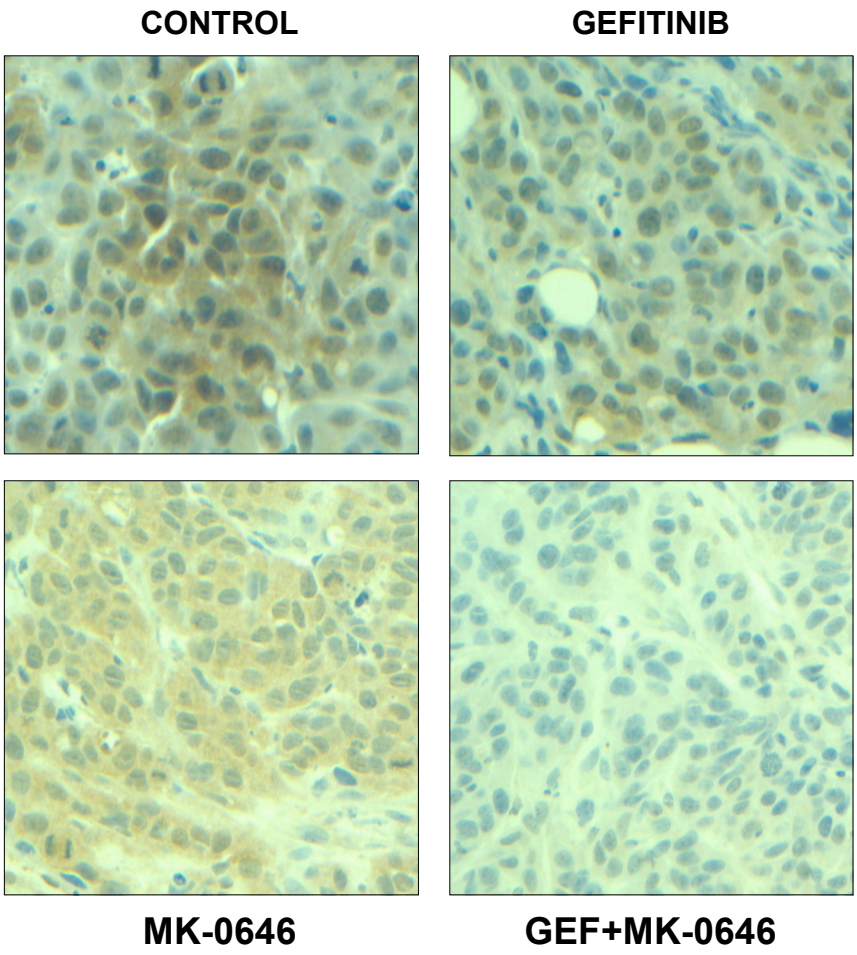
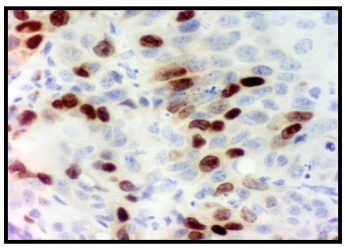
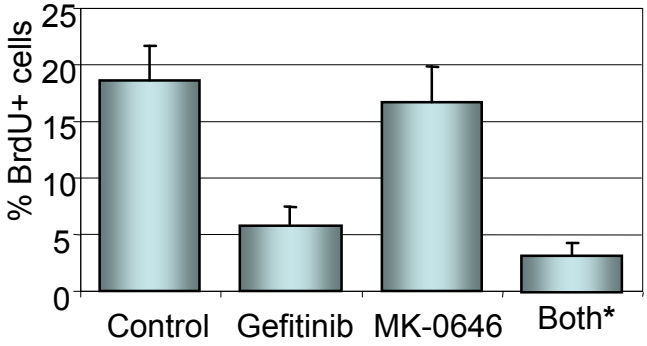


Guix et al., Supplemental Figure 8

A





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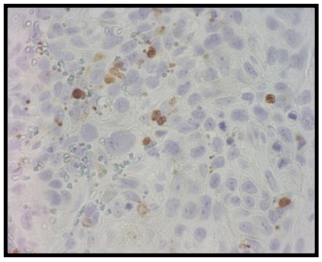

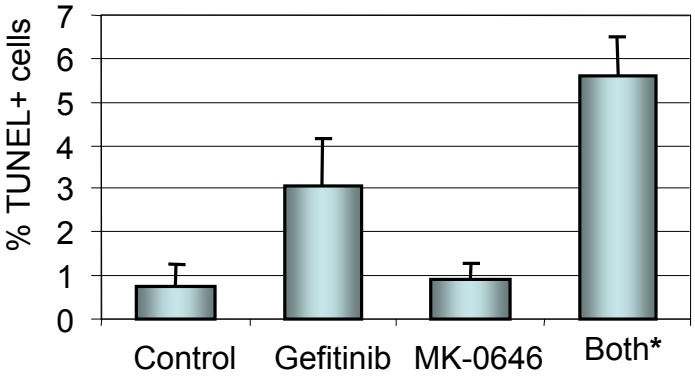

Guix et al., Supplemental Figure 9

Supplemental Figure legends.

Supplemental Figure 1. A431 GR cells are resistant to erlotinib and cetuximab. ***A)*** Parental and A431 GR cells were grown for 72 h in 0.5% FBS containing medium with or without gefitinib or erlotinib at the indicated μM concentrations. Cell number was determined in a coulter counter. Each data point represents the mean ± SD of 3 wells. Results are presented as percent of untreated group. Student's *t*-test was used for statistical comparisons. ***B)*** Parental and GR A431 cells were grown in Matrigel in the absence or presence of gefitinib 1 μM or cetuximab 10μg/ml. *Upper panel)* Pictures were taken after 8 days. *Lower panel)* Cell numbers from Matrigel experiment. Cells were harvested by trypsinization and then counted. Cell numbers are represented as percent of untreated.

Supplemental Figure 2. PTEN expression is maintained in the A431 GR cells. Parental and GR cells were exposed to gefitinib for 1 or 24 hours or vehicle control. Cell lysates were prepared and probed with the indicated antibodies. Each western blot of the A431 and A431 GR extracts for each of these antibodies is the same exposure from a single gel and blot; an irrelevant lane between the A431 and A431 GR extracts was omitted from the figure.

Supplemental Figure 3. PI3K inhibitors block the growth of A431 cells resistant to EGFR inhibitors. A431 GR cells were grown in 12 well plates in 0.5% FBS containing medium for 72 h with or without gefitinib (1μM) or PI-103 (1μM), harvested by

trypsinization and cell numbers were determined with a Coulter counter. Bars represent the mean ± SD of 3 wells.

Supplemental Figure 4. IGFBP-3 knockdown leads to mild gefitinib resistance. A431 parental cells were infected with lentivirus encoding either GFP, scrambled shRNA or IGFBP-3 shRNA. ***A)*** Cells were selected in puromycin and lysates were probed with antibodies against IGFBP-3 and GAPDH. ***B)*** The infected A431 cells were subjected to a cell growth assay in 0.5% FBS in the absence or presence of gefitinib (1μM) as in Supplemental Fig. 1A. Cell numbers are presented as percent of untreated. Student's *t*-test was used for statistical comparisons.

Supplemental Figure 5. IGF-IR tyrosine kinase inhibition resensitizes cetuximab-resistant A431 cells to cetuximab. A) Lysates from parental and cetuximab-resistant (CR) cells (Materials and Methods) were prepared and tested in an immunoblot procedure with the indicated antibodies. The 42-kDa IGFBP3 band was not detectable in A431 CR cells. **B)** Subconfluent monolayers of A431 CR cells were grown in 6-well plates in 0.5% FBS containing medium in the presence or absence of cetuximab (100 nM), AEW541 (1 μM), or both. After 72 h, cells were trypsinized and cell numbers determined with a Coulter counter. Bars represent the mean ± S.E. of 3 wells.

Supplemental Figure 6. HN11 GR cells lose expression of IGFBP-4. Protein lysates were prepared from A431, A431 GR, HN11 and HN11 GR cells. The lysates were probed with antibodies against IGFBP-4 and GAPDH.

Supplemental Figure 7. A431 GR2 cells require EGFR and IGF-1R blockade to inhibit PI3K/Akt signaling and block cell growth. A second, independent gefitinib resistant A431 cell line, A431 GR2, was developed as described in the Materials and Methods. ***A)*** Cell proliferation assay of A431 GR2 cells as in Fig 3E in the presence of vehicle control, gefitinib, AEW541 or their combination at the indicated concentrations. Student's *t*-test was used for statistical comparisons. ***B)*** A431 GR2 cells were treated with single-agent gefitinib, AEW541, Mk-0646 or combinations of gefitinib and AEW541 or Mk-0646 at the indicated concentrations for for 6 hours. Cells were lysed and extracts were probed with the indicated antibodies.

Supplementary Figure 8. Combined blockade of IGF-IR and EGFR synergistically inhibits AKT phosphorylation activity in vivo. Mice were treated as indicated in Fig. 7. Seventy-two hours after initiation of treatment (2 doses of MK-0646 for the arms containing the antibody), 3 tumors from each treatment group were harvested, and tumors were fixed in paraformaldehyde or flash frozen in liquid N_2. Tumor lysates were analyzed by western blot analysis using the indicated antibodies. **b)** Five μm-thick tumor sections from tumors allocated to the indicated treatment arms were subjected to IHC with an S473 P-Akt antibody as described (50). We followed the following scoring system for P-Akt, which measures both the percentage of stained nuclei and the intensity of cytoplasmic staining, combining them into one score: percent positive nuclei + 1/3 (cytoplasmic intensity x 100). The cytoplasmic staining intensity for P-Akt was graded as follows: 0=none; 1=weak; 2=moderate; 3=strong. Median scores ± SE (*n*=3) were as

follows: control, 113.3±3.3; gefitinib, 82.7±14.3; MK-0646, 86.1±12.1; both inhibitors, 43.2±9.2 (p=0.01).

Supplementary Figure 9. Inhibition of IGF-IR with MK-0646 enhances the anti-tumor effect of gefitinib *in vivo*. Xenograft tumors were treated as in Supplemental Figure 8. BrdU (150 mg/kg) was administered 2 h prior to mouse sacrifice. **Top:** Tumor sections from paraffin-embedded tumor blocks were subjected to IHC with a BrdU antibody as described (46). Shown is a representative image from control tumors. The number of proliferating cells was determined by counting 10 random high-power fields (at 400x) and expressed as percentage of BrdU-positive cells. Student's *t*-test was used for statistical comparisons. *, p=0.04 compared to gefitinib alone. **Bottom:** Tumor sections were subjected to terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate-biotin nick-end labeling (TUNEL) analysis as described (50). Shown is a representative image from tumors receiving combined treatment. TUNEL-positive cells were counted as for BrdU-positive cells. *, p=0.01 compared to gefitinib alone. For both panels, each bar represents the mean ± S.E. of 3 mice per group.