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Supplemental Information

**Requirement for Interaction of PI3-Kinase p110 α
with RAS in Lung Tumor Maintenance**

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SUPPLEMENTAL DATA

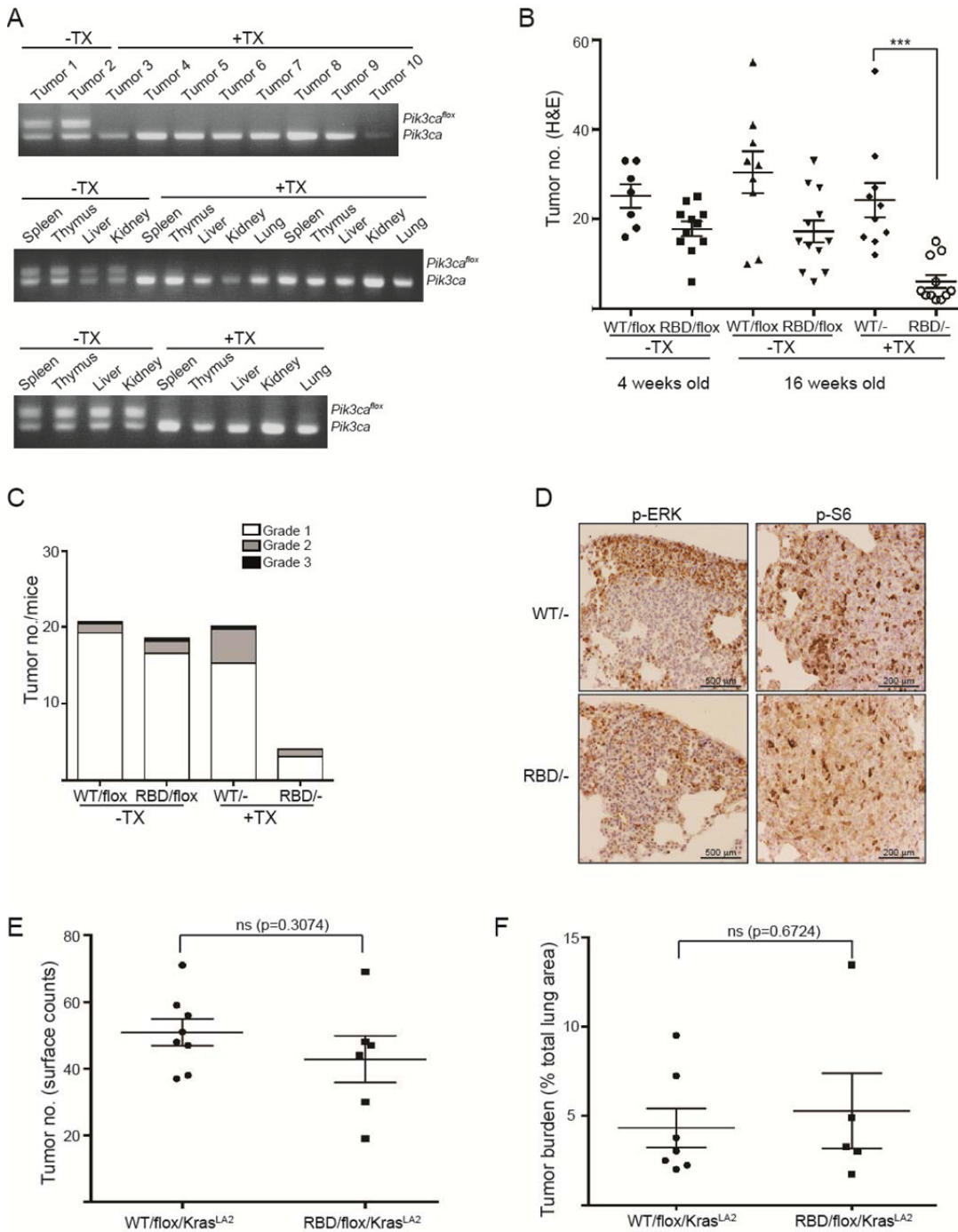


Figure S1, related to Figure 1. Expression of p110 α -RBD induces tumor regression in early stage tumors.

(A) PCR showing efficiency of recombination in tumors and in different tissues at the end of tamoxifen treatment

(B) Analysis of the number of tumors in tamoxifen treated and untreated mice in H&E stained sections.

(C) Quantification of tumor grade in 16-week-old mice treated and untreated with tamoxifen.

(D) p-ERK and p-S6 staining of lung sections from *Pik3ca*^{WT/-} mice and *Pik3ca*^{RBD/-} mice.

(E) Mice lacking the *Rosa26-CreERT2* locus were left for tumor development for 16 weeks and then sacrificed. The graph represents number of tumors on the lung surface of *Kras*^{LA2};*Pik3ca*^{WT/flox} and *Kras*^{LA2};*Pik3ca*^{RBD/flox} mice.

(F) Analysis of tumor burden from H&E slides of *Kras*^{LA2};*Pik3ca*^{WT/flox} and *Kras*^{LA2};*Pik3ca*^{RBD/flox} mice. Mice were 16-weeks old when sacrificed.

Error bars indicate mean \pm SEM. (Significance using Student's T test *** $p < 0.001$).

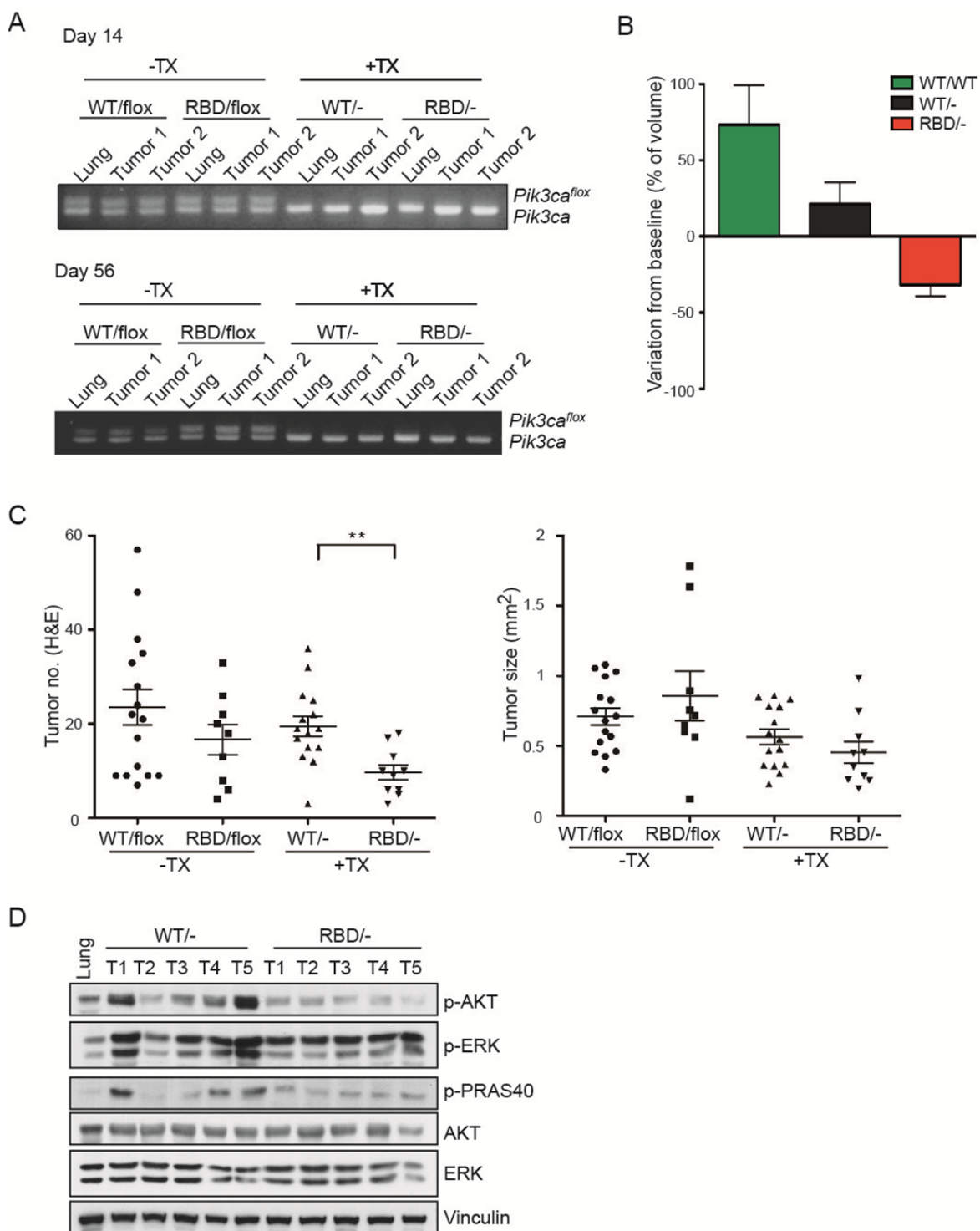


Figure S2, related to Figure 2. Expression of p110 α -RBD in established tumors reduces tumor burden.

(A) PCR for the presence of the WT and floxed *Pik3ca* allele after tamoxifen treatment and 8 weeks later. Tamoxifen treated animals show loss of the floxed *Pik3ca* allele in both the lungs and tumors.

(B) Comparison of the change in tumor volume in *Pik3ca*^{WT/WT}, *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} mice.

(C) Quantification of tumor number and tumor size in histological sections from *Pik3ca*^{WT/flox}, *Pik3ca*^{RBD/flox}, *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} mice at 16 weeks of age.

(D) Western blot analysis of AKT phosphorylation in tumors extracted from the lungs of *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} mice 8 weeks after the commencement of tamoxifen treatment.

Error bars indicate mean \pm SEM. (Significance using Student's T test ** $p < 0.01$).

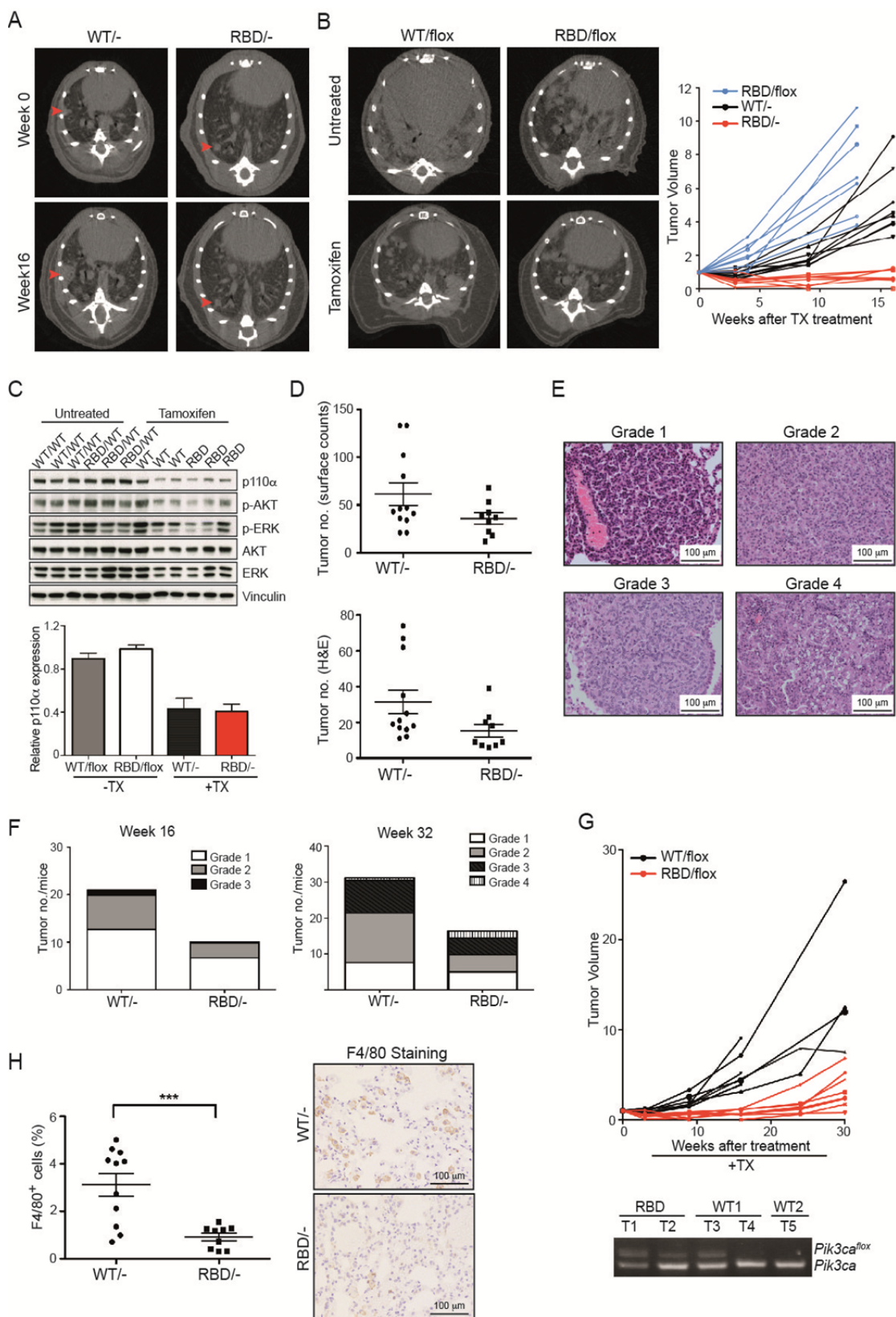


Figure S3, related to Figure 3. p110 α -RBD inhibits tumor progression.

(A) Representative transaxial images of lungs from *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} mice before tamoxifen treatment and 16 weeks later.

(B) Representative transaxial images of lungs from *Pik3ca*^{WT/flox}, *Pik3ca*^{RBD/flox}, *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} mice. Analysis of tumor growth using micro CT imaging in untreated *Pik3ca*^{RBD/flox} mice and in *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} mice is shown on the right. Some of the individual tumors in *Pik3ca*^{RBD/flox} mice could no longer be isolated from surrounding tumors after 13 weeks. Individual tumors in *Pik3ca*^{WT/flox} mice could not be accurately isolated from surrounding tumors during this experiment.

(C) Western blot analysis of p110 α , activated AKT and activated ERK levels 14 days after the start of tamoxifen treatment. Graph of p110 α expression levels is shown below, expression was quantified using ImageJ software and normalised to the loading control vinculin.

(D) Quantification of tumor multiplicity on the pleural surface and in histological sections from *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} mice 16 weeks after the commencement of tamoxifen treatment.

(E) Representative images of grade 1-4 tumors as enumerated in Figures S1, S3 and S5.

(F) Assessment of tumor grade in *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} samples taken at 16 weeks and > 32 weeks after the commencement of tamoxifen treatment.

(G) Continued analysis of tumor growth using micro CT imaging in tamoxifen treated *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} mice up to 30 weeks after the commencement of treatment. Some of the individual tumors in *Pik3ca*^{WT/-} could no longer be

isolated from surrounding tumor burden after 16 weeks. Black lines represent tumors from *Pik3ca*^{WT/-} mice and red lines represent tumors from *Pik3ca*^{RBD/-} mice. *Pik3ca*^{WT/-} n=3 animals, *Pik3ca*^{RBD/-} n=3 animals.

(H) Quantification of F4/80+ cells in lung samples from *Pik3ca*^{WT/-} and *Pik3ca*^{RBD/-} mice. Representative images are shown on the right.

Error bars indicate mean \pm SEM. (Significance using Student's T test *** p < 0.001).

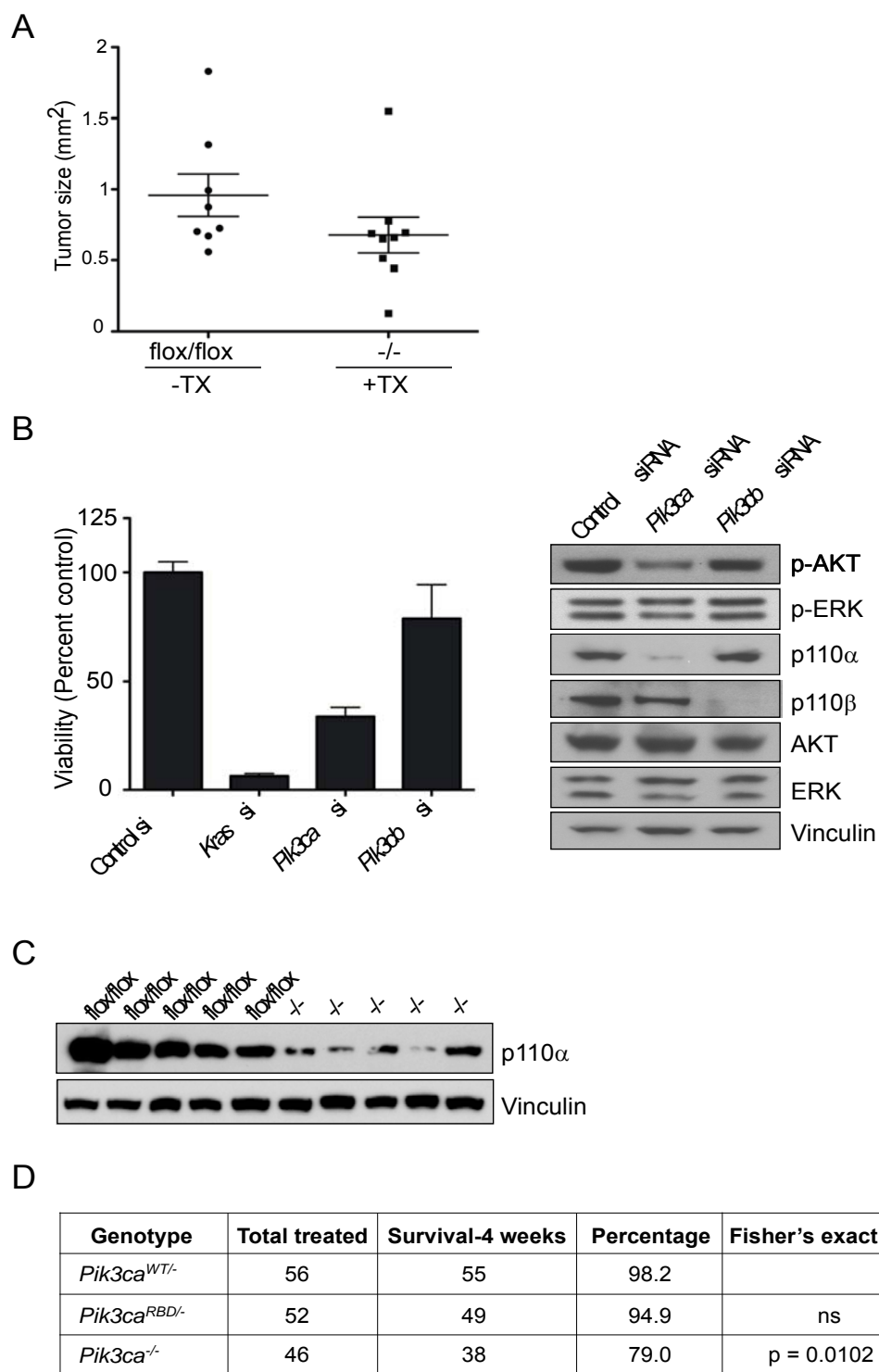


Figure S4, related to Figure 5. Complete removal of p110 α in established tumors similarly reduces tumor burden.

- (A) Quantification of tumor size in histological sections from *Pik3ca*^{flox/flox} and *p110 α* ^{-/-} mice at 16 weeks of age.
- (B) Quantification of the effect of siRNA-mediated ablation of *Kras*, *p110 α* or *p110 β* expression on proliferation of LKR13 cells using cell titre blue assay 72 hr after transfection. Western blot analysis of the effect of siRNA-mediated *p110 α* ablation on AKT phosphorylation in LKR13 cells is shown on the right.
- (C) Western blot assessment of *p110 α* loss in tumor samples 12 weeks after the commencement of tamoxifen treatment.
- (D) Survival rates of *Pik3ca*^{WT/-}, *Pik3ca*^{RBD/-} and *Pik3ca*^{-/-} mice 4 weeks after the commencement of tamoxifen treatment.

Error bars indicate mean \pm SEM.

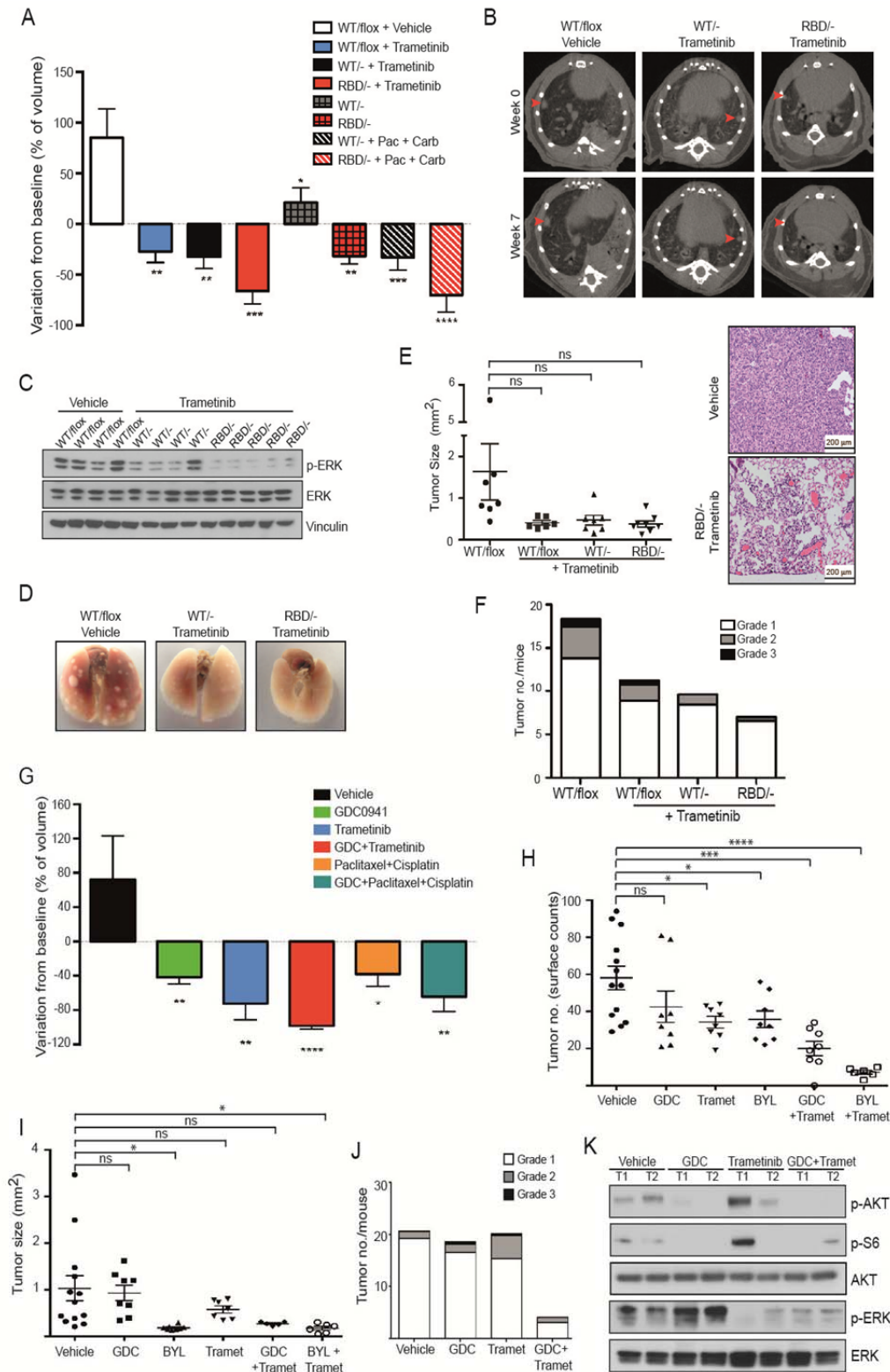


Figure S5, related to Figure 6. Combined PI 3-kinase inhibition with MEK inhibition induces tumor regression.

(A) Comparison of the change in tumor volume in mice treated as described in the graph.

(B) Representative transaxial images of lungs from vehicle treated *Pik3ca*^{WT/flox} mice, trametinib treated *Pik3ca*^{WT/-} mice or trametinib treated *Pik3ca*^{RBD/-} mice.

(C) Western blot analysis of ERK phosphorylation in lung samples 7 hr after the final trametinib treatment.

(D) Representative lung images from mice treated as described in (B).

(E) Quantification of tumor size in lung samples from vehicle treated *Pik3ca*^{WT/flox} mice, trametinib treated *Pik3ca*^{WT/flox} mice, trametinib treated *Pik3ca*^{WT/-} mice, or trametinib treated *Pik3ca*^{RBD/-} mice. Representative images of tumors from vehicle treated *Pik3ca*^{WT/flox} mice and trametinib treated *Pik3ca*^{RBD/-} mice are shown.

(F) Quantification of tumor grade in lung samples from mice treated as described in (E).

(G) Comparison of tumor volume changes observed in micro CT scans from *Kras*^{LA2} mice treated with GDC0941 and/or trametinib, carboplatin plus paclitaxel (standard of care treatment) and GDC0941 in conjunction with carboplatin plus paclitaxel.

(H) Quantification of number of tumors on the lung surface of *Kras*^{LA2} mice treated with vehicle, GDC0941 (GDC), BYL719 (BYL), trametinib and the combined GDC plus trametinib and BYL plus trametinib.

(I) Analysis of tumor size in H&E stained sections in *Kras*^{LA2} mice treated with vehicle, GDC, BYL, trametinib and the combined GDC plus trametinib and BYL plus trametinib.

(J) Analysis of tumor grade in *Kras*^{LA2} mice treated with vehicle, GDC and/or trametinib.

(K) Western blot analysis of p-ERK and p-AKT in *Kras*^{LA2} mouse lungs treated with GDC0941, trametinib, the combination of both drugs, or vehicle alone.

Error bars indicate mean \pm SEM. (Significance using Student's T test * $p < 0.05$

** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Micro CT scanning

Typically mice were scanned before tamoxifen/drug treatment and at specific intervals after tamoxifen/drug treatment. Micro CT analysis of anaesthetized animals was performed using the SkyScan 1176 micro CT scanner (Bruker micro CT). Animal temperature was maintained at 37°C and physiological monitoring was recorded. Scanning parameters: image isotropic pixel size 35 µm, source voltage 50 kV, source current 500 mA, with 0.5 mm Aluminium filter, 60 ms exposure, 0.7 degrees rotation stem over 180 degrees. Motion artefacts were corrected by retrospective gating using the RespGate software (Farncombe, 2008) before reconstruction (N-Recon program as per manufacturers' instructions SkyScan).

From reconstructed images, the tumor volume or lung (air) volume (mm³) was quantified by using CTAnalyser software (Skyscan version 1.10.11.0). Tumor volumes were calculated relative to the initial volume before tamoxifen or drug treatment. For total lung volume analysis, a region of interest was drawn around the thoracic cage and the air within this region was segmented and quantified by batch processing and produced representative 3D models of lungs. To get the relative percentage of air, regions of interest were drawn separately on the right and the left lung lobes before calculating the respective volumes as described above.

Tumor transplantation

Aged (tamoxifen) untreated mice (> 7months old) were culled and tumors were carefully isolated from the lungs. Different tumors from different mice from the same genotype were pooled. Tumors were then mechanically disaggregated and treated with dispase/collagenase (2 mg/ml) for 1hr at 37°C under constant rotation. Digested tissues were then placed on ice and 25 µg/ml DNase was added and samples incubated on ice for 5 min. Samples were then filtered through a 70 µM strainer, centrifuged for 10 seconds and supernatant was discharged. Pellets were resuspended in 200 µl of red blood cell lysis buffer (0.15 M NH₄Cl, 10mM KHCO₃, 0.1mM EDTA). After 90 seconds 800 µl of DMEM without serum were added. 200 µl of FBS was then added to the bottom of the tube and samples were centrifuged for another 10 seconds. Supernatant was removed and pellets resuspended in PBS containing 10% BSA. Cells were then stained with phycoerythrin labelled anti-CD45 and anti-CD31 antibodies (1:200 dilution) (R&D systems, Minneapolis, MN, USA). DAPI was also added (1:400). Samples were sorted and the negative unstained population was collected. Cells were then centrifuged for 4 minutes at 1300 rpm and resuspended in PBS so that we had 100000 cells per 50 µl. NuNu mice were anesthetized and a catheter (BD Insyte, BD, Franklin Lakes, NJ USA) was introduced via their trachea. 50 µl of the tumoral cell suspension was introduced through the probe directly into the lungs. Mice were left to recover and after a period of 8 weeks they were micro CT scanned every 6 weeks.

Immunohistochemical staining and analysis

For immunostaining, fixed and paraffin embedded sections were de-waxed in xylene, dehydrated by passage through graded alcohols to water. If required for antigen retrieval, sections were microwaved in citrate buffer pH 6 for 15 minutes and then transferred to PBS. Endogenous peroxidase was blocked using 1.6% hydrogen peroxide in PBS for 10 minutes followed by washing in distilled water. Normal serum diluted to 10% in 1% BSA was used to block non-specific staining in the tissue for 30 minutes. Slides were incubated with the appropriate primary antibody dilution in 1% BSA for 1 hour at room temperature. Sections were washed in PBS prior to applying secondary antibody (biotinylated) for 45 min at room temperature. Sections were then washed in PBS and then incubated in ABC (Vector Laboratories PK-6100) for 30 minutes. Following washing in PBS, DAB solution was applied for 2-5 minutes with development of the colour reaction being monitored microscopically. Slides were washed in tap water, stained with a light haematoxylin, dehydrated, cleared and then mounted. For analysis of F4/80 immunostaining, samples were scanned using an Ariol microscope (Leica Biosystems) and the number of positive cells in each lung sample was determined using Ariol image analysis software. For quantification of cell death in tumors, TUNEL staining was carried out on lung samples. The number of TUNEL positive cells per tumor was quantified and tumors with a score ≥ 2 were designated TUNEL positive.

Glucose Tolerance Tests

Kras^{WT} mice were treated with tamoxifen at 14 weeks of age and glucose tolerance tests were carried out 5 weeks after the commencement of treatment. Mice were fasted overnight and were then injected with glucose (1g/kg). Blood samples were taken prior to injection and at 15, 30, 90 and 120 mins after injection. Blood glucose levels were measured using an Accu-Chek Mobile blood glucose monitor.

siRNA transfection

LKR13 cells grown in DMEM/10% FCS were reverse transfected with 50nM Dharmacon siRNA SMARTpools on a 96-well plate scale using DharmaFECT transfection reagent as per manufacturers instructions. After 72 hr cell viability was assessed by incubation with Cell Titre Blue Reagent (Promega) for 1.5 hr and fluorescence was measured on an Envision plate reader (Perkin-Elmer). Alternatively, lysates were prepared and ERK and AKT phosphorylation was determined using Western Immunoblotting.

SUPPLEMENTAL REFERENCE

Farncombe, T. H. (2008). Software-based respiratory gating for small animal conebeam CT. *Med Phys* 35, 1785-1792.