Supplemental figure legends

Figure S1. Variable expression of the three AKT isoforms in human glioblastomas. Heatmap showing gene expression of AKT1, 2 and 3 in 189 human glioblastomas. Tumors with genomic amplification of EGFR are indicated by black bars under the heatmap. Gene expression data from 189 TCGA glioblastoma samples that also had available genomic SNP6 array data available was downloaded from the TCGA data portal (http://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp). The heatmap was generated using GeneMaths, with array normalization using all of the genes on the array. The samples with *EGFR* amplification were previously identified (Table S8 from ref (2)).

Figure S2. *Pten* deletion and EGFRvIII expression accelerated PMA proliferation. Growth during serial passage of early passage PMAs from $Pten^{cKO}$ mice (A) or $Pten^{cKO};p53^{cKO}$ mice (B) alone or with expression of EGFRvIII (C and D). Expression of EGFRvIII further accelerates proliferation of PMAs established from all genotypes analyzed, whereas (E) knock-down of Akt3 did not alter PMA proliferation. Shown are mean cumulative population doublings ± SEM with the number (n) of individual cultures for each group shown in brackets.

Figure S3. Deletion of *Akt1* did not rescue neuronal hypertrophy induced by Pten deletion. Mice deficient for *Akt1* were bred with $Pten^{cKO}$ mice where cre recombinase directed *Pten* deletion in granule neurons of the dentate gyrus. (A) Paraffin sections of dentate gyrus granule neurons were stained using IHC for Pten (brown) and nuclei were counterstained using hematoxylin (blue) from wild-type (WT), $Akt1^{KO}$, $Pten^{cKO}$ or $Pten^{cKO};Akt1^{KO}$ mice. Note that the increased size of *Pten* negative neurons (lower panels) is not altered upon deletion of *Akt1* (lower right). (B) The nuclear size of *Pten* wildtype or conditional knockout (cKO) granule neurons of the dentate gyrus, from *Akt1* WT or KO brains, was measured using Bioquant image analysis software. Shown is the mean ± SD of 65 cells from seven mice per group for $Pten^{cKO}$ and four mice per group for wild-type mice.

Figure S4. Deletion of *Pten* and *p53* with EGFRvIII expression was required for anchorage independent growth of astrocytes. Early passage PMAs were cultured from $Pten^{WT}$ or $Pten^{cKO}$ with or without *p53* deletion and transduced with control (GFP) or EGFRvIII expressing retrovirus. Cells were plated in soft agar and incubated for 10 days

before colonies were photographed. Shown are overlays of phase contrast and GFP fluorescence images. Scale bar indicates 50μm.

Figure S5. Akt1 and Akt2 were not required for anchorage independent growth of transformed PMAs. Early passage PMAs from *Pten^{cKO};p53^{cKO}* mice were cultured and transduced with retrovirus to express EGFRvIII before plating in soft agar. (A) Overlays of representative phase contrast and GFP fluorescence of colonies from *Pten^{cKO};p53^{cKO}*;EGFRvIII PMAs that were *Akt1^{WT}*, *Akt1^{KO}* alone or *Akt1^{KO}* transduced with control (pLKO) or Akt2-specific shRNA-expressing lentivirus. Scale bar represents 50 μm. (B) Quantification of (A). Colonies greater than 50μm were counted after 13-14 days. Graphed is the mean ± SEM from three independently isolated cultures for each group.

Figure S6. Deletion of *Akt1* did not alter proliferation of *Pten* wild-type tumors. (A) Immunohistochemistry for Ki67 (brown) on tumors resulting from intracranial implantation of *p53^{cKO};EGFRvIII* PMAs that were wild-type (WT) or knockout (KO) for *Akt1*. Slides were counterstained with hematoxylin (blue). (B) The percentage of Ki67 positive cells was determined by analysis of four representative fields of view from three individual tumors for each group. Shown is the mean ± SEM.

Figure S7. Akt2 or Akt3 knock-down decreased phospho-Akt levels *in vivo*. Intracranial tumors derived from PMAs were lysed and analyzed by western blot for the indicated proteins. Shown are representative tumors from *p53^{cKO};EGFRvIII* or *Pten^{cKO};p53^{cKO};EGFRvIII* astrocytes that were (A) wild-type or knockout for *Akt1*, infected with control (pLKO) or lentivirus expressing either (B) Akt2-specific or (C) Akt3-specific shRNA. Note that phosphorylated Akt (S473 and T308) is not significantly different between Akt1 wild-type or knockout tumors, although it is elevated in Pten-deficient tumors as expected, however phospho-Akt S473 levels are decreased in Akt2 and Akt3 knockdown tumors regardless of Pten expression. The presence of Pten protein in the *Pten*-deficient tumors illustrates contamination of normal brain in the tissue samples and reflects the invasive nature of these tumors. Similarly the Akt1 signal in the *Akt1* knockout tumors is due to contamination of normal brain from the recipient nude mice. Immunoblots in (B) are from the same gel and autoradiography film exposure but several lanes were removed for clarity.

Figure S1

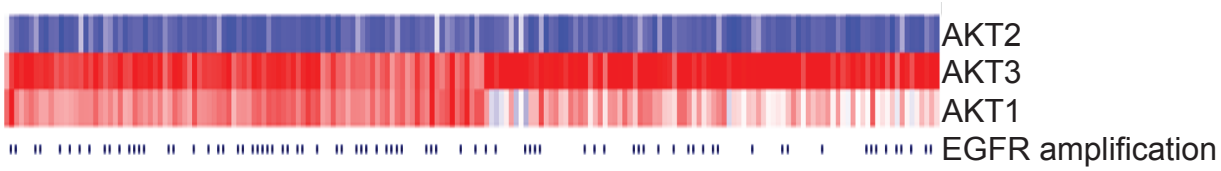


Figure S2

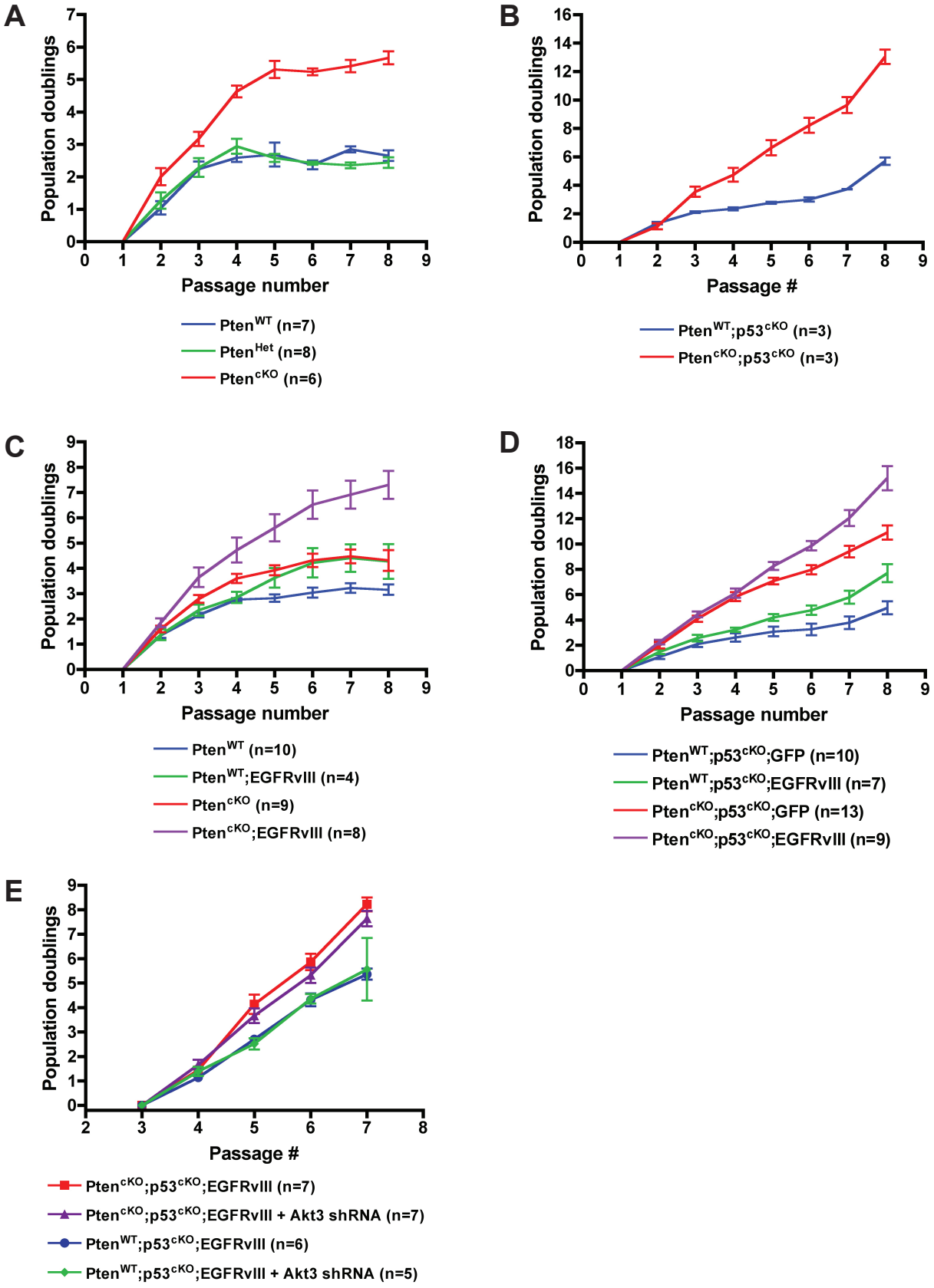
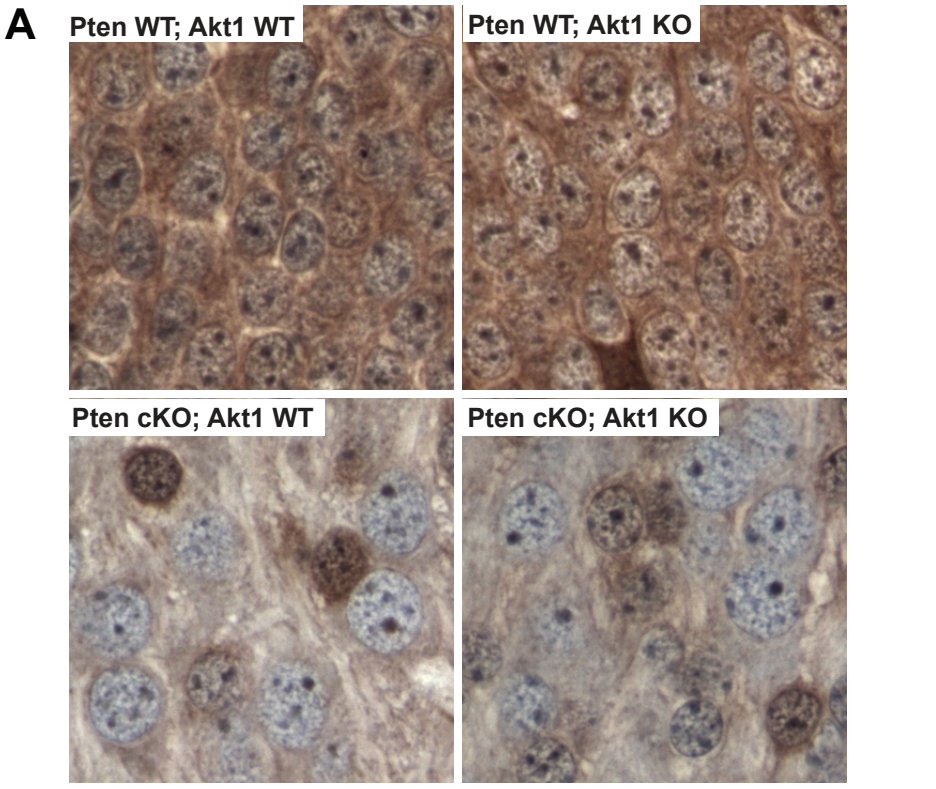

A
Population doublings
Passage number
Pten^WT (n=7)
Pten^Het (n=8)
Pten^cKO (n=6)
B
Population doublings
Passage #
Pten^WT;p53^cKO (n=3)
Pten^cKO;p53^cKO (n=3)
C
Population doublings
Passage number
Pten^WT (n=10)
Pten^WT;EGFRvIII (n=4)
Pten^cKO (n=9)
Pten^cKO;EGFRvIII (n=8)
D
Population doublings
Passage number
Pten^WT;p53^cKO;GFP (n=10)
Pten^WT;p53^cKO;EGFRvIII (n=7)
Pten^cKO;p53^cKO;GFP (n=13)
Pten^cKO;p53^cKO;EGFRvIII (n=9)
E
Population doublings
Passage #
Pten^cKO;p53^cKO;EGFRvIII (n=7)
Pten^cKO;p53^cKO;EGFRvIII + Akt3 shRNA (n=7)
Pten^WT;p53^cKO;EGFRvIII (n=6)
Pten^WT;p53^cKO;EGFRvIII + Akt3 shRNA (n=5)

Figure S3


A
Pten WT; Akt1 WT
Pten WT; Akt1 KO
Pten cKO; Akt1 WT
Pten cKO; Akt1 KO

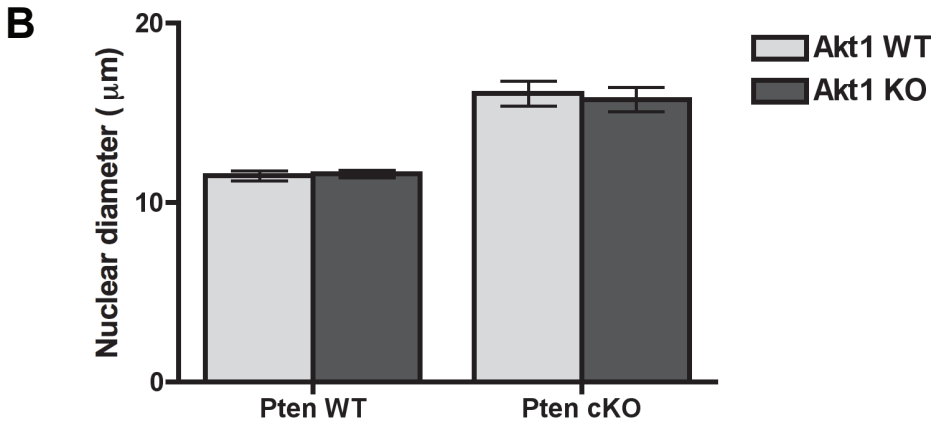

B
Nuclear diameter (μm)
20
10
0
Pten WT
Pten cKO
Akt1 WT
Akt1 KO

Figure S4

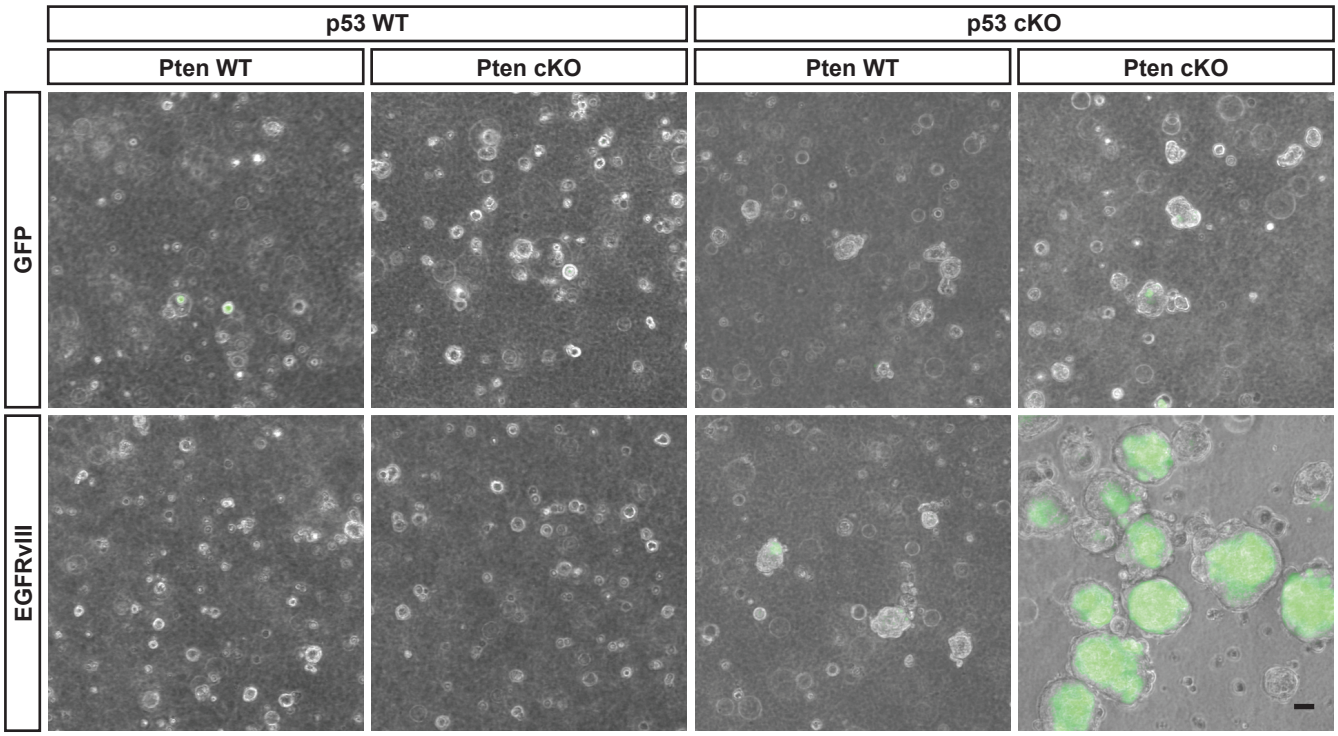
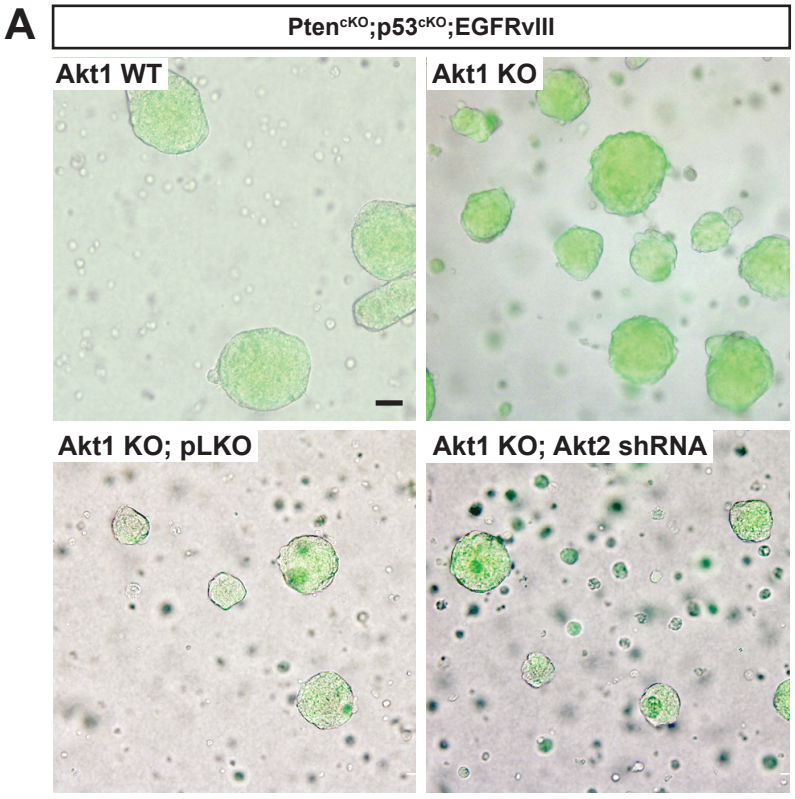


Figure S5


A
Pten^cKO;p53^cKO;EGFRvIII
Akt1 WT
Akt1 KO
Akt1 KO; pLKO
Akt1 KO; Akt2 shRNA

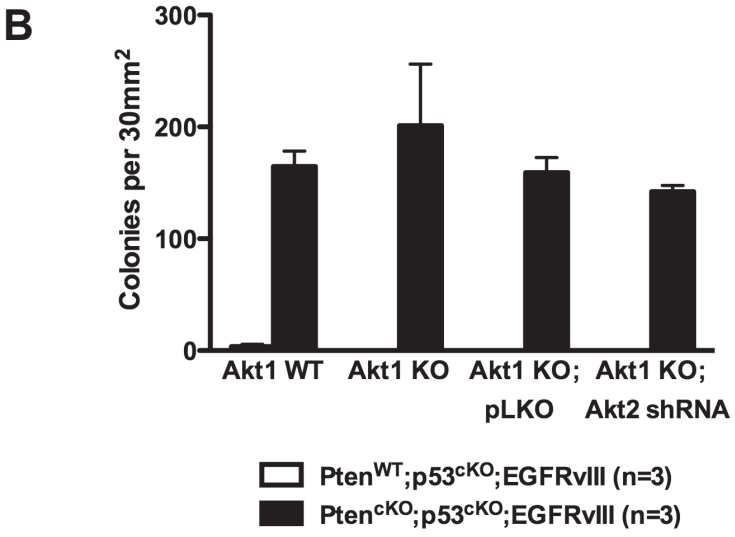
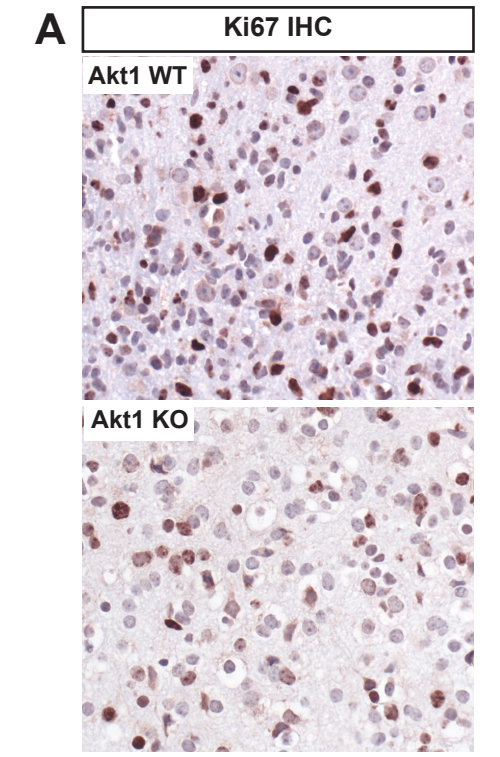

B
Colonies per 30mm2
300
200
100
0
Akt1 WT
Akt1 KO
Akt1 KO; pLKO
Akt1 KO; Akt2 shRNA
Pten^WT;p53^cKO;EGFRvIII (n=3)
Pten^cKO;p53^cKO;EGFRvIII (n=3)

Figure S6


A
Ki67 IHC
Akt1 WT
Akt1 KO

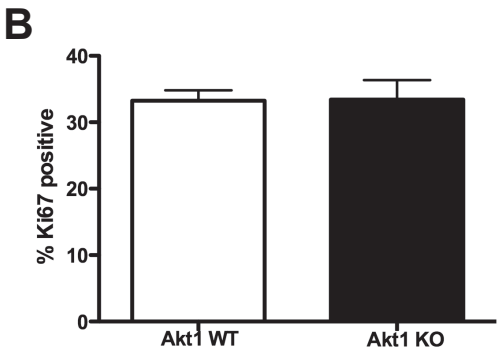
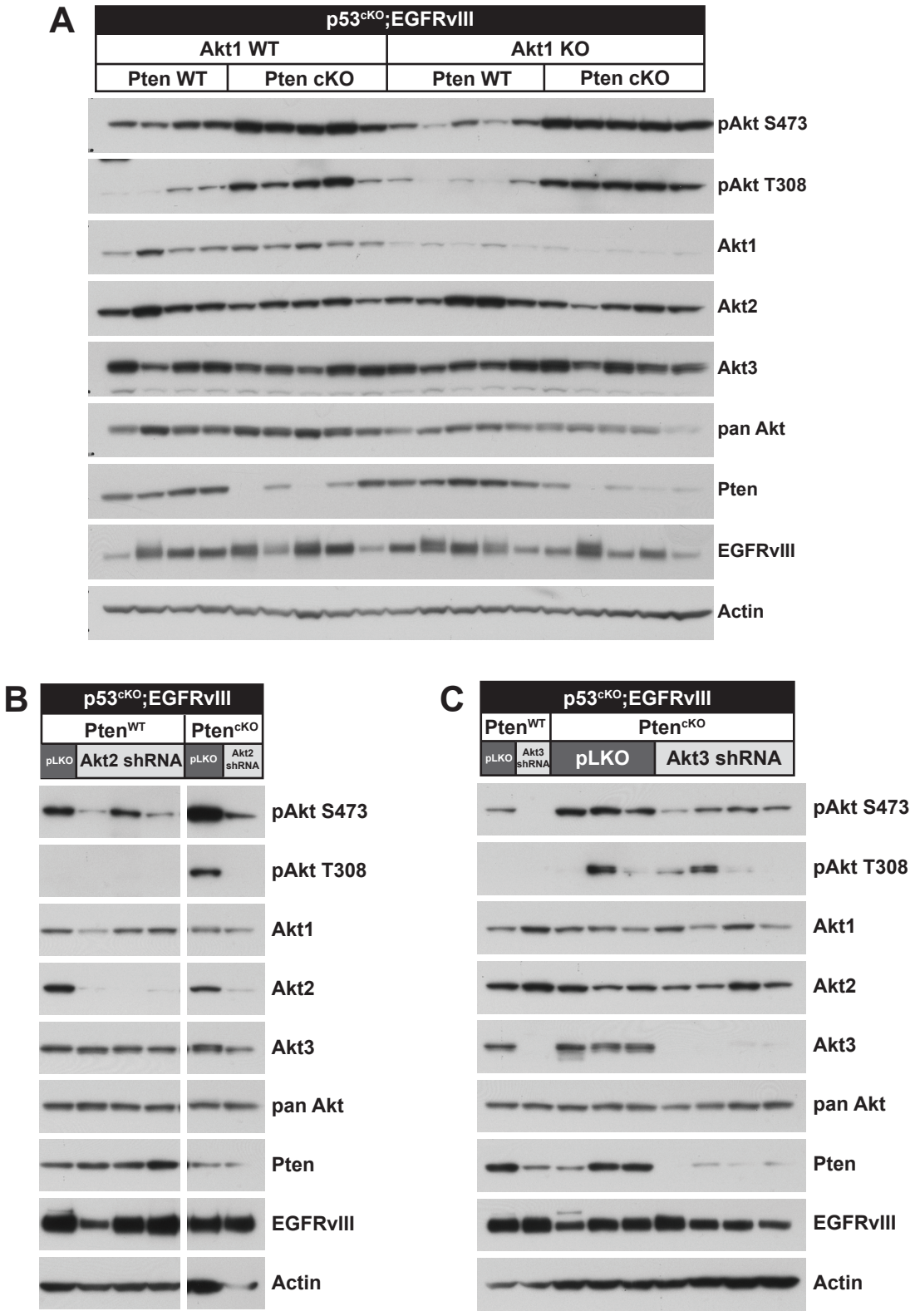

B
% Ki67 positive
40
30
20
10
0
Akt1 WT
Akt1 KO

Figure S7


A
p53cKO;EGFRvIII
Akt1 WT
Akt1 KO
Pten WT
Pten cKO
Pten WT
Pten cKO
pAkt S473
pAkt T308
Akt1
Akt2
Akt3
pan Akt
Pten
EGFRvIII
Actin
B
p53cKO;EGFRvIII
PtenWT
PtencKO
pLKO
Akt2 shRNA
pLKO
Akt2 shRNA
pAkt S473
pAkt T308
Akt1
Akt2
Akt3
pan Akt
Pten
EGFRvIII
Actin
C
p53cKO;EGFRvIII
PtenWT
PtencKO
pLKO
Akt3 shRNA
pLKO
Akt3 shRNA
pAkt S473
pAkt T308
Akt1
Akt2
Akt3
pan Akt
Pten
EGFRvIII
Actin

T**able S1. Summary of the tumors resulting from intracranial implantation of PMAs.** Indicated are the *Pten* and *p53* genotypes for each culture and if the cells were infected with EGFRvIII-expressing retrovirus. Indicated are the numbers of tumors obtained from the number of mice implanted. Median survival of recipient mice is listed as days following implantation of 10^5 cells per mouse.

Genotype	**Tumors / number of mice**	**Median latency (days)**
Pten^{WT};EGFRvIII	0/3	-
Pten^{WT};p53^{cKO}	0/5	-
Pten^{WT};p53^{cKO};EGFRvIII	34/34	47.5
Pten^{cKO};EGFRvIII	0/6	-
Pten^{cKO};p53^{cKO}	3/6	216
Pten^{cKO};p53^{cKO};EGFRvIII	33/33	26

Supplemental methods

Primers

For amplification of EGFRvIII:

Forward: ATGTCGACACCATGCGACCCTCCGGGACG

Reverse: TATGCGGCCGCGTCATGCTCCAATAAATTCACTGCTT

For amplification of Akt1:

Forward: TATAGTCGACCATGAACGACGTAGCCATTGTG

Reverse: TTTTGTCGACTCAGGCTGTGCCACTGGC

For amplification of Akt3:

Forward: AGAATTCACCATGAGCGATGTTACCATTGTG

Reverse: TCAGATCTCATTCCCGTCCGCTTGCC

For mutation of Akt3 (K177A):

Forward:

GCAAGTGGAAAATACTATGCTATGGCCATTCTGAAGAAAGAAGTCATTATTG

Reverse:

CAATAATGACTTCTTTCTTCAGAATGGCCATAGCATAGTATTTTCCACTTGC

For mutation of Akt3-specific shRNA binding sequence (Akt3 and K177A):

Forward: CGCCCAGACCATCACCATCACACCACCTGAAAAGTATG

Reverse: GATGGTGATGGTCTGGGCGGTAAATTCTTCATCAAAATATC