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Supplemental Information

c-RAF Ablation Induces Regression of Advanced *Kras*/*Trp*53 Mutant Lung Adenocarcinomas by a Mechanism Independent of MAPK Signaling

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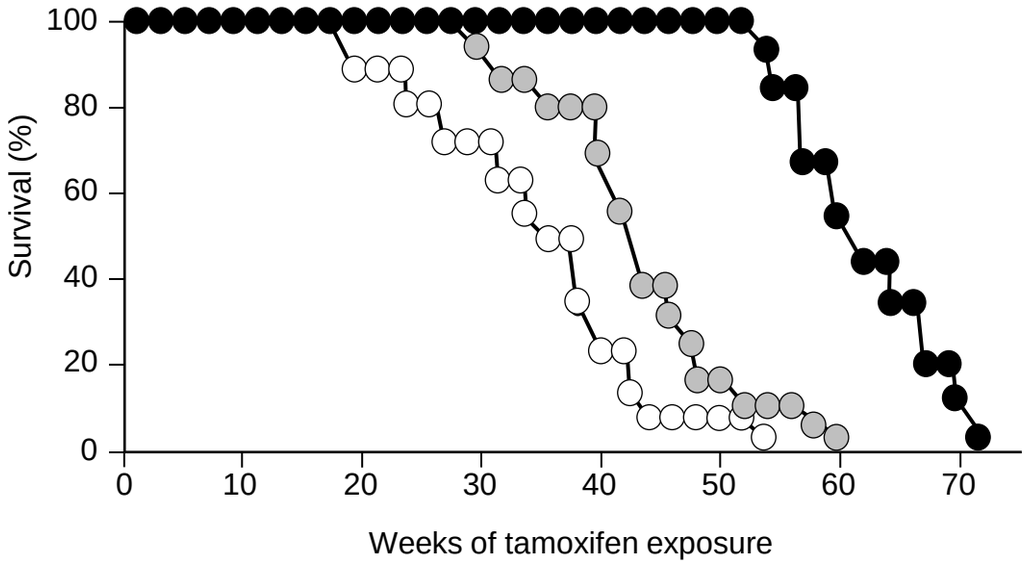
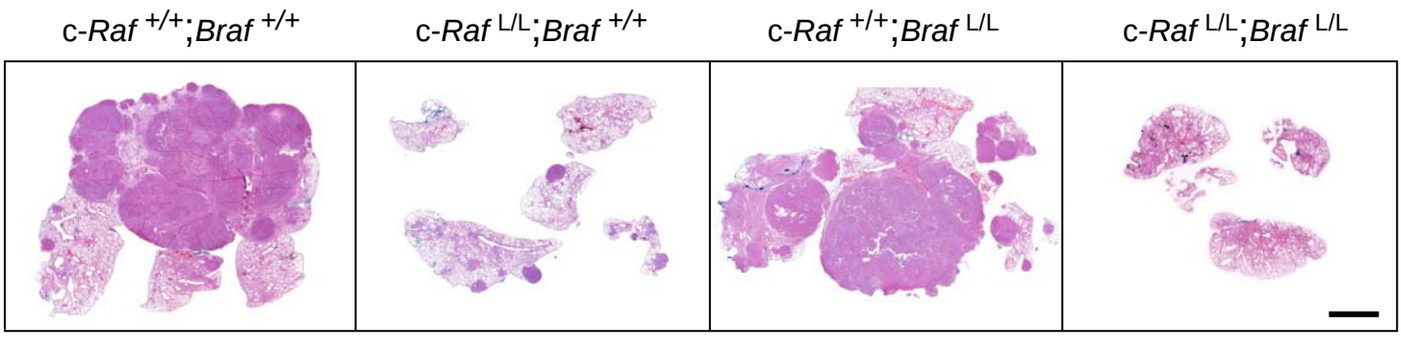
A


100
80
60
40
20
0
Survival (%)
0
10
20
30
40
50
60
70
Weeks of tamoxifen exposure

B


c-Raf +/+;Braf +/+
c-Raf L/L;Braf +/+
c-Raf +/+;Braf L/L
c-Raf L/L;Braf L/L

C

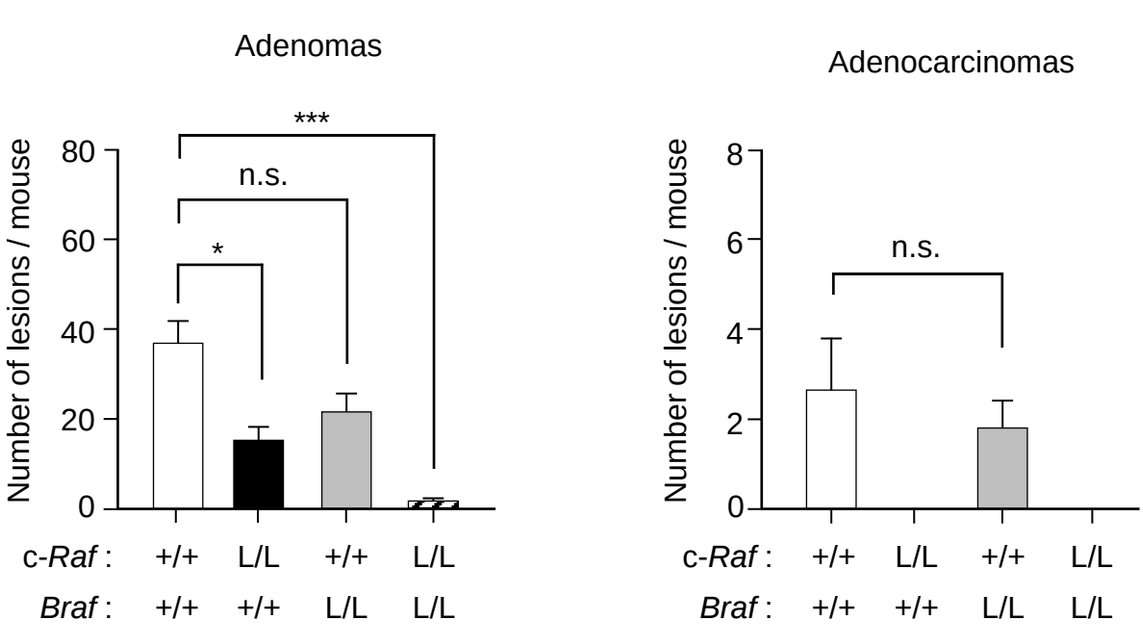

Adenomas
Number of lesions / mouse
80
60
40
20
0
***
n.s.
*
c-Raf : +/+ L/L +/+ L/L
Braf : +/+ +/+ L/L L/L
Adenocarcinomas
Number of lesions / mouse
8
6
4
2
0
n.s.
c-Raf : +/+ L/L +/+ L/L
Braf : +/+ +/+ L/L L/L

Figure S1. c-RAF ablation prevents progression of established *Kras*^{G12V} driven lung tumors. Related to Figure 3.

(**A**) Survival of *Kras*$^{+/FSFG12V}$;*RERT*$^{ert/ert}$ (n=18) (open circles), *Kras*$^{+/FSFG12V}$;*RERT*$^{ert/ert}$;*Braf*$^{L/L}$ (n=29) (solid grey circles), *Kras*$^{+/FSFG12V}$;*RERT*$^{ert/ert}$;c-*Raf*$^{L/L}$ (n=20) (solid black circles) CT^{+} tumor-bearing mice exposed to TMX for the indicated time. Mice were sacrificed at humane end point.

(**B**) H&E staining of paraffin sections of whole lungs obtained from representative *Kras*$^{+/FSFG12V}$;*RERT*$^{ert/ert}$;c-*Raf*$^{+/+}$;*Braf*$^{+/+}$, *Kras*$^{+/FSFG12V}$;*RERT*$^{ert/ert}$;c-*Raf*$^{L/L}$;*Braf*$^{+/+}$, *Kras*$^{+/FSFG12V}$;*RERT*$^{ert/ert}$;c-*Raf*$^{+/+}$;*Braf*$^{L/L}$ and *Kras*$^{+/FSFG12V}$;*RERT*$^{ert/ert}$;c-*Raf*$^{L/L}$;*Braf*$^{L/L}$ tumor bearing mice after 4 months of TMX exposure and sacrificed at humane end point. Scale bar, 5 mm.

(**C**) (Left) Quantification of number of adenomas and (Right) adenocarcinomas per mouse in *Kras*$^{+/FSFG12V}$;*RERT*$^{ert/ert}$;c-*Raf*$^{+/+}$;*Braf*$^{+/+}$ (open bar), *Kras*$^{+/FSFG12V}$;*RERT*$^{ert/ert}$;c-*Raf*$^{L/L}$;*Braf*$^{+/+}$ (solid black bar), *Kras*$^{+/FSFG12V}$;*RERT*$^{ert/ert}$;c-*Raf*$^{+/+}$;*Braf*$^{L/L}$ (solid grey bar) and *Kras*$^{+/FSFG12V}$;*RERT*$^{ert/ert}$;c-*Raf*$^{L/L}$;*Braf*$^{L/L}$ (stripped bar) tumor-bearing mice (n=6 per genotype) after 4 months of TMX exposure. Error bars indicate mean ± SEM. p values were calculated using the unpaired Student´s T test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$. n.s.: not significant

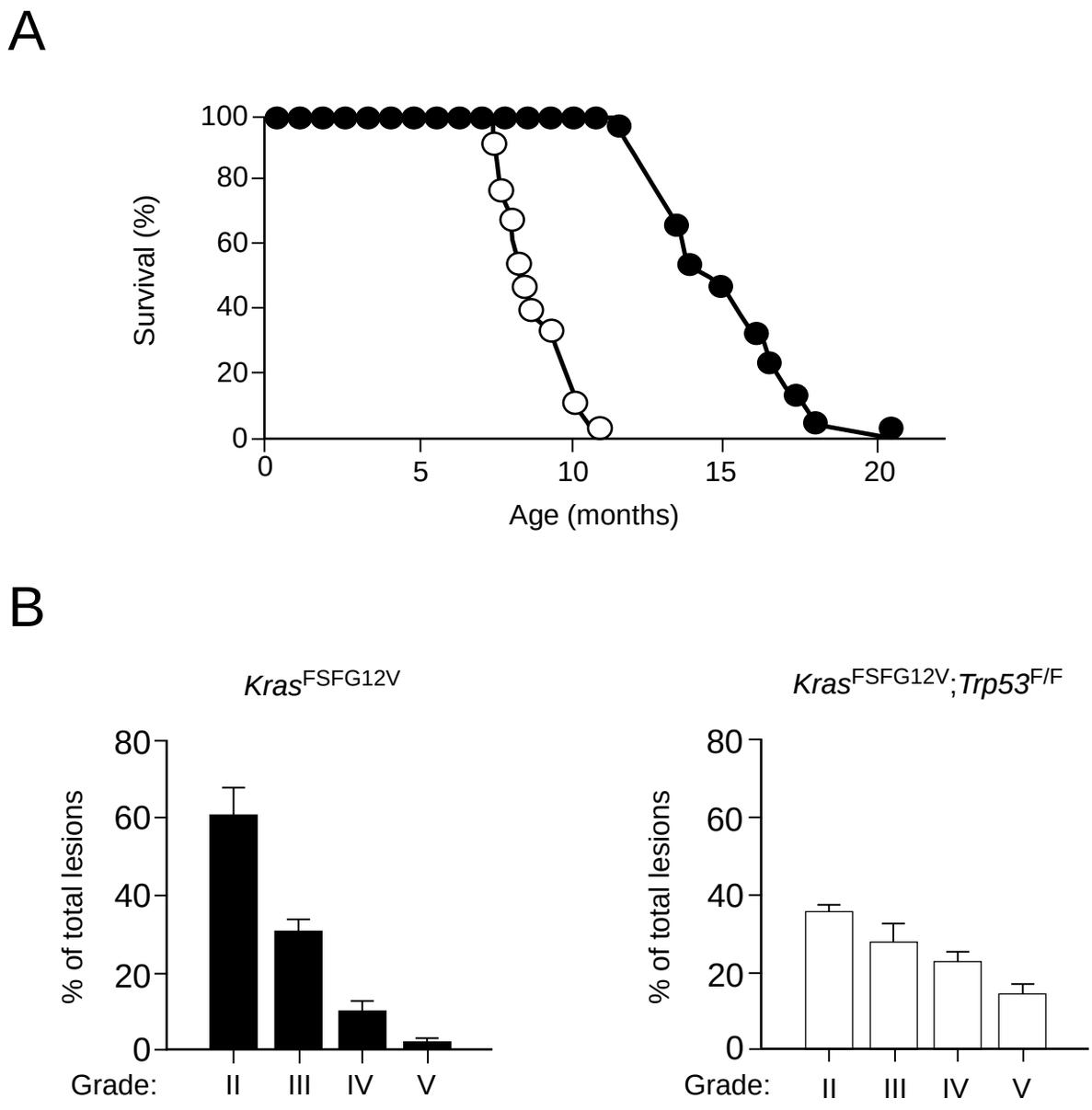
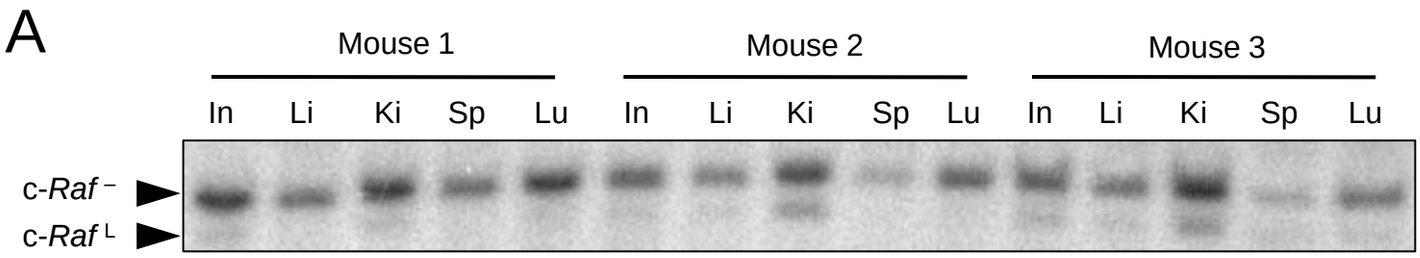
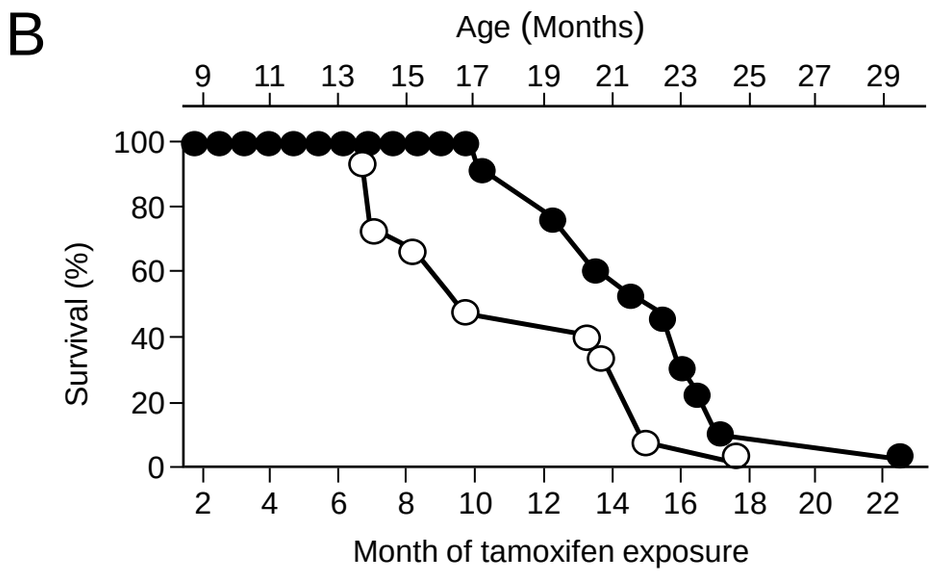


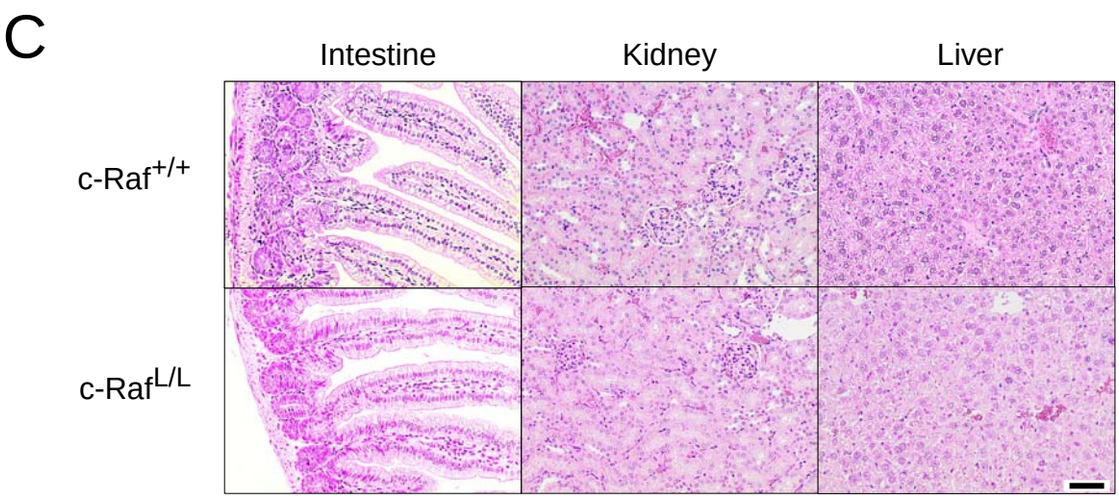
Figure S2. Survival and tumor grade in *Kras*$^{+/FSFG12V}$ and *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$ mice upon infection with Ad-Flp particles. Related to Figure 3.

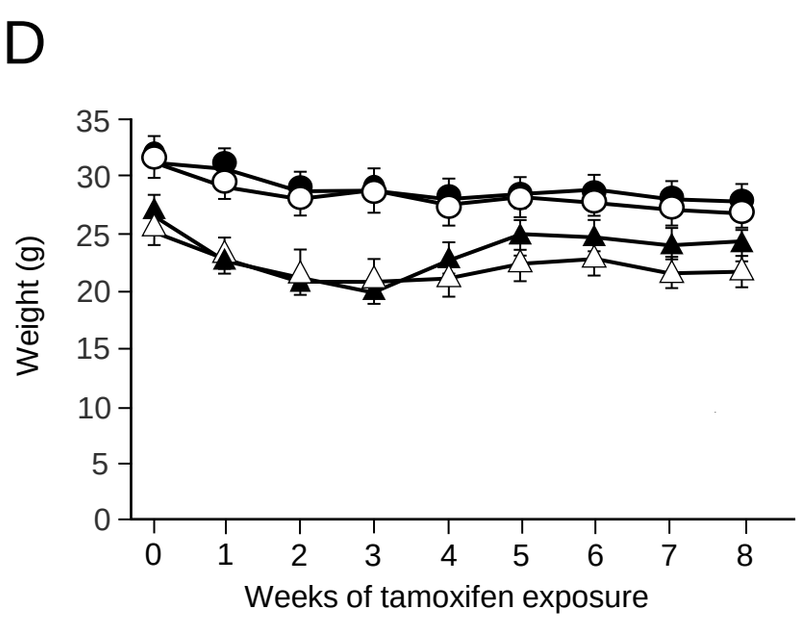
(**A**) Survival of *Kras*$^{+/FSFG12V}$ (solid circles) (n=26) and *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$ (open circles) (n=21) infected with 10^6 pfu of Ad-Flp/mouse at 2 months of age.

(**B**) Grading of tumors present in (Left) *Kras*$^{+/FSFG12V}$ (solid bars) and (Right) *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$ (open bars) mice (n=6 per genotype) infected as indicated in (a) and sacrificed at humane end point. Results are indicated as percentage of grade II to V lesions. Error bars indicate mean ± SEM.


A
Mouse 1
Mouse 2
Mouse 3
In Li Ki Sp Lu In Li Ki Sp Lu In Li Ki Sp Lu
c-Raf –
c-Raf L


B
Age (Months)
9 11 13 15 17 19 21 23 25 27 29
100
80
60
40
20
0
Survival (%)
2 4 6 8 10 12 14 16 18 20 22
Month of tamoxifen exposure


C
Intestine
Kidney
Liver
c-Raf+/+
c-RafL/L


D
35
30
25
20
15
10
5
0
Weight (g)
0 1 2 3 4 5 6 7 8
Weeks of tamoxifen exposure


E
c-Raf +/+
c-Raf L/L

Figure S3. c-RAF systemic ablation results in acceptable toxicity. Related to Figure 3.

(**A**) Southern blot analysis of *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{L/L}$ tissues after 2 months of TMX exposure (n=3 mice). Migration of the diagnostic DNA fragments corresponding to the un-recombined c-*Raf*^{L} (3.1 kbp) alleles as well as to the recombined c-*Raf*$^{-}$ (3.5 kbp) alleles are indicated by arrowheads. I: intestine, L: liver, K: kidney, S: spleen, Lu: lung.

(**B**) Survival of *Kras*$^{+/+}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{+/+}$ (solid circles) (n=13) and *Kras*$^{+/+}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{L/L}$ (open circles) (n=15) mice exposed to TMX *ad libitum*. Upper scale shows the animal age in months. Downer scale shows the time in months of TMX exposure.

(**C**) Representative photos of H&E stained histological sections of paraffin embedded organs of *Kras*$^{+/+}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{+/+}$ (up) and *Kras*$^{+/+}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{L/L}$ (down) mice at humane end point after continuous exposure to TMX. Scale bar, 0.05 mm.

(**D**) Body weight change in grams (g) of lung tumor-bearing male mice (circles) and female mice (triangles) exposed to TMX for 2 months. *Kras*$^{+/LSLG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{+/+}$ (solid circles (n=11) and solid triangles (n=9)) and *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{L/L}$ (open circles (n=13) and open triangles (n=14)). Error bars indicate mean ± SEM.

(**E**) Representative photos of tumor-bearing *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{+/+}$ (Left) and *Kras*$^{+/LSLG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{L/L}$ (Right) mice after 2 months of TMX exposure.

Fur loss in *Kras*$^{+/LSLG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{L/L}$ mouse is caused by barbering behavior, a normal phenomenon observed in mice.

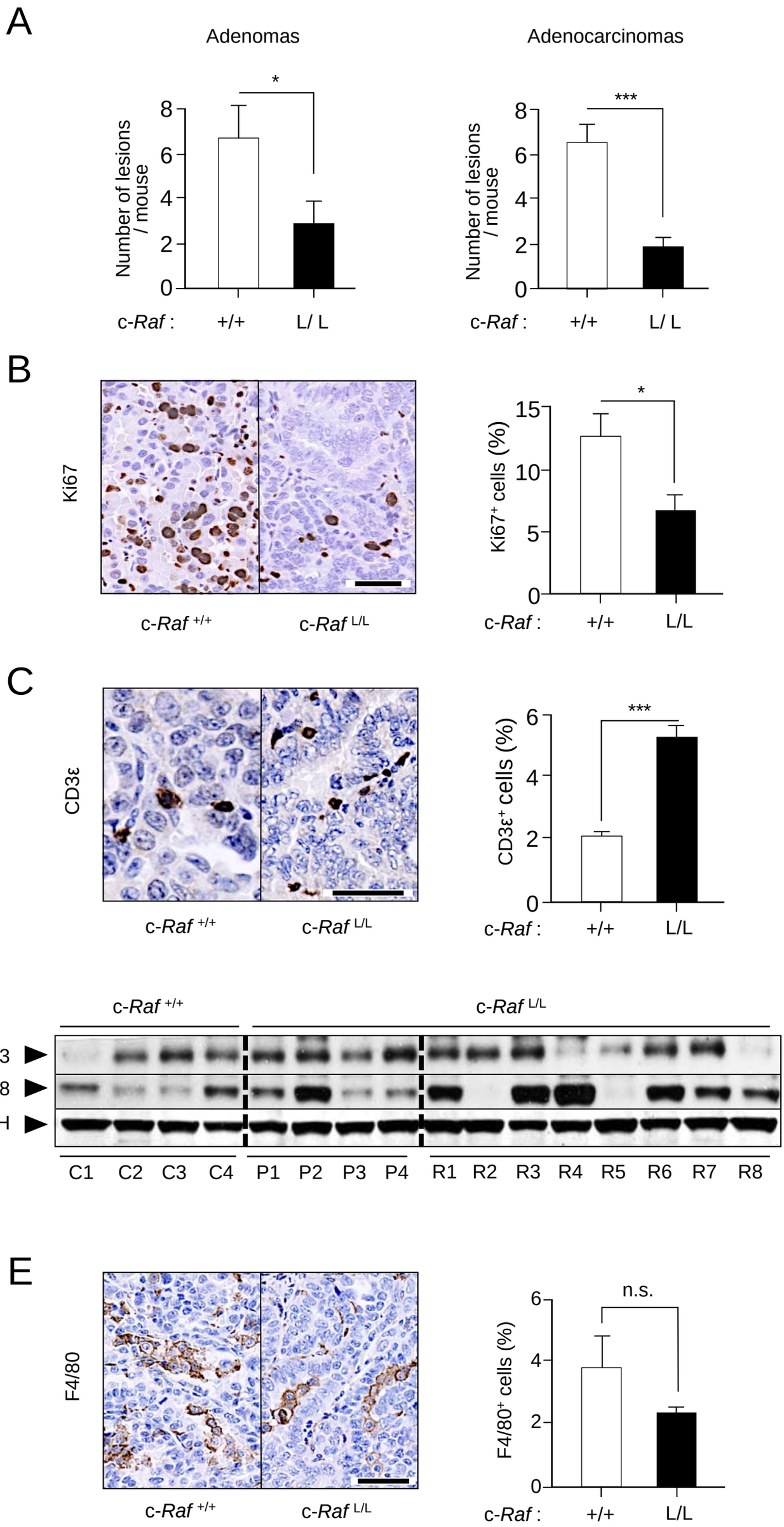

A
Adenomas
Adenocarcinomas
Number of lesions / mouse
*
***
c-Raf :
+/+
L/ L
B
Ki67
Ki67+ cells (%)
c-Raf +/+
c-Raf L/L
L/L
C
CD3ε
CD3ε+ cells (%)
D
CD3
CD8
GAPDH
C1 C2 C3 C4 P1 P2 P3 P4 R1 R2 R3 R4 R5 R6 R7 R8
E
F4/80
F4/80+ cells (%)
n.s.

Figure S4. c-RAF ablation prevents progression of established *Kras*^{G12V}/*Trp53* mutant lung tumors. Related to Figure 3.

(**A**) (Left) Quantification of number of adenomas and (Right) adenocarcinomas per mouse in *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{+/+}$ (open bar) and *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{L/L}$ tumor-bearing mice (n=6 per genotype) after 2 months of TMX exposure.

(B) (Left) Ki67 staining and (Right) quantification of Ki67^{+} cells in representative sections of paraffin embedded tumors of *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{+/+}$ (+/+, open bar) and *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{L/L}$ (L/L, solid bar) mice (n=6 per genotype) treated with TMX for 2 months. Scale bar, 0.1 mm.

(**C**) (Left) CD3ε staining and (Right) quantification of CD3ε cells in representative sections of paraffin embedded tumors of *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{+/+}$ (+/+, open bar) and *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{L/L}$ (L/L, solid bar) mice (n=6 per genotype) after 2 months of TMX exposure. Scale bar, 0.05 mm.

(**D**) Western blot analysis of CD3ε, and CD8, expression in lysates derived from individual tumors (C1 to C4) of control *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{+/+}$ mice and of *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$; h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{L/L}$ mice exposed to TMX for 2 months. Progressor (P1-P4) and regressor (R1-R8) tumors analyzed are indicated. GAPDH was used as loading control. Migration of the above proteins is indicated by arrowheads.

(**E**) (Left) F4/80 staining and (Right) quantification of F4/80^{+} cells in representative sections of paraffin embedded tumors of *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{+/+}$ (+/+, open bar) and *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{L/L}$ (L/L, solid bar) mice (n=6 per genotype) after 2 months of TMX exposure. Scale bar, 0.05 mm.

Error bars indicate mean ± SEM. p values were calculated using the unpaired Student´s T test. *p < 0.05 and ***p < 0.001. n.s.: not significant.

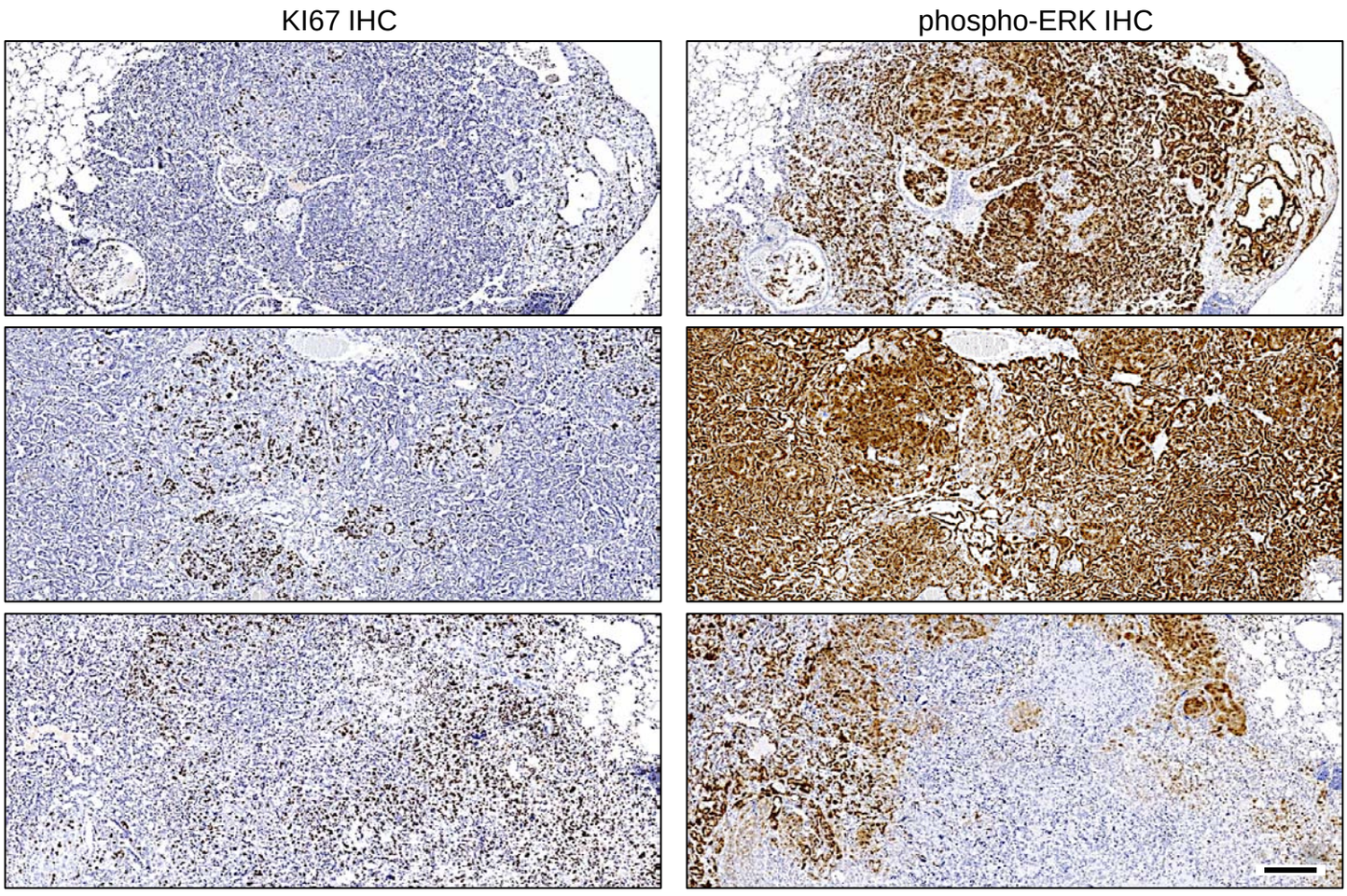


Figure S5. Phospho-ERK expression does not correlate with proliferation in *Kras*^{G12V}/*Trp53* mutant tumors after ablation of c-RAF. Related to Figure 4.

(Left) Ki67 staining and (Right) phospho-ERK staining in 3 representative sections of paraffin embedded *Kras*$^{+/FSFG12V}$;*Trp53*$^{F/F}$;h*UBC-CreERT2*$^{+/T}$;c-*Raf*$^{L/L}$ tumors form 3 independent animals at 2 month of TMX exposure. Scale bar, 0.2 mm.

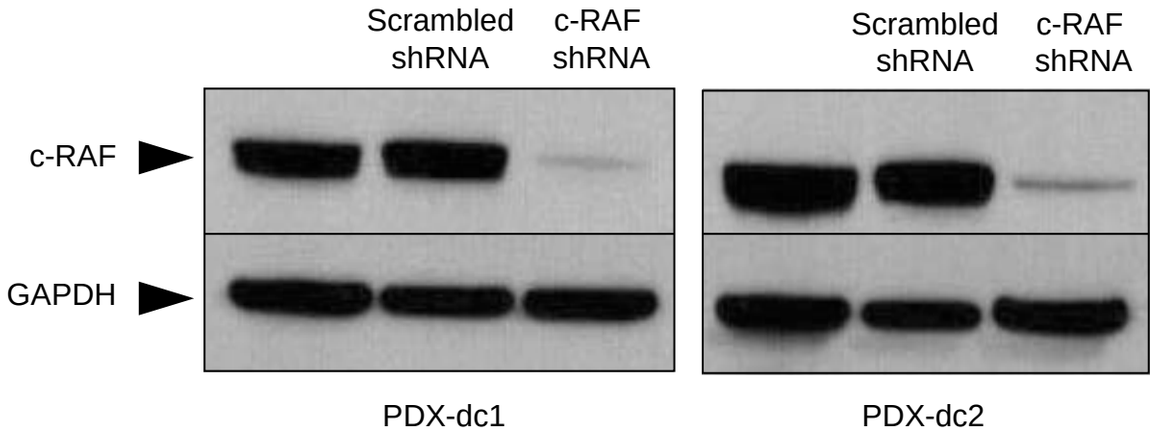


Figure S6. Effective c-RAF down-regulation in human tumor cells. Related to Figure 6.
Western blot analysis of c-RAF in 2 PDX-derived cell lines (PDX-dc1 and PDX-dc2) not infected and infected with lentiviral supernatants expressing a Scrambled shRNA, as control, and a shRNA against c-RAF at 2 weeks after the selection. GAPDH was used as loading control. Migration of the above mentioned proteins are indicated by arrowheads.