SUPPLEMENTARY INFORMATION

Differential effects of oncogenic K-Ras and N-Ras on proliferation, differentiation, and tumor progression in the colon

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Supplementary Notes

Expression of *Kras* and *Nras* in human colon crypts

In Fig. 1 we analyzed the expression of *Kras* and *Nras* along the length of the murine colonic crypt by performing Taqman analysis on cDNA derived from laser captured frozen sections. We also performed this experiment on human tissue, but instead used formalin-fixed, paraffin-embedded tissue as the source of the RNA (Supplementary Methods). The expression patterns of *Kras* and *Nras* were similar in human and mouse (Supplementary Fig. 1a).

Generation of *Nras*$^{LSL\text{-}G12D}$ mice

To study oncogenic N-Ras *in vivo*, we generated a Cre-dependent conditional allele by gene targeting in V26.2 murine embryonic stem (ES) cells derived from the C57BL/6 strain (Supplementary Fig. 1b). In the absence of Cre recombinase, the insertion of a floxed stop element (LSL) into the first intron constitutes a null allele. Upon expression of Cre, the stop element is excised and the activated allele of N-Ras (N-Ras^{G12D}) is expressed (Fig. 1). Because the mutant allele is expressed from its endogenous promoter, N-Ras^{G12D} is expressed at the same level and in the same pattern as the wild-type. Primers used for genotyping all mouse strains in this study are listed in Supplementary Table 1.

Activation of K-Ras in adults

In Fig. 1, we showed that expression of activated K-Ras in the intestinal epithelium produced chronic hyperplasia. In this experiment, we activated our conditional allele of *Kras* by crossing to mice carrying the *Fabpl-Cre* transgene [1]. Because this transgene becomes active in the embryonic intestinal epithelium, we sought to determine whether expression of K-Ras^{G12D} solely in the adult epithelium produced the same phenotype. To this end, we crossed our *Kras* conditional mice to animals carrying a tamoxifen-sensitive form of Cre recombinase (CreER^{T2}) driven from the *Rosa26* promoter [2]. When we activated K-Ras in the adult colonic epithelium, we observed a phenotype similar to our initial analysis, indicating that the original K-Ras^{G12D} phenotype was not due to the embryonic expression of the *Fabpl-Cre* transgene (Supplementary Fig. 1c-e).

Inhibition of the Mek kinase *in vivo*

In Fig. 2, we used a small molecule inhibitor to demonstrate that the hyperplasic phenotype associated with K-Ras^{G12D} requires functional Mek. Initially, we performed pilot studies to determine the optimal dosing regiment for CI-1040, a Mek inhibitor [3]. Our pilot studies indicated that CI-1040 was highly effective in suppressing Mek activity *in vivo* in the colonic epithelium when given as two doses of 150 mg/kg separated by 12 hours (Supplementary Fig. 1f,g).

DSS time course

Treatment of mice with dextran sodium sulfate (DSS) is a common way to induce apoptosis specifically within the colonic epithelium [4]. In the course of our experiments, we performed a time course to determine how the colonic epithelium responds to this chemical. In our experiment, epithelial apoptosis reached an apex at 4 days and the epithelial damage was clearly apparent by 5 days (Supplementary Fig. 2a-d).

N-Ras over-expressing cells

In Figs. 1-3, we demonstrated that K-Ras^{G12D} and N-Ras^{G12D} differentially affected proliferation and apoptosis in the colonic epithelium. Although we found by Taqman analysis that the two genes were expressed at similar levels, it remained a formal possibility that the inability of activated N-Ras to promote hyper-proliferation was due to the fact that it was expressed at a

lower level than K-Ras. Using colon cancer cells that over-express N-Ras^{G12V} [ref 5], we found that high levels of mutant N-Ras did not promote proliferation, but did suppress apoptosis (Supplementary Fig. 2e,f).

Apc/*Kras* double mutant mice have reduced lifespan

We noted that expression of activated K-Ras in Apc-mutant colonic tumors had a major effect on tumor histology (Fig. 4). Nevertheless, these animals express K-Ras^{G12D} throughout the entire colonic epithelium that results in hyperplasia (Fig. 1). This widespread hyperplasia led to the development of many more tumors in double mutant animals compared to those expressing wild-type K-Ras, leading to a dramatic reduction in lifespan (Supplementary Fig. 3a).

Quantitative analysis of signaling in mouse tumors

In Fig. 1, we used quantitative western blotting to analyze signaling through canonical Ras effector pathways in colonic epithelium expressing activated K-Ras or N-Ras. We have performed a similar experiment in colonic tumors expressing activated forms of Ras (Supplementary Fig. 3b). Consistent with the results of standard western analysis (Fig. 5), K-Ras^{G12D} appeared to activate Mek, but not Erk, and to down-regulate Akt signaling. N-Ras^{G12D} did not activate any of the pathways in a statistically significant manner, but did appear down-regulate Akt signaling.

Erk is not activated in human colon cancers

In Fig. 1, we demonstrated that phospho-Erk was detectable by immunohistochemistry only in the terminally differentiated cells at the tops of crypts expressing K-Ras^{G12D}. In colonic tumors, no increase in phospho-Erk was detectable after activation of K-Ras (Fig. 5). We also could not detect significant levels of phospho-Erk by immunohistochemistry in any tumors from a panel of primary human colon cancers, although adjacent normal tissue sometimes exhibited high levels of phospho-Erk near the tops of the crypts (Supplementary Fig. 4a).

Ras signaling in human cell lines

To perform mechanistic studies of K-Ras effector pathways, we have used somatic cell genetics in human colorectal cancer cell lines. These studies utilize a set of isogenic cell lines that differ

only in their K-Ras mutation states. The parental cell line, DLD-1, is mutant for K-Ras (*Kras*^{G13D}/+) and the isogenic derivative, DKs-8, retains the wild-type allele but has lost of the G13D allele by virtue of gene targeting (*Kras*$^{+/-}$) [6]. In this isogenic pair of cell lines, mutant K-Ras transmitted downstream signals almost identical to our autochthonous mouse colon tumors (Supplementary Fig. 4b).

Somatic genetic analysis of Mkp3 function

We noted by western blotting that activated K-Ras up-regulated the expression level of Mkp3, an Erk phosphatase (Fig. 5). Using Taqman analysis, we found that K-Ras^{G13D} did not affect expression of the related gene *Mkp1* (Supplementary Fig. 4c). We used lentiviral delivery of shRNA to knockdown Mkp3 levels in colon cancer cell lines (Supplementary Fig. 4d) and found that Mkp3 knockdown affected the growth rate of DLD-1 cells expressing activated K-Ras (Fig. 5). This was not true in DKs-8 cells expressing wild-type K-Ras (Supplementary Fig. 4e). shRNA target sequences within the Mkp3 gene are listed in Supplementary Table 1.

CI-1040 and AZ628 similarly affect Erk activation in DLD-1 cells

Cells expressing activated K-Ras appeared to be sensitive to inhibition of Raf, but not to inhibition of Mek (Fig. 6). By contrast, cells expressing activated B-Raf were sensitive to inhibition of both Mek and Raf (Fig. 6). We found that CI-1040 and AZ628 both suppressed Erk activation in DLD-1 cells, though only AZ628 affected viability in this cell line (Supplementary Fig. 4f). Alternately, HT-29 cells were equally sensitive to CI-1040 and AZ628, but AZ628 was much more effective at suppressing Erk activation. (Supplementary Fig. 4f).

DLD-1 cells are sensitized to Sorafenib compared to DKs-8 cells

We showed that AZ628, a novel Raf inhibitor, affected the viability of DLD-1 cells, but not the isogenic cell line DKs-8 (Fig. 6). To confirm that this phenotype effect was due to inhibition of Raf, we treated cells with Sorafenib, a chemically distinct Raf inhibitor [7]. As with AZ628, DLD-1 cells were sensitized to inhibition of Raf by Sorafenib when compared to the isogenic DKs-8 cells (Supplementary Fig. 4g).

Supplementary methods

Mouse strains and treatments

For Mek inhibition, animals were treated twice daily with 150 or 100 mg/kg CI-1040 resuspended in 0.5% hydroxypropyl methylcellulose (Sigma). Animals treated for 24 hours were given two doses of 150 mg/kg. In animals treated twice daily for 1 week, the maximum tolerated dose (MTD) was 100 mg/kg. To induce colonic apoptosis, the normal drinking water was replaced with 2.5% dextran sodium sulfate (DSS) for a period of 7 days. To induce K-Ras^{G12D} expression in the adult colonic epithelium, animals carrying R26-CreER^{T2} [2] were given 3 injections of tamoxifen (9 mg per 40 g weight) suspended in sterile corn oil. Injections were separated by 48 hours. Animals were sacrificed and analyzed 2 weeks after the final injection.

Laser capture and RNA isolation

To analyze gene expression in the human colonic epithelium, 8 μm sections were cut from formalin-fixed, paraffin-embedded tissues. Tissues corresponding to the top and bottom 30 μm of crypts were isolated by laser capture using an Arcturus microdissection system. RNA was isolated using the Ambion RecoverAll™ kit and Taqman analysis was performed as described previously [8].

Supplementary References

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2. Ventura, A. et al. Restoration of p53 function leads to tumour regression in vivo. *Nature* **445**, 661-5 (2007).
3. Sebolt-Leopold, J.S. et al. Blockade of the MAP kinase pathway suppresses growth of colon tumors in vivo. *Nat Med* **5**, 810-6 (1999).
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Supplementary Table 1

Genotyping primers

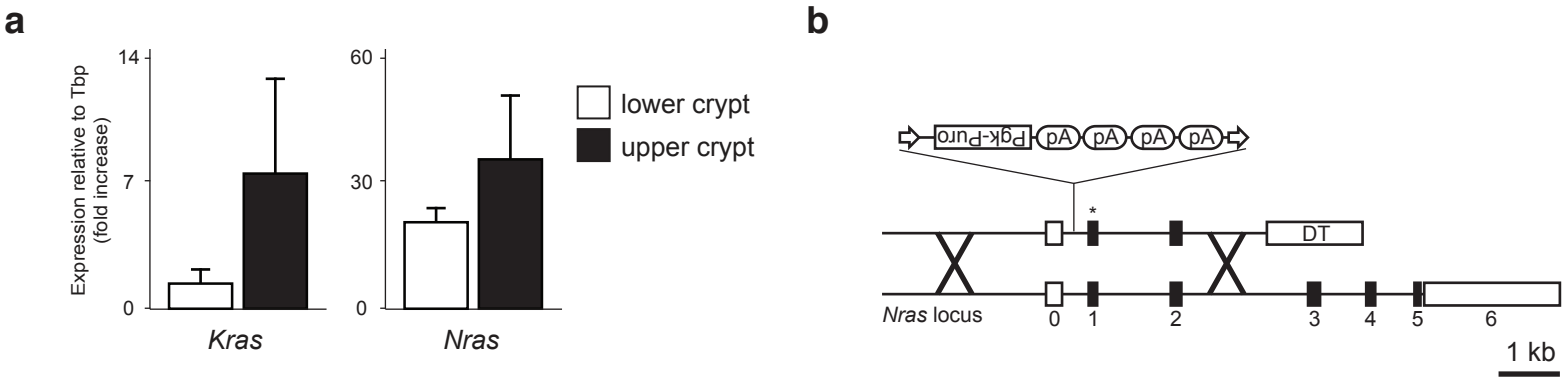
Gene/allele	Primer 1	Primer 2	Primer 3
Fabpl-Cre	CCTGATCCTGGCAATTTCG	GGACTCACTAATGTTTGCTG	
Villin-Cre	CAAGCCTGGCTCGACGGCC	CGCATAACCAGTGAAACAGCATTGC	
R26-CreER^{T2}	AAAGTCGCTCTGAGTTGTTAT	GGAGCGGGAGAAATGGATATG	CCTGATCCTGGCAATTTCG
R26R	GGAGCGGGAGAAATGGATATG	GCGAAGAGTTTGTCCTCAACC	AAAGTCGCTCTGAGTTGTTAT
Kras$^{LSL-G12D}$	GTCTTTCCCCAGCACAGTGC	CTCTGGCCTACGCCACCAGCTC	AGCTAGCCACCATGGCTTGAGTAAGTCTGCA
Nras$^{LSL-G12D}$	AGACGCGGAGACTTGGCGAGC	GCTGGATCGTCAAGGCGCTTTTCC	AGCTAGCCACCATGGCTTGAGTAAGTCTGCA
Apc^{2lox14}	GATGGGTCTGTAGTCTGGG	GGCTCAGCGTTTTCCTAATG	

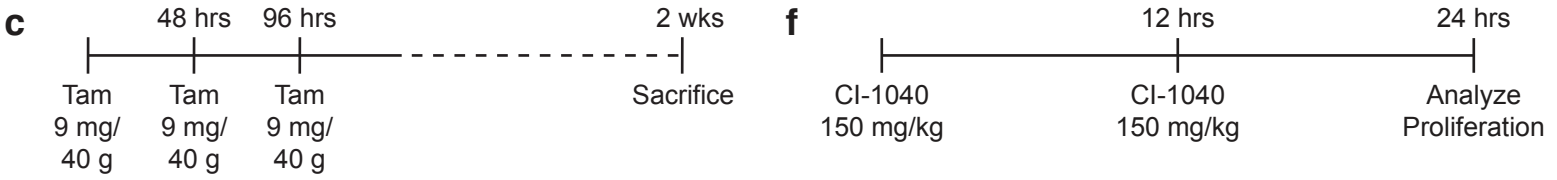
shRNA target sequences

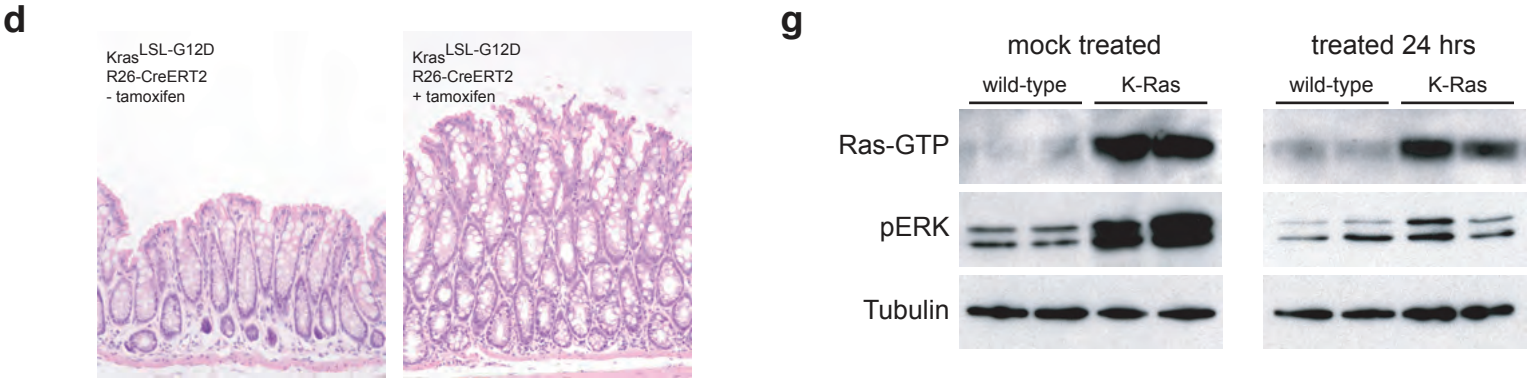
Mkp3 kd1	5'-GCAGGAGAGTTTAAATACA-3'
Mkp3 kd2	5'-GTTGGTTTCTTTCCTTTAA-3'

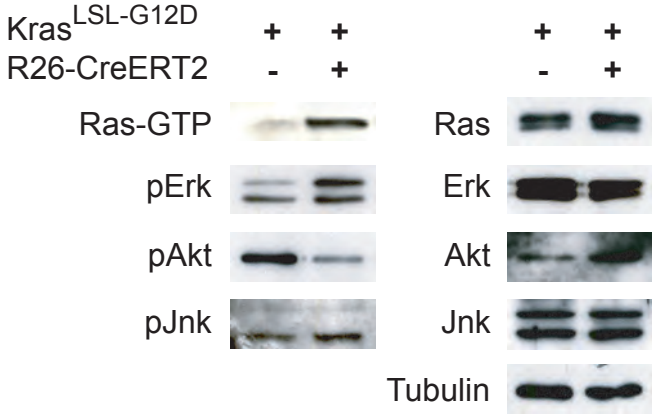
Supplementary Figure 1 (a) Expression analysis of *Kras* and *Nras* in the human colonic epithelium. In this experiment, RNA was isolated from formalin-fixed paraffin-embedded tissues after laser capture microdissection. Both K-Ras and N-Ras are more highly expressed at the top of the human colonic crypt ($p < 0.01$), although the difference is greater for K-Ras. **(b)** Schematic representation of $Nras^{LSL\text{-}G12D}$ targeting in murine embryonic stem cells. The lox-stop-lox element (LSL) is inserted into the first intron, upstream of the G12D activating mutation in the first coding exon. **(c)** Schematic representation of the tamoxifen (Tam) treatment scheme for *R26-CreER*^{T2} animals. Animals were treated at 8 weeks of age. **(d)** H&E staining of colons from untreated and treated animals. Treatment of *R26-CreER*^{T2} ; $Kras^{LSL\text{-}G12D}$/+ animals activated K-Ras and induced hyperplasia. **(e)** Signaling downstream of K-Ras^{G12D} after activation in adults. Similar to signaling in *Fabpl-Cre* ; $Kras^{LSL\text{-}G12D}$/+ animals, activation of K-Ras in adults led to up-regulation of phospho-Erk and down-regulation of phospho-Akt. **(f)** Treatment of mice with CI-1040. Two doses of 150 mg/kg were separated by 12 hours. Proliferative indices were assessed 24 hours after the first injection. **(g)** Confirmation of CI-1040 activity *in vivo*. In mock treated animals, colons expressing K-Ras^{G12D} had high levels of activated Erk. By contrast, treatment with CI-1040 led to attenuated Erk activation.

Supplementary Figure 1



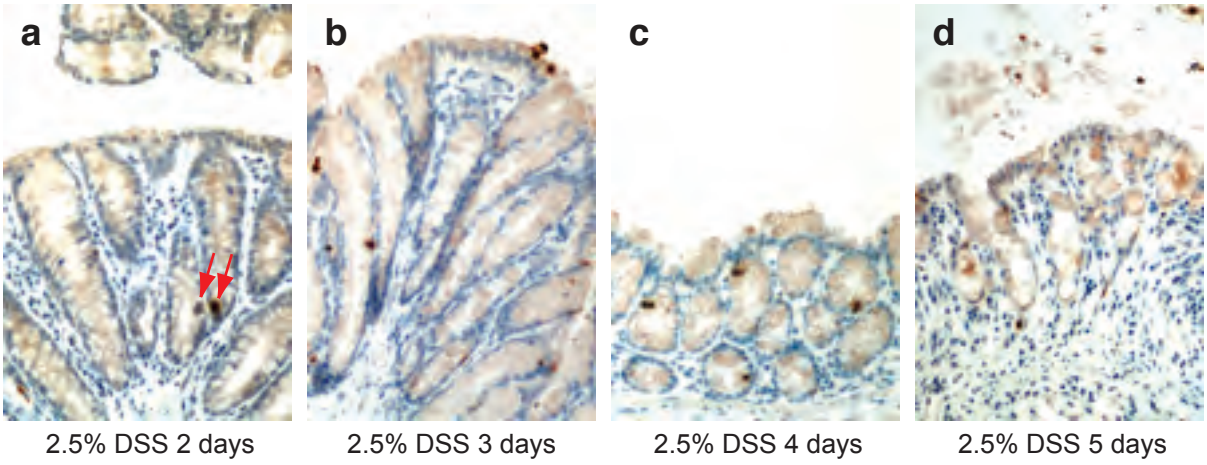


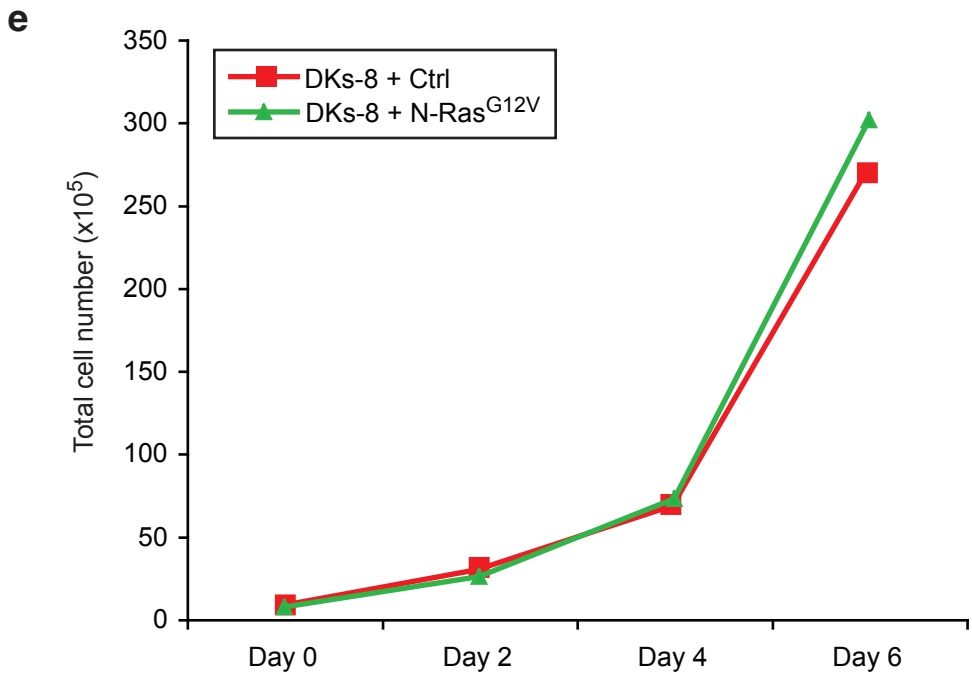


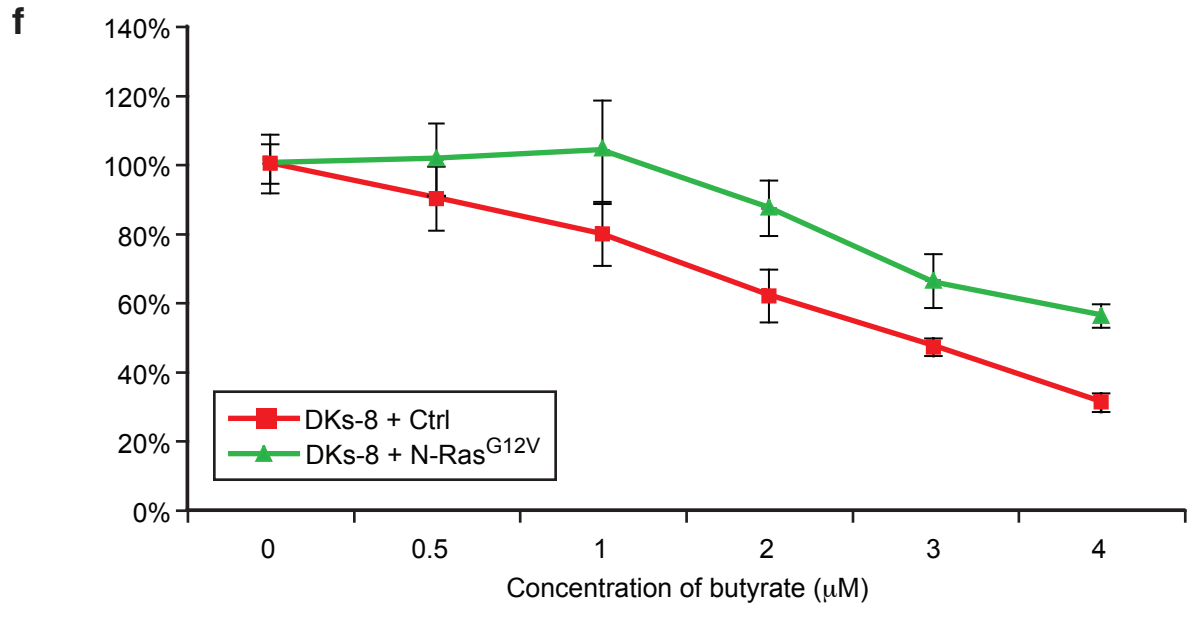


Supplementary Figure 2 (a-d) Time course of apoptosis in wild-type animals treated with 2.5% DSS in the drinking water. Cleaved caspase 3 (CC3) positive cells are apparent 2 days after treatment begins (red arrows) and the number of CC3 positive cells reaches it maximum level around 4 days after treatment. By 5 days, the epithelial damage becomes apparent and the number of apoptotic epithelial cells begins to decline. **(e)** Growth curves for wild-type cells (DKs-8 + Ctrl) and isogenic cells expressing mutationally activated N-Ras from a retroviral promoter (DKs-8 + N-Ras^{G12V}). Over-expression of mutant N-Ras does not alter the growth properties of the cells. **(f)** Over-expressed mutant N-Ras confers resistance to butyrate-induced apoptosis. Cells were treated with various concentrations of butyrate and assayed for viability after 24 hours. Wild-type cells (red) show a dose-dependent decrease in viability. At all concentrations mutant N-Ras (green line) confers resistance to butyrate-induced apoptosis.

Supplementary Figure 2

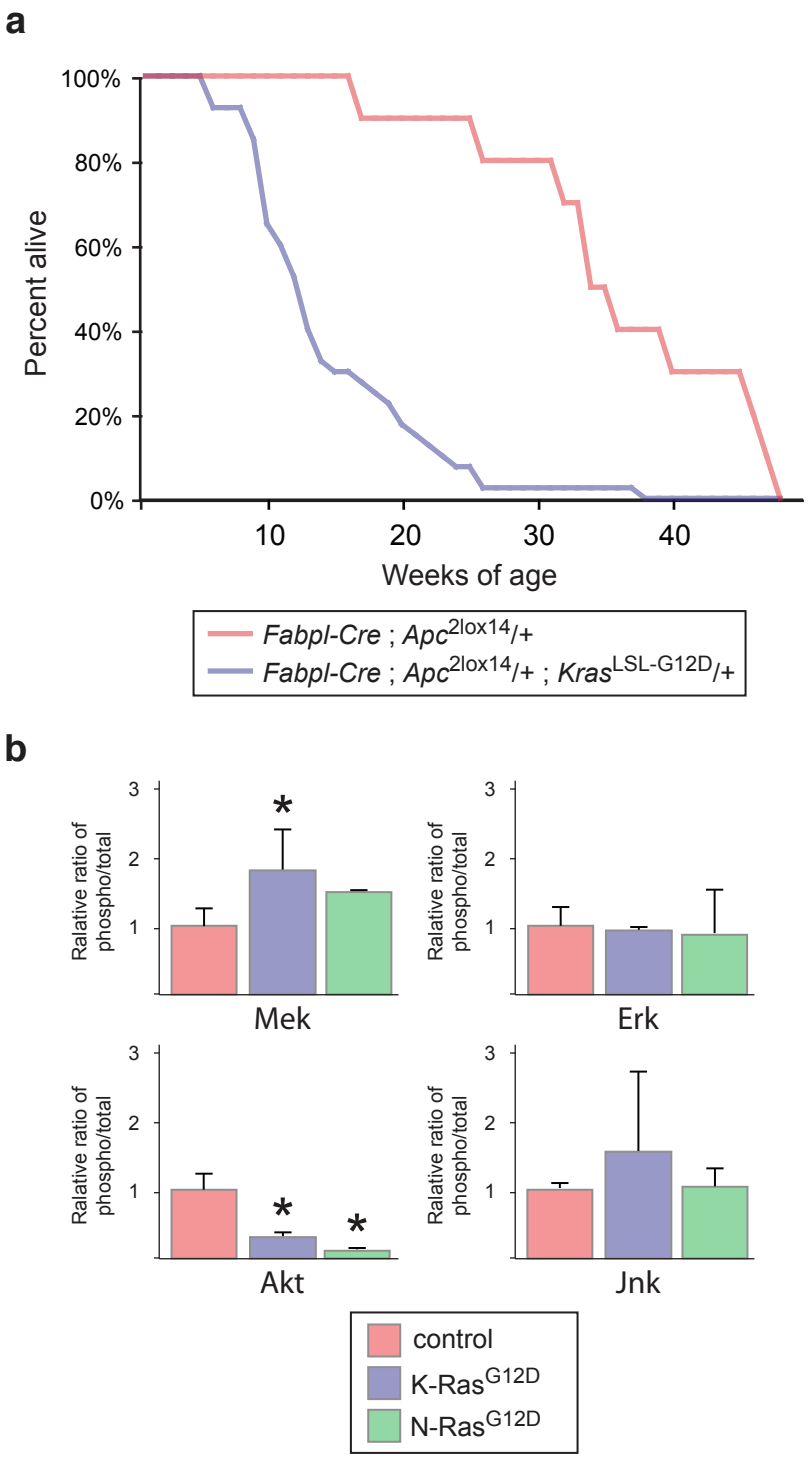






Supplementary Figure 3 (a) Survival curve of tumor-bearing animals. Animals doubly mutant for *Apc* and *Kras* have a significantly reduced lifespan (average survival = 14 weeks) compared to animals mutant for *Apc* alone (average survival = 34 weeks). **(b)** Quantification of signaling in colonic tumors of varying Ras genotype. Signaling in colonic tumors is very similar to signaling in the normal colonic epithelium (Fig. 1f), with the exception that K-Ras^{G12D} does not activate Erk in the tumor epithelium.

Supplementary Figure 3



Supplementary Figure 4 (a) Immunohistochemical detection of phospho-Erk in primary human tissues. Adenocarcinomas do not stain positively for phospho-Erk (n = 18), while the differentiated cells at the top of the "normal" colonic crypts are occasionally positive (4/18 adjacent normal samples). Scale = 100 μm. **(b)** Quantitation of signaling in isogenic human colon cancer cell lines. Similar to our results with autochthonous mouse colon tumors, mutant K-Ras activates Mek, but not Erk, in DLD-1 (*Kras*^{G13D}/+) compared to DKs-8 (*Kras*$^{+/-}$). K-Ras^{G13D} also down-regulates Akt, as in mouse tumors. One difference between the mouse and human cells is that mutant K-Ras appears to suppress Jnk in the cell lines, but had no significant effect in mouse tumors. **(c)** Taqman analysis of *Mkp3* expression. DLD-1 cells expressing K-Ras^{G13D} exhibit a 5-fold increase in *Mkp3* expression relative to DKs-8 cells expressing wild-type K-Ras. Two independent shRNAs knock down *Mkp3* expression in both DLD-1 and DKs-8 cells. The shRNAs do not affect expression of the related gene *Mkp1*. **(d)** shRNA-mediated knockdown of Mkp3. Two unique shRNAs (kd1 and kd2) were used to suppress Mkp3 expression. **(e)** Growth curves in Mkp3 knockdown cells. Reduced Mkp3 expression does not affect growth in DKs-8 cells expressing wild-type K-Ras. **(f)** Quantitation of signaling in cells treated with CI-1040 and AZ628. For this experiment, both drugs were used at a concentration of 1 μM. Although CI-1040 and AZ628 have similar effects on the steady state levels of phospho-Erk in DLD-1 cells, only AZ628 affects viability of these cells. **(g)** Inhibition of Raf with Sorafenib. DLD-1 cells expressing mutant K-Ras are hypersensitive (compared to DKs-8) to inhibition of Raf Sorafenib ($p = 0.001$ at 10 μM). Interestingly, and unlike AZ628, cells expressing mutant K-Ras are as sensitive to Sorafenib as are cells expressing mutant B-Raf.

Supplementary Figure 4