IRAK4 mediates colitis-induced tumorigenesis and chemoresistance in colorectal cancer

Supplementary Data

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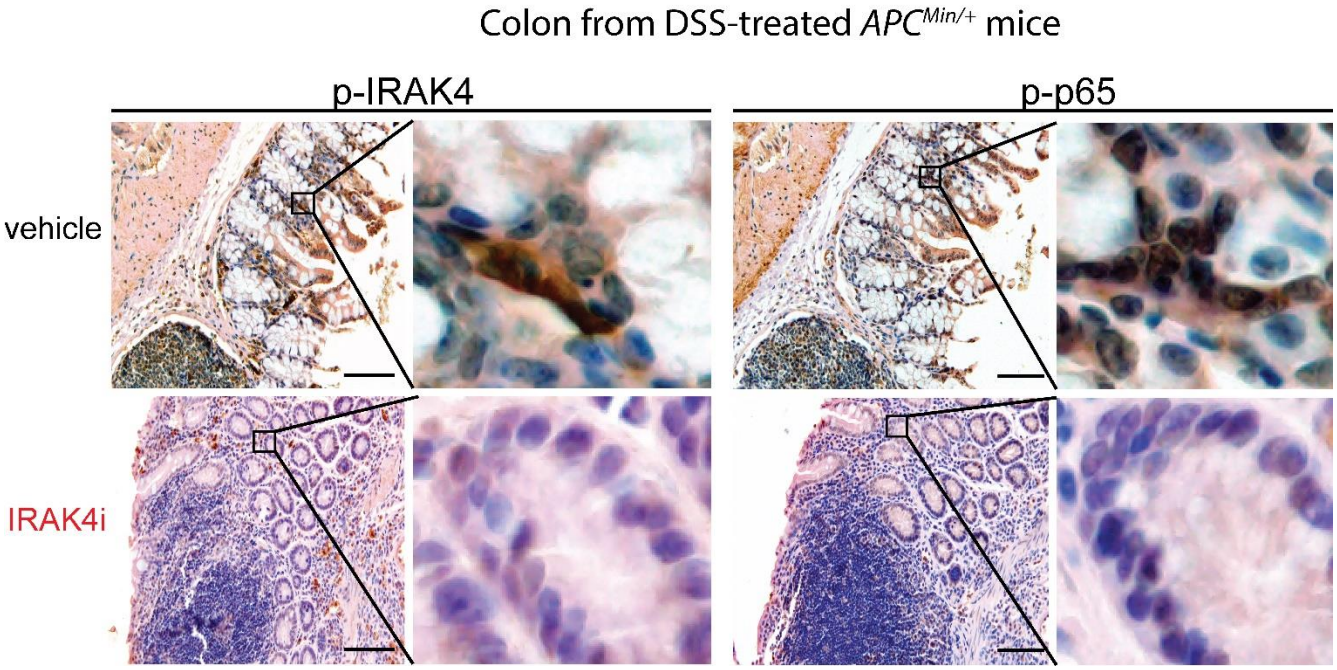
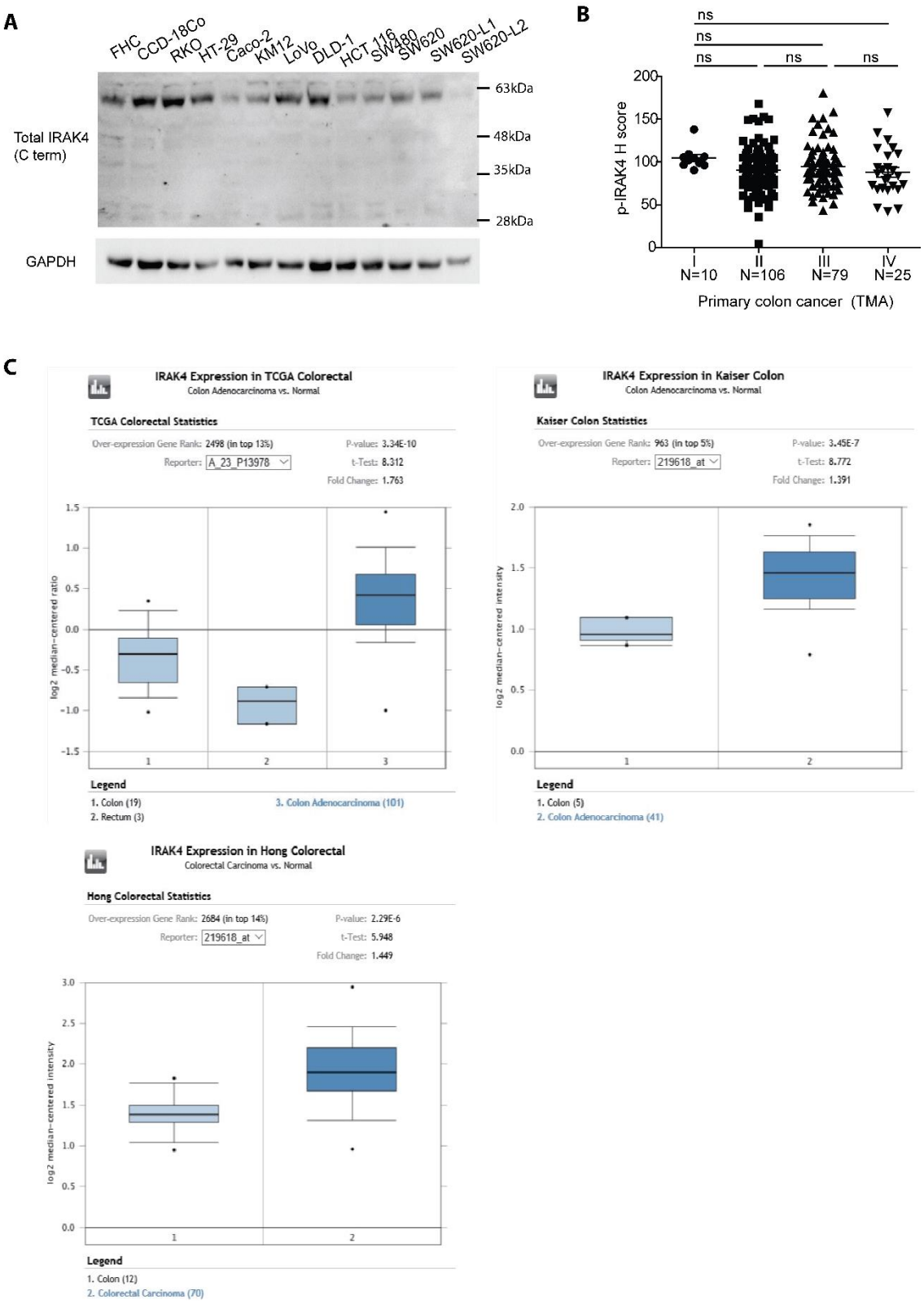
The authors have declared that no conflict of interest exists.

Supplementary Figures and Tables



Supplementary Figure 1. IRAK4i suppresses p-IRAK4 and p-p65 staining in colon tissue
Representative IHC pictures of the indicated markers from colonic tissues from DSS-treated *APC*$^{Min/+}$ mice followed by vehicle or IRAK4i treatment for 4 weeks. Suppression of p-IRAK4 and p-p65 supports on-target effect of IRAK4i. Colonic epithelium near a lymphoid aggregate was selected for presentation.

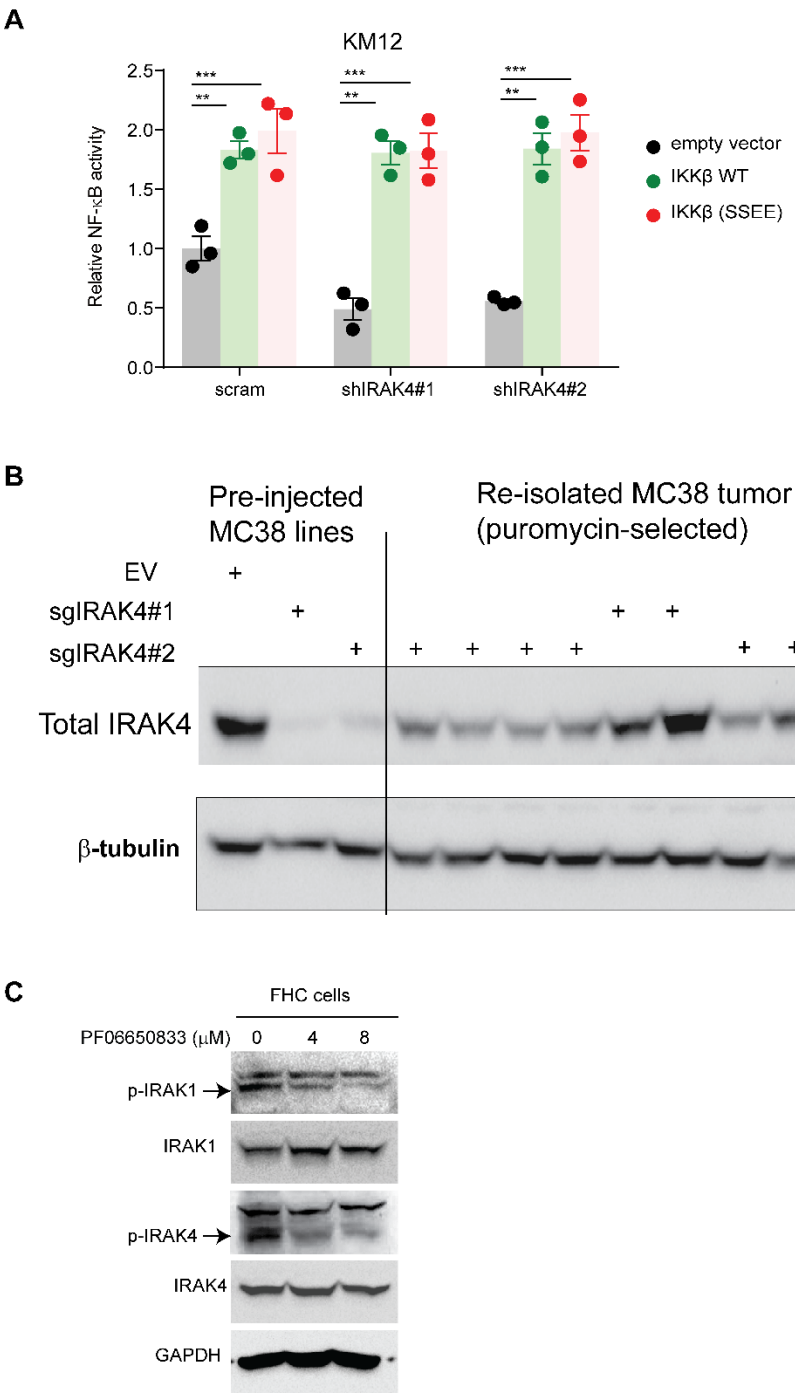


Supplementary Figure 2. IRAK4 expression is upregulated in CRC samples

(A) Western blots showing lack of definite short (~32kDa) IRAK4 band in tested CRC lines using a commercial antibody raised against the C-terminus of IRAK4 (Abcam #5985).

(B) Comparison of p-IRAK4 IHC intensities by H scores in CRC samples of different clinical stages from a pool of 5 commercial TMAs (Tukey's multiple comparison test, ns: not significant).

(C) Comparison of IRAK4 mRNA expression between normal and colon cancer tissues from three different datasets in Oncomine.

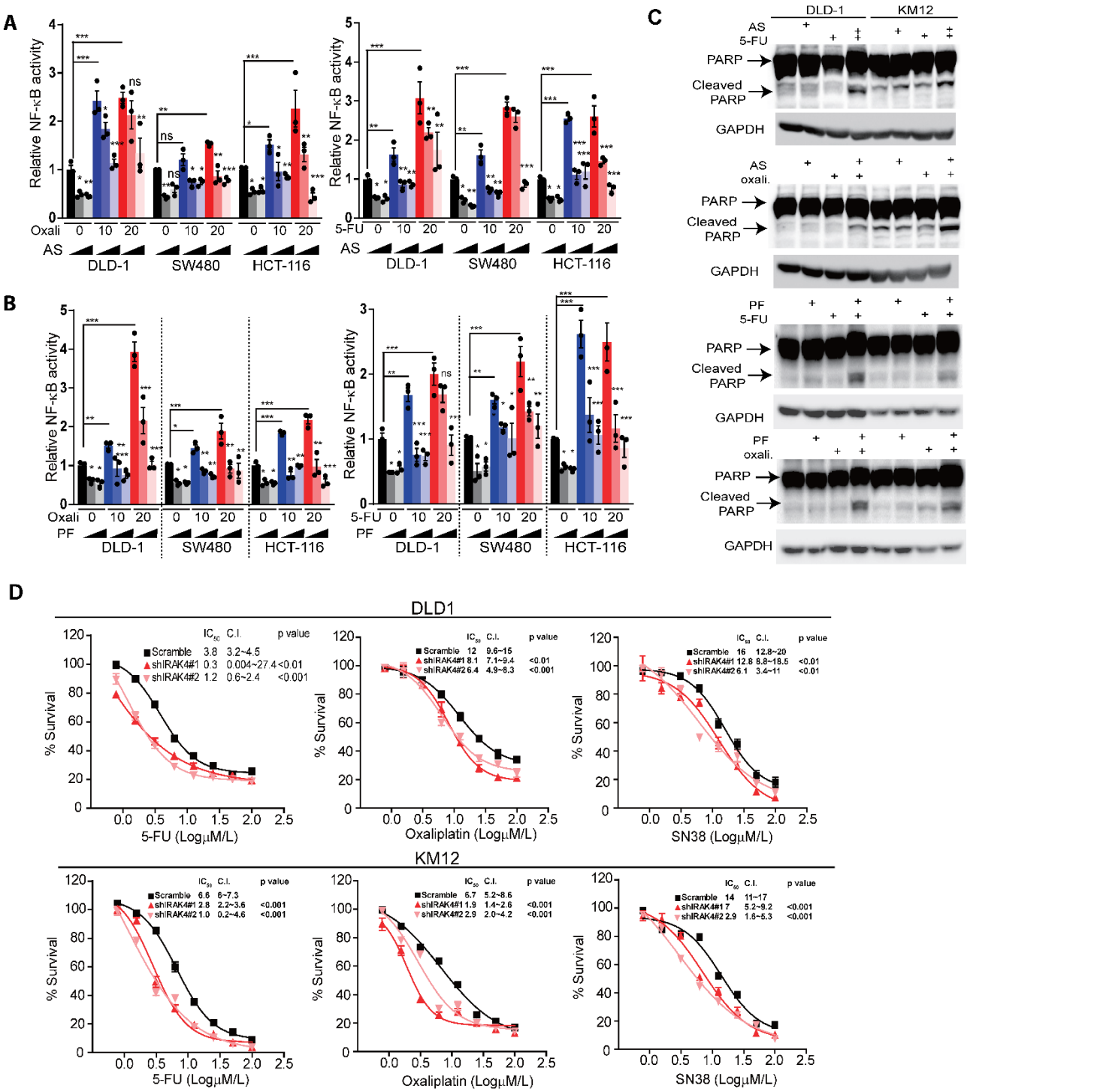


Supplementary Figure 3. Essential role of IRAK4 in NF-κB activity and tumorigenesis

(A) NF-κB luciferase reporter assay of the indicated KM12 cells transfected with empty vector, wild-type (WT) or constitutively activated (S177E/S181E) IKKβ. Data presented as mean ± SEM from one of two experiments done in triplicates (ANOVA, **p<0.01, ***p<0.001).

(B) Western bots showing restoration of IRAK4 expression in re-cultured and puromycin-selected MC38 tumor lines harvested from terminal mice in experiments **Fig. 4F and 4G.**

(C) Western blots showing suppressive effect of IRAK4i PF06650833 on p-IRAK4 and p-IRAK1 in normal colon cell line FHC.

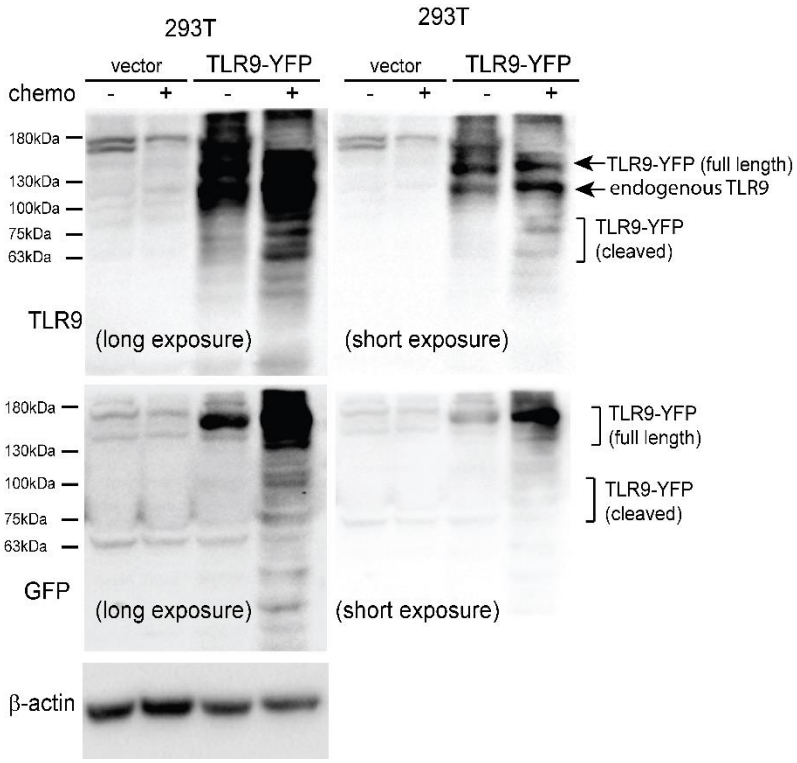


Supplementary Figure 4. IRAK4 inhibition suppresses NF-κB and potentiates chemotherapy

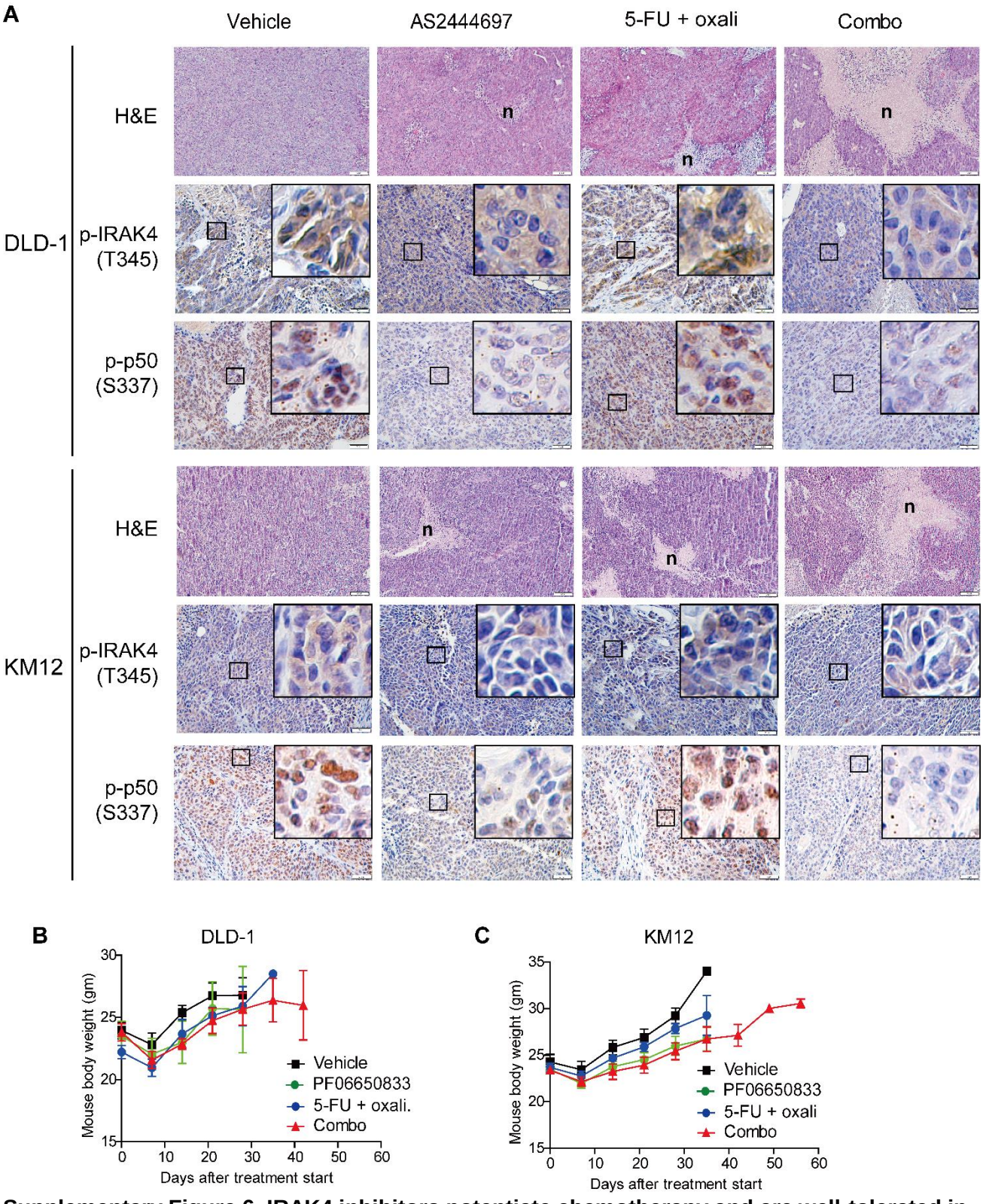
(A) (B) NF-κB luciferase reporter assay of three different CRC cell lines treated with oxaliplatin or 5-FU (0, 10 or 20μM) and AS2444697 (AS) or PF06650833 (PF) at 0, 4, 8 μM overnight. Data represent one of three sets of experiment each done in triplicates and presented as mean ± SEM (*p<0.05, **p<0.01, ***p<0.001).

(C) Western blots showing increased PARP cleavage in two different CRC cell lines treated for 24 hours with IRAK4 inhibitors (both at 4μM) and 5-FU or oxaliplatin (both at 10μM).

(D) Dose-response curves based on Alamar Blue viability assay on two CRC cell lines stably expressing scramble or IRAK4 shRNAs incubated in the indicated chemotherapeutic agent at 8 different concentrations over 5 days. Data represent one of three sets of experiment each done in triplicates and presented as mean ± SEM (ANOVA).



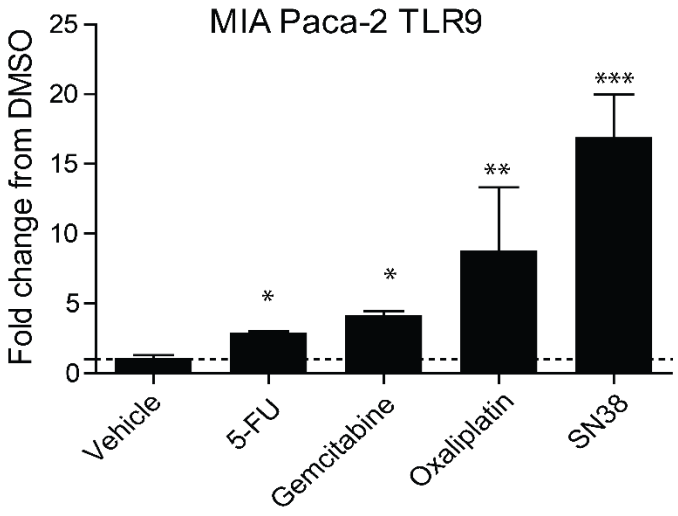
Supplementary Figure 5. Chemotherapy induces TLR9 expression and cleavage
Western blots showing enhanced protein level of full length and cleaved form of TLR9-YFP in 293T cells transfected with TLR9-YFP in 293T cells. To ensure equal basal expression, 293T cells were transfected with TLR9-YFP, and 24 hours later split into halves for treatment with DMSO or 5-FU + oxaliplatin (10μM each) overnight before being harvested for lysis. Experiment was conducted three times with similar results.



Supplementary Figure 6. IRAK4 inhibitors potentiate chemotherapy and are well-tolerated in mice

(A) Representative H&E and p-IRAK4, p-p50 IHC images from DLD-1 and KM12 tumors harvested from experiment in Fig. 7C and 7G, respectively. (n: necrotic areas)

(B), (C) Serial measurements of body weight of mice bearing DLD-1 or KM12 tumors treated as indicated following treatment start. Data presented as means ± SEM.



Supplementary Figure 7. Chemotherapy induces TLR9 expression in pancreatic cancer cells
qPCR showing fold change of TLR9 mRNA levels in MIA Paca-2 cells treated overnight with various chemotherapeutic agents (all in 10μM). Data represents one of two sets of experiments done in biological duplicates and technical triplicates and presented as means ± SEM (ANOVA, *p<0.05, **p<0.01, ***p<0.001)

Supplementary Table 1. Clinicopathologic characteristics of all Stage IV colon cancer patients analyzed (N=204).

Characteristics	p-IRAK H-score low-medium (N=136)	p-IRAK H-score High (N=68)	*P*
Sex			
Male	77	40	0.88
Female	59	28	
Age, years			
Median	58.9	59.1	0.48
Range	26-84	28.5-84.7	
Presentation of liver metastasis			
Synchronous	58	35	0.42
Metachronous	68	30	
Unknown	10	3	
Number of liver metastasis			
Median	2	2	0.16
Mean	2.2	2.5	
Range	1-14	1-10	
Neoadjuvant chemotherapy			
Yes	25	16	0.48
No	93	45	
Unknown	18	7	
Extent of liver surgery			
Less than lobectomy	70	29	0.30
Lobectomy	66	39	
Resection status			
Complete	112	61	0.22
Incomplete/unverified	24	7	
Liver margin			
Negative	124	61	0.8
Positive	12	7	
Median overall survival (resection to death, years)	3.81 (HR 0.69, 95% CI 0.49-0.98)	2.90	**0.038** (Log-rank) **0.20** (Wilcoxon)

Supplementary Table 2. Antibodies used in this study (Methods)

Name	Host	Clone#	Company	Dilution
p-IRAK4(T345/S346), WB	Rabbit	D6D7	Cell Signaling	1:1000
Total IRAK4	Rabbit	4363	Cell Signaling	1:1000
Total IRAK4 (C-terminus)	Rabbit	ab5985	Abcam	1:500
p-IRAK1(T209)	Rabbit	N/A	Cell Signaling	1:1000
Total IRAK1	Rabbit	D51G7	Cell Signaling	1:1000
p-IKKα/β(S176/180)	Rabbit	N/A	Cell Signaling	1:1000
Total IKKβ	Rabbit	2C8	Cell Signaling	1:1000
p-NF-κB/p65(S536)	Rabbit	ab86299	Abcam	1:250
Total NF-κB/p65	Rabbit	D14E12	Cell Signaling	1:1000
p-NF-κB/p50(S337)	Mouse	A-8	Santa Cruz	1:200
Total NF-κB p105/p50	Rabbit	N/A	Cell Signaling	1:1000
GAPDH	Mouse	0411	Santa Cruz	1:1000
α-tubulin	Mouse	B-7	Santa Cruz	1:500
Histone H3	Rabbit	D1H2	Cell Signaling	1:1000
PARP	Rabbit	N/A	Cell Signaling	1:1000
Ki-67	Mouse	8D5	Cell Signaling	1:50
Cleaved Caspase-3	Rabbit	5A1E	Cell Signaling	1:200
Pan-cytokeratin	Mouse	C11	Cell Signaling	1:400
β-tubulin	Rabbit	9F3	Cell Signaling	1:2000
p-IRAK4 (T345), IHC and IF	Mouse	A8A8	ABNOVA	1:250~500
TLR9	Rabbit	D9M9H D2C9	Cell Signaling	1:1000