SUPPLEMENTAL INFORMATION

EXPERIMENTAL PROCDEURES

Derivation of the Ras Dependency Index (RDI)

Weighted averages for relative cell densities for MOIs of 5 and 1 with the KRAS A and B shRNAs were calculated. The inverse of these averages was then calculated. This number was multiplied by the transduction efficiency for each respective cell line (the proportion of cells expressing the control shRNA following puromycin selection compared those not treated with puromycin), yielding the RDI value. An RDI of 2 is calibrated as a 50% reduction in cellular proliferation following KRAS depletion.

Lentiviral shRNA Experiments

293T cells were seeded (3ml at density of 2 x 10^5 cells per ml) in duplicate wells of a 6 well plate per shRNA construct. Constructs were from the Broad RNAi Consortium. Lentiviral particles were generated using a three-plasmid system, as described previously (Moffat et al., 2006; Naldini et al., 1996). To standardize lentiviral transduction assays, viral titers were measured in a benchmark cell line, A549. For growth assays, titers corresponding to multiplicities of infection (MOIs) of 5 and 1 in A549 cells were employed. For KRAS knockdown, cells were plated on day zero at 3x10^4 cells/ml in 96 well plates (100 µl per well) or 6 well plates (3 ml per well). Cells were spin infected, as described previously (Moffat et al., 2006). 24 hours post-infection, cells were treated with 1 µg/ml puromycin for 3 days to eliminate uninfected cells. Media was replaced and cells were grown for 2 more days, then fixed with 4% formaldehyde and stained with 1 µM Syto60 dye (Invitrogen Inc) for 1 hour. Syto60 fluorescence was quantified with a LiCor fluorescence scanner in the IR700 channel. Alternatively, cells were harvested for

western blot analysis by lysing in MLB (20mM Tris HCl pH7.5, 150mM NaCl, 10mM $MgCl_2$, 1% NP-40, 0.25% Na deoxycholate, 10% Glycerol, supplemented with Complete Protease Inhibitor Cocktail, 1mM Na Vanadate and 25mM NaF). Lysates were normalized for total protein using Pierce BCA reagent and resolved by SDS-PAGE followed by transfer to PVDF.

KRAS and *BRAF* Genotyping

To determine the mutation states of *KRAS* and *BRAF* in colorectal cancer cell lines used in this study, total RNA was extracted from cells with the RNeasy Kit (Qiagen). RNA was reverse transcribed with an Applied Biosystems Reverse Transcriptase Kit. *KRAS* exon4 was sequenced from cDNA with the following primers: forward: CCA TTT CGG ACT GGG AGC GAG C and reverse: CCT ACT AGG ACC ATA GGT ACA TCT TC. *BRAF* exon15 was sequenced with TCA TAA TGC TTG CTC TGA TAG GA (F) and GGC CAA AAA TTT AAT CAG TGG (R).

In vivo Pharmacology with Xenografted Mouse Tumors

Human colorectal cancer tumor cells were trypsinized and resuspended as single cell suspensions at $3x10^7$ cells per ml in PBS. 100µL ($3x10^6$ cells total) of this suspension were injected into opposite left and right flanks of NOD/SCID mice. All mice were housed in a pathogen-free environment at the Massachusetts General Hospital and were handled in strict accordance with Good Animal Practice as defined by the Office of Laboratory Animal Welfare, and all animal experiments were done with approval from Massachusetts General Hospital Subcommittee on Research Animal Care. Tumor size was monitored daily and once tumor volume had reached approximately $200mm^3$, treatment with 5Z-7-oxozeaenol was initiated (7 to 14 days post-implantation). Mice were injected daily with 15mg/kg 5Z-7-oxozeaenol. The drug was resuspended as a 25mg/ml

stock in DMSO. This was further diluted 10-fold in Arachis Oil (Sigma Inc.) to yield a 2.5mg/ml stock in 10% DMSO. Approximately 120µl of this stock was delivered to 20g mice intraperitoneally. Alternatively, 10% DMSO in Arachis Oil was delivered as a vehicle control.

Western blotting and antibodies

The following antibodies were used for western blotting: KRAS OP-24, Pan-Ras OP-40 (Calbiochem); PARP (BD Pharmigen, 4C10-5); BMP-7 (Abcam); phospho-ERK, Axin2, phospho Smad1 and total Smad1/5/8, phospho- and total AMPK, phospho- and total AKT, cleaved Caspase-3 (Cell Signaling); GAPDH (Chemicon); E-Cadherin, β-catenin (BD Pharmigen) Syk, TAK1, total ERK1 (Santa Cruz). For secreted BMP-7 levels, $1x10^6$ HT29 cells stably expressing ER-KRAS(12V) were plated in 10cm dishes. 24h post-plating, 10ml serum-free DME/F12 medium (Gibco) was added. Conditioned media was collected 24h post-induction of ER-KRAS(12V) with 4-HT and concentrated to 500µL using Amicon® Ultra-4 Centrifugal Filter Units with 3kDa membranes. To assess BMP-7 levels, 60µL of this concentrated conditioned medium was used for western blotting.

TOP-FLASH Reporter Assays

For data shown in Figure 4B, cells were plated in 12-well tissue culture plates at a density of $5x10^4$ cells/ml and 1ml per well. Cells were then co-transfected with either 0.5µg FOP-FLASH or TOP-FLASH plasmids (kind gift from Vijay Yajnik, Massachusetts General Hospital) plus 50ng of pRL-TK (expressing Renilla luciferase). Normalized luciferase activity was obtained by using the Dual-Luciferase Reporter Assay System (Promega Inc). For data shown elsewhere, stable cell lines expressing the TOP-FLASH reporter were generated by transducing cells with 7TFP (see below) recombinant lentiviruses and selecting with 2µg/ml puromycin for 5 days.

Plasmid Constructs

Lentiviral vectors were used throughout this study. 7TFP, encoding a 7xTcf-FFLUC was obtained from Addgene (Fuerer and Nusse, 2010). Human epitope-tagged *HA-NRAS(G12V)*, *V5-KRAS4A(G12V)* and *HA-KRAS4B(G12V)* were cloned into pLenti-PGK-Hygro (Campeau et al., 2009) by Gateway cloning (Invitrogen Inc.) – LR reaction from pDONR-223 Entry clones. Human *BMPR1A* was cloned into pDONR-223 by Gateway Cloning with a mutated stop codon. The Q233D missense mutation was introduced using the Stratagene Quickchange site-directed mutagenesis kit. *BMPR1A(Q233D)* was cloned into pLenti6-CMV-V5 by Gateway cloning to allow for expression of a C-terminal in-frame V5 fusion construct. Constitutively-active CTNNB1 was generated via Gateway cloning into pDONR-223 and subsequent site-directed mutagenesis to generate a S33Y/S45A double mutant. This mutant was subcloned into the pWPI lentiviral expression.

Immunofluorescence microscopy

Cells were fixed in EM grade 4% formaldehyde and permeabilized with 0.1% Triton X-100. Staining with primary antibodies was carried out overnight at 4˚C. For mouse monoclonal antibodies, an Alexa594-conjugated goat anti-mouse secondary antibody was used (Molecular Probes). For rabbit polyclonal antibodies, Alexa-488 conjugated goat anti-rabbit secondary antibody was used (Molecular Probes). Nuclei were visualized using DAPI. Micrographs were either captured on an IX81 Spinning Disk Deconvolution Microscope equipped with 100X Plan-Apo Oil objective (Figures 5H, 6G) or a Zeiss Laser Confocal Microscope equipped with a 63X Plan-Apo Oil objective (Figures 5D and S4B). Digital images were processed with Slidebook, Zeiss LSM Browser and Adobe Photoshop CS4.

Reconstitution experiments

HT29, SW620 or SKCO1 cells were infected with recombinant lentiviruses encoding either BMPR1A-CA and CTNNB1-CA or vector control (containing the ccDB gene). For BMPR1A-CA stable expression, cells were selected in 5µg/ml Blasticidin for 7 days and pooled clones were established. Stable expression was verified using the V5 epitope tag on the BMPR1A transgene product. For CTNNB1-CA, the pWPI recombinant lentiviruses encode GFP driven by IRES. Thus, stable cell clones were obtaining by FACS live cell sorting to obtain the top 10% of GFP expressing cells. The SW620-CTNNB1-CA stable cell clones were passaged 1:5 every 2 days and assayed for KRAS dependency after the fifth passage.

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. Computational analyses of KRAS dependency in CRC cell lines.

(A) Heatmap representation of hierarchical clustering analysis of median-centered log2 transformed probe intensities for the KRAS Dependency Gene Set across a panel of 40 CRC cell lines of various genotypes.

(B) Panther Molecular Function classifications for DEP genes, using the DAVID gene ontology algorithm.

(C) KEGG pathway enrichment in the DEP genes from the KRAS Dependency Gene Set using the DAVID algorithm.

(D) Viral titration curve for SW837 and SW620 cells. Cells were infected with lentiviruses encoding control shGFP and treated with puromycin to select for infected cells, 24h post-infection. Relative cell density was quantitated 6 days post-infection. Data are presented as the mean of three experiments +/- SEM.

(E) Representative examples of kinase knockdown assays. Scans of cells fixed and stained with syto-60 dye in 96-well plates following knockdown of indicated kinases with 5 different shRNAs per gene (shA through shE). shGFP is shown as a control.

(F) Quantitation of well intensities for the scans shown in Figure S2A. Representative examples of effects on growth and viability following shRNA knockdown of kinase expression for *MAP3K7, VRK2* and *CHUK* in SW837 and SW620 cells. Five individual shRNAs were used (shA through shE), represented by different colors. Relative cell densities are shown, normalized to shGFP control expressing cells for 3 different viral titers (MOIs of 4, 2 and 1). Data are represented as the mean of triplicates +/- SEM.

Figure S2. Pharmacological profiling of TAK1 inhibitor sensitivity in colorectal, pancreatic and lung cancer cell lines.

(A) 5Z-7-oxozeaenol IC50 values for colorectal cancer cell lines of various genotypes as well as 2 “normal” epithelial cell lines, MCF10A and MDCK (light gray bars). Data are represented as the mean of 3 independent experiments +/- SEM.

(B) 5Z-7-oxozeaenol IC50 values for *KRAS* mutant PDAC and NSCLC cell lines.

Figure S3. K-means clustering and CRC patient clustering analyses of the KRAS dependency gene set.

(A) K-means clustering (k=3) of CRC cell lines. Node averages are depicted in the heat map, representing median-centered values.

(B) Correlations between 5Z-7-Oxozeaenol IC50 values (µM) and Expression Scores for Nodes 0 and 8 from the K-means clustering analysis.

(C) Comparison of average expression scores for Nodes 0 and 8 genes for CRC patients genotyped for *APC* and *KRAS* mutations.

(D) Comparison of expression of two Wnt target genes *MYC* and *TCF7* in CRC patients.

(E) Correlation between TAK1 dependency gene expression and the RDI values for a panel of 12 *KRAS* mutant CRC cell lines.

Figure S4. Regulation of canonical Wnt signaling by KRAS and TAK1.

(A) Imaging of raw luciferase activity showing TOP-FLASH reporter activity in SKCO1 cells following KRAS depletion.

(B) Laser confocal micrographs showing E-cadherin (red) and β-catenin (green) localization in representative KRAS-independent and KRAS-dependent cell lines following treatment with either DMSO vehicle or 5μM 5Z-7-oxozeaenol. DAPI-stained nuclei are shown in blue. Scale bar = 20 μm.

(C) Raw well scans showing cell growth following treatment of HT29 cells expressing oncogenic mutants of the indicated Ras proteins at various doses of 5Z-7-oxozeaenol.

(D) Imaging of TOP-FLASH activity of C2BBe1 cells expressing mutant KRAS (G12V) at two different viral titers (MOI-1 and MOI-5) and treated with various concentrations of 5Z-7-oxozeaenol.

(E) Imaging and quantitation of TOP-FLASH activity of C2BBe1 and HT29 cells expressing mutant KRAS (G12V) at varying viral titers and pre-treated with the indicated concentrations of 5Z-7-oxozeaenol.

(F) TOP-FLASH reporter activity in *KRAS* mutant PDAC cell lines following inhibition of GSK-3 kinase with increasing concentrations of the small molecule inhibitor BIO. PANC-

1 (red text) are KRAS-independent cells and YAPC (green text) are KRAS-dependent cells. Luminescence counts (photons/sec) are plotted on the y-axis. Data are representative of three independent experiments +/- SEM.

(G) TOP-FLASH reporter dose-response relationships in PANC-1 and YAPC cells following combined treatment with GSK-3 and TAK1 inhibitors (BIO and 5Z-7-Oxozeaenol, respectively). Luminescence counts (photons/sec) are plotted on the y-axis. Data are represented as the means of triplicates +/- SEM.

(H) Effects of combined GSK-3 and TAK1 inhibition on proliferation and viability of PANC-1 and YAPC cells. Relative cell density following 3 days of combination treatment is shown. Data are represented as the means of three independent experiments +/- SEM.

Figure S5. Effects of BMP7 depletion on SW837 cells.

(A) Effects on proliferation and viability of SW837 cells following depletion of BMP7 with five individual shRNAs. Data are plotted relative to shGFP control expressing cells. Data are represented as the mean of 3 independent experiments +/- SEM.

(B) Effects of BMP7 disruption on PARP and caspase-3 cleavage, 4 days post-infection with shRNA expressing lentiviruses. GAPDH serves as a loading control.

Figure S6. Relationships between β-catenin/BMPR1A/NF-κB activity and KRAS/TAK1 dependency.

(A) Introduction of constitutively-active β-catenin (CTNNB1-CA) to SW620 cells and related effects on KRAS dependency as measured by the RDI. Data are representative of 3 independent experiments +/- SEM.

(B) Effects of KRAS depletion by lentiviral shRNA delivery on apoptosis as measured by caspase 3 and PARP cleavage in vector control or CTNNB1-CA expressing SW620 cells. Total β-catenin expression levels and effects on the Wnt target Axin2 are also shown. GAPDH is shown as a gel loading control. Data are representative of 2 independent experiments.

(C) Effects of CTNNB1-CA expression on TAK1 dependency as assessed by IC50 values for 5Z-7-oxozeaenol in SW620 cells.

(D) Effects of constitutively-active BMP receptor (BMPR1A-CA) on KRAS dependency in SW620 and SKCO1 cells, as measured by the RDI. Panel on the right shows V5 expression of V5-epitope tagged BMPR1A in HT29 cells compared to SW620 cells.

(E) Effects of TAK1 inhibition with 5Z-7-oxozeaenol on NF-κB luciferase reporter activity in SW620/SKCO1 KRAS-dependent cells (left panels) or in HT29 cells -/+ activated KRAS (induced activation with 1μM 4HT).

Table S1. Differentially expressed probe sets comparing KRAS-independent to KRAS-dependent CRC cells. Probe sets with p-values <0.05 and fold expression either >2 (DEP genes) or <0.5 (IND genes) were selected. DEP score is the product of –log (p-value) and log (fold difference) for each probe set. DEP score >0 indicates association with KRAS dependency whereas <0 indicates association with KRAS independency.

Table S2. IC50 values for 5Z-7-oxozeaenol (μM) for CRC cell lines used in this study, with corresponding genotypes for *KRAS, BRAF, APC* and *CTNNB1*. * denotes KRAS-dependent cell lines. WT = wild-type; nd = not determined.