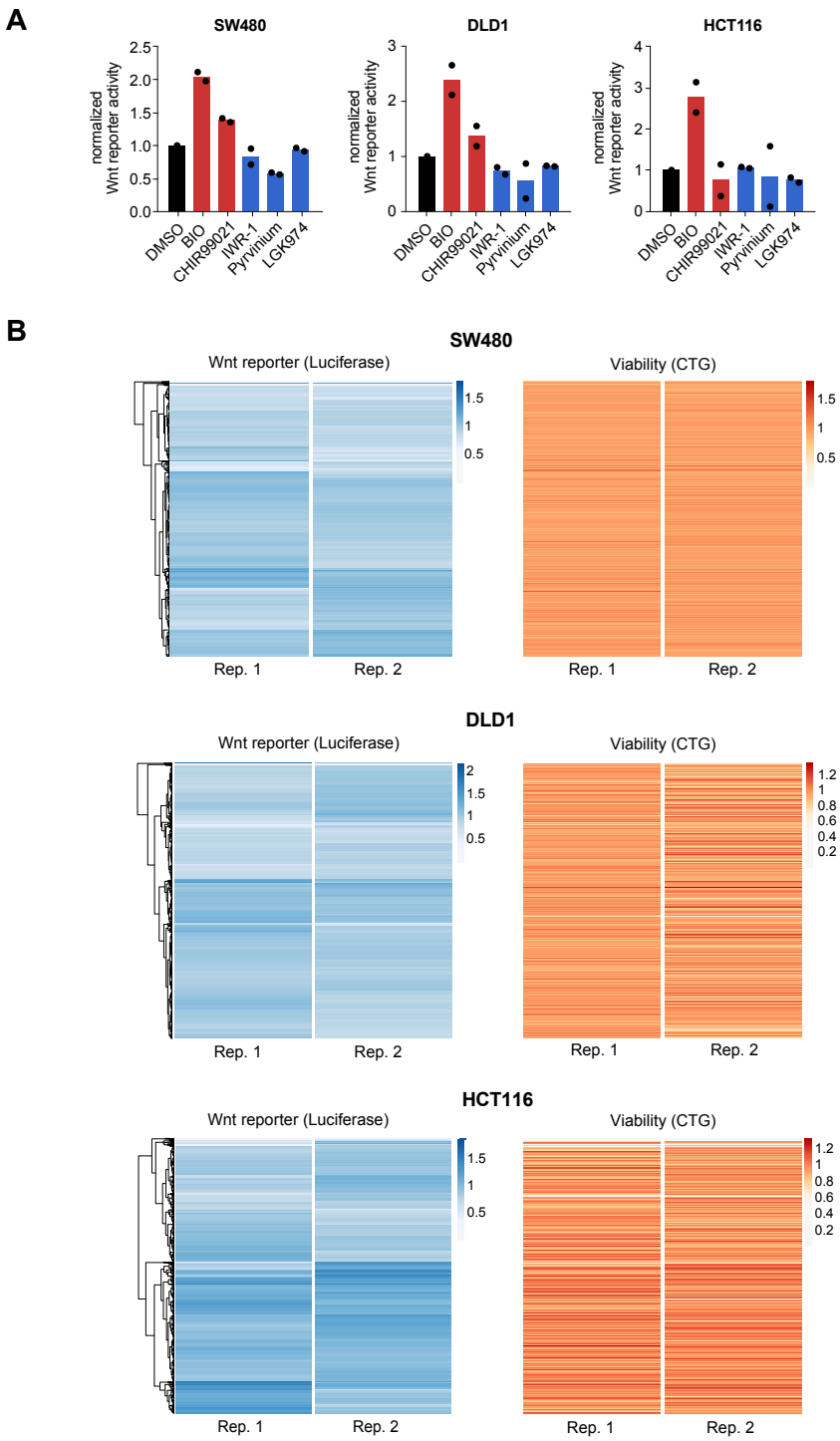


Supplementary Information

MEK inhibitors activate Wnt signalling and induce stem cell plasticity in colorectal cancer

Zhan et al.

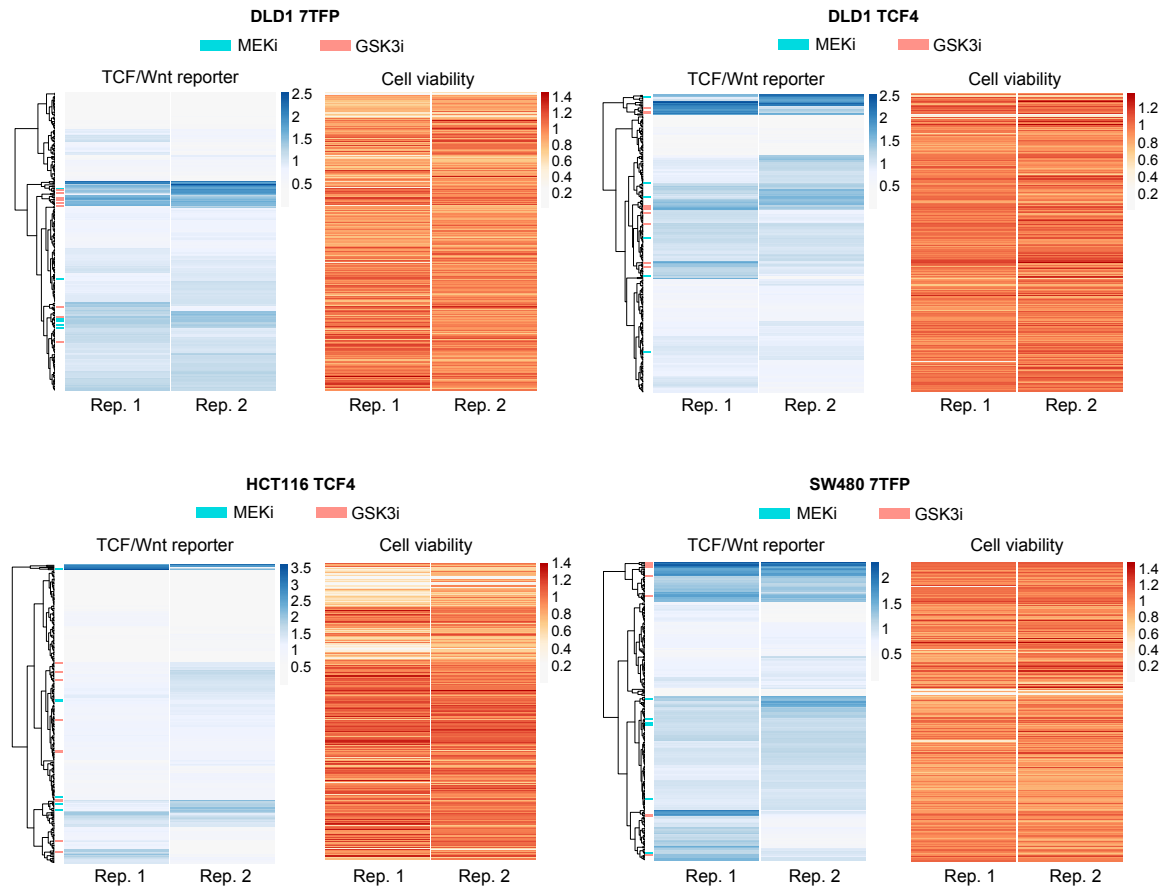
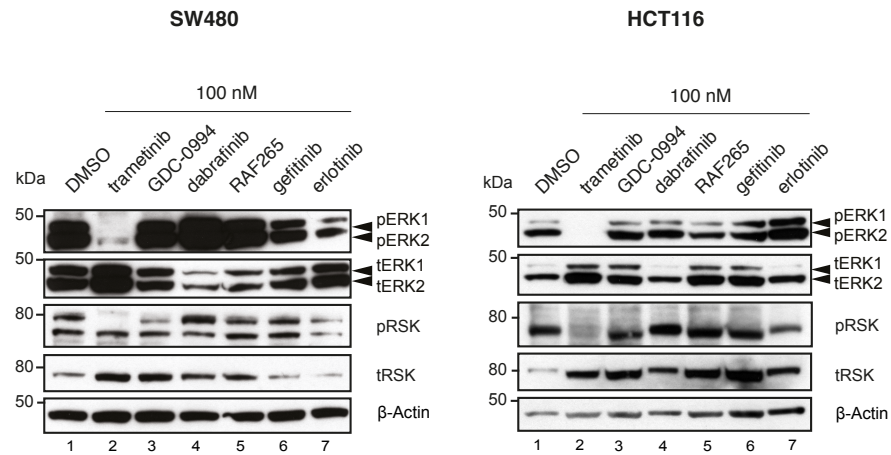
Supplementary Figures



Supplementary Figure 1: Pretests and extended results of the exploratory compound screen for modifiers of Wnt signaling.

(A) Performance of small molecule Wnt activators and inhibitors in reporter cell lines. Wnt reporter cell lines SW480-7TFP, DLD1-7TFP and HCT116-TCF4 were treated with the GSK3B inhibitors BIO and CHIR99021, the tankyrase inhibitor IWR-1, the casein kinase 1 alpha activator pyrvinium and the porcupine inhibitor LGK974 at a concentration of 10 μ M for 24 h, followed by measurement of cell viability and Wnt reporter activity. Data from two independent experiments are presented.

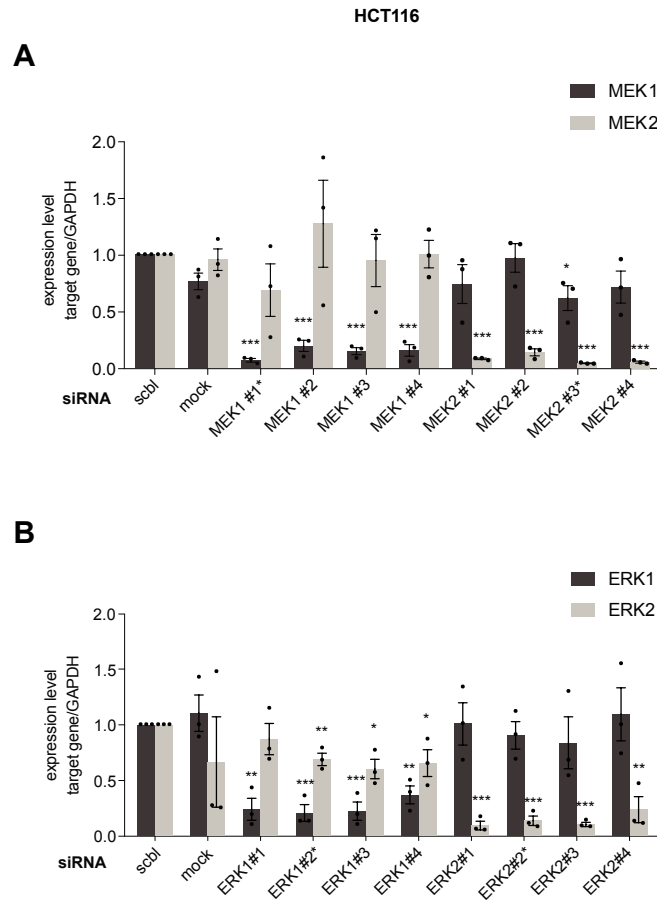
(B) Heatmaps showing TCF/Wnt and viability reporter signals of large exploratory compound screens. The heat maps represent the luciferase (Wnt, left map) and CTG (viability, right map) signals after normalization to the median signal of all samples of the respective plates. Results of both independent biological replicates are shown.

A**B**

Supplementary Figure 2: Extended results of the kinase inhibitor screen for modifiers of Wnt signaling

A) Heatmaps showing TCF/Wnt and viability reporter signals of focused kinase inhibitor screens. The heat maps represent the luciferase (Wnt, left map) and CTG (viability, right map) signals after normalization to the median signal of all samples of the respective plates. Results of both independent biological replicates are shown.

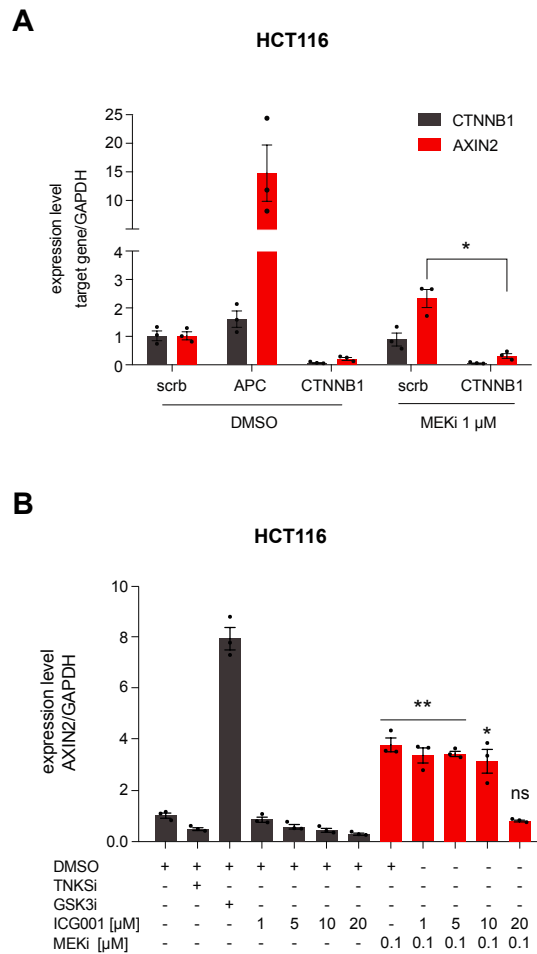
(B) Efficiency of different RAS pathway inhibitors on reducing ERK and RSK phosphorylation. SW480 and HCT116 cells were treated for 24 h with 100 nM of the MEK inhibitor trametinib, the ERK inhibitor GDC-0994, the RAF1/BRAF inhibitors dabrafenib and RAF265 and the EGFR inhibitors gefitinib and erlotinib for 24 h. Cells were then lysed for Western Blot analysis. A representative image of three independent biological replicates is shown.



Supplementary Figure 3: Performance of individual siRNAs targeting MEK1/2 and ERK1/2.

(A-B) Efficiency of individual siRNAs targeting MEK1/2 (A) and ERK1/2 (B). HCT116 cells were transfected with the indicated siRNAs for 72 h, followed by measurement of expression levels of target gene by qPCR. siRNA selected for combinatorial knockdown are marked by (*).

(A-B) Data from three independent experiments are presented as mean $\pm$ s.e.m. ** $p < 0.01$, *** $p < 0.001$, two-sided Student's t-test.

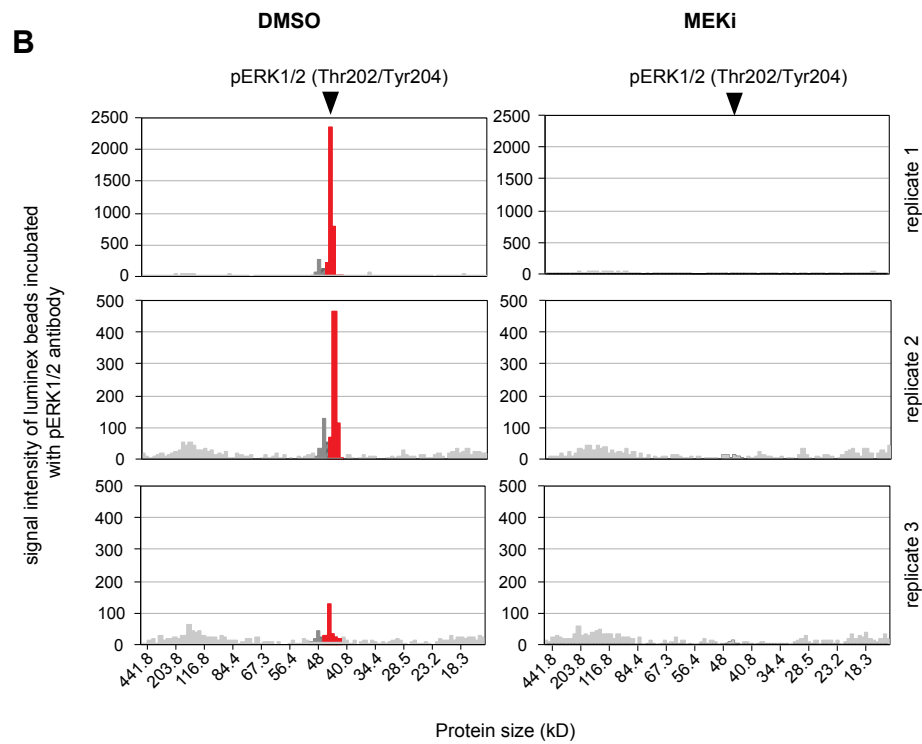
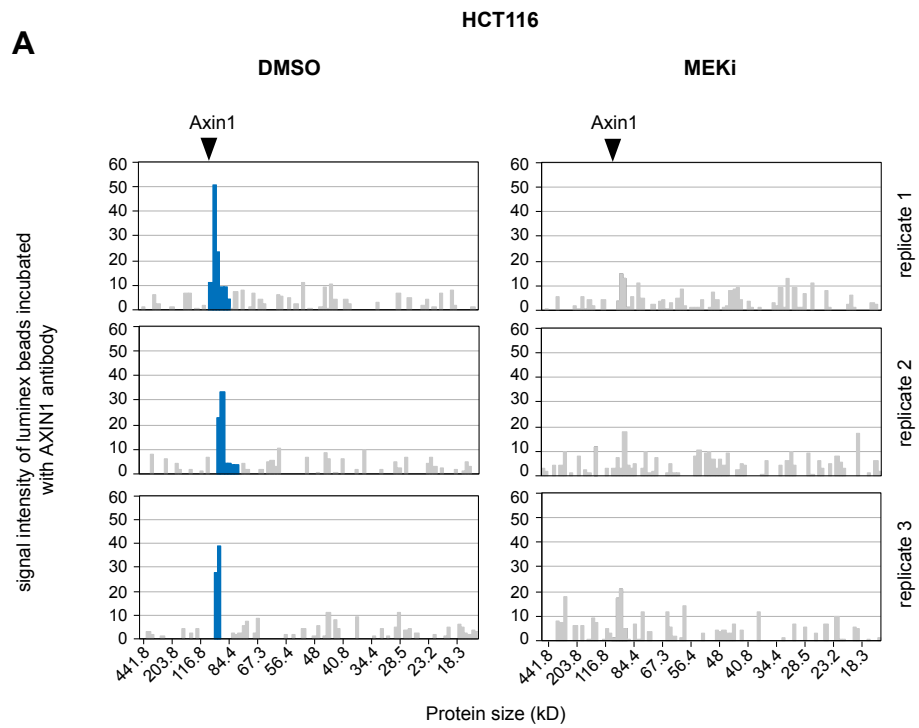


Supplementary Figure 4: MEK inhibitor induced Wnt activation is dependent on beta-catenin.

(A) siRNA mediated knockdown of beta-catenin (CTNNB1) abolishes the effect of MEK inhibition on Wnt activity. HCT116 cells were transfected with the indicated siRNAs for 48 h, followed by 24 h of incubation with trametinib. Expression levels of target genes were measured by qPCR.

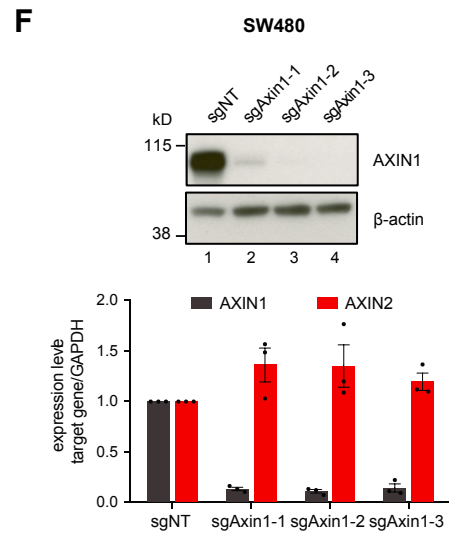
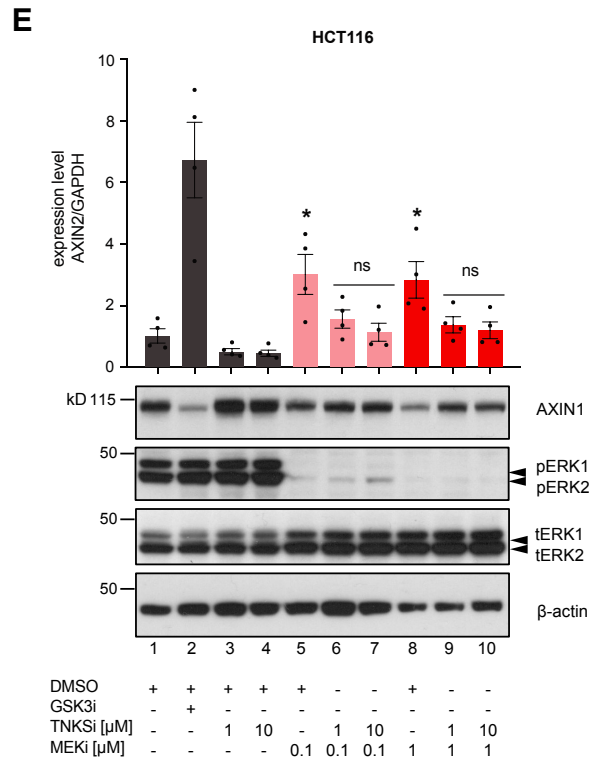
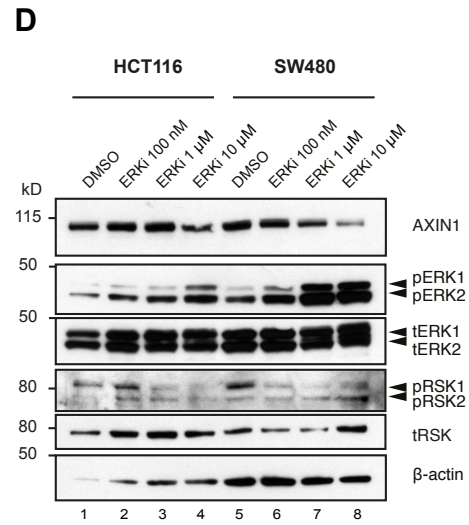
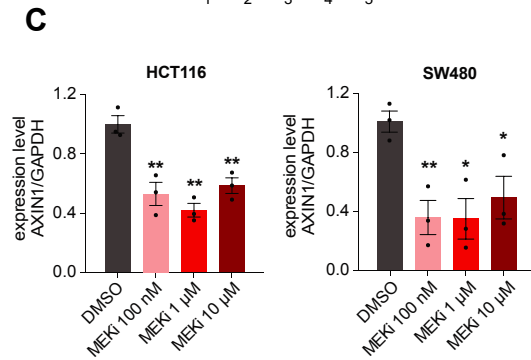
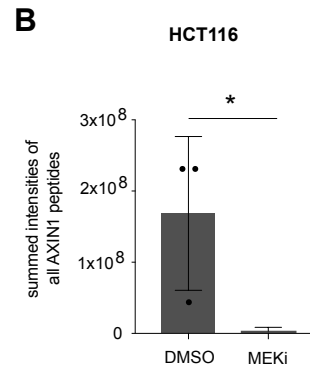
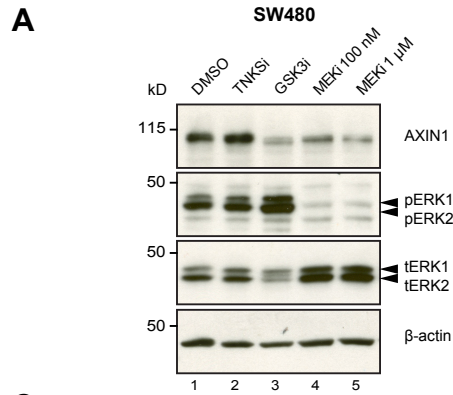
(B) Pharmacological inhibition of CTNNB1/CBP interaction abolishes the effect of MEK inhibition on Wnt activity. HCT116 cells were treated for 24 h with the MEK inhibitor trametinib alone or together with different concentrations of ICG-001, a small molecule inhibitor of CTNNB1/CBP interaction. *AXIN2* expression was measured by qPCR. At high concentrations of ICG-001, the stimulating effect of trametinib on *AXIN2* expression is abolished.

(A-B) Data from three independent experiments are presented as mean $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$, two-sided Student's t-test.



Supplementary Figure 5: DigiWest demonstrates reduction of AXIN1 protein levels upon trametinib treatment.

(A) HCT116 cells were treated for 24 h with 1 μ M of trametinib and then lysed and processed for DigiWest analysis. The signal intensity of Luminex beads incubated with an anti-AXIN1 (blue) and anti-phosphoERK1/2 (Thr202/Tyr204) antibody (red) is shown for all three biological replicates.



Supplementary Figure 6: AXIN1 protein levels determine the effect of trametinib on Wnt signaling.

(A) Western Blot showing reduction of AXIN1 protein levels in SW480 cells upon MEK inhibition. Colorectal cancer cells were treated for 24 h with the indicated compounds and then lysed for Western Blot analysis. A representative image of three independent biological replicates is shown.

(B) Affinity purification of AXIN1 and subsequent mass spectrometry analysis reveals an approximately 50-fold reduction in AXIN1 abundance upon treatment with trametinib. Data from three independent experiments are presented as mean $\pm$ s.e.m. * $p < 0.05$, one-sided Student's t-test.

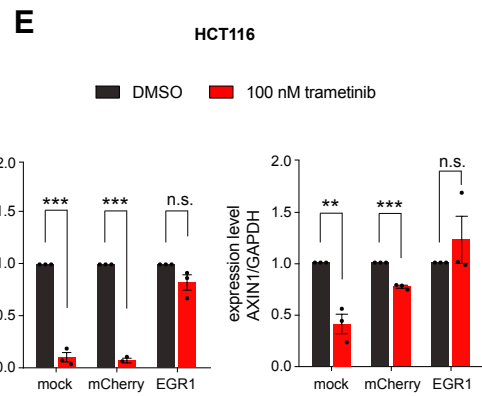
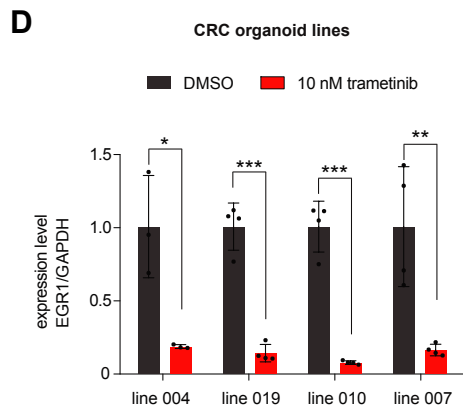
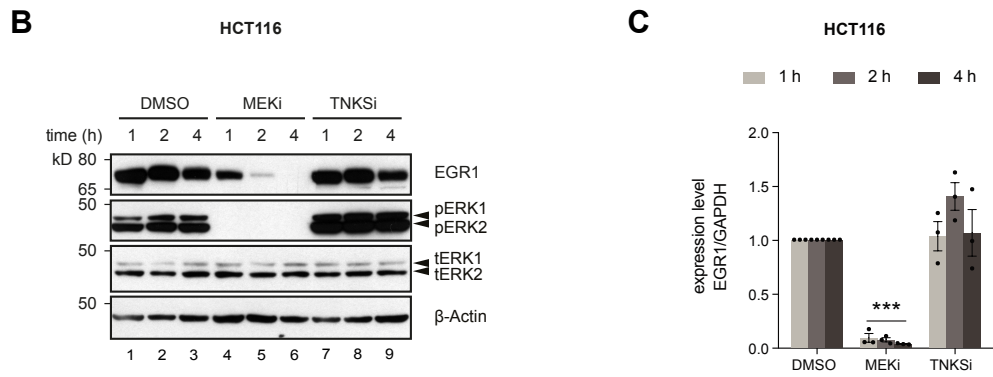
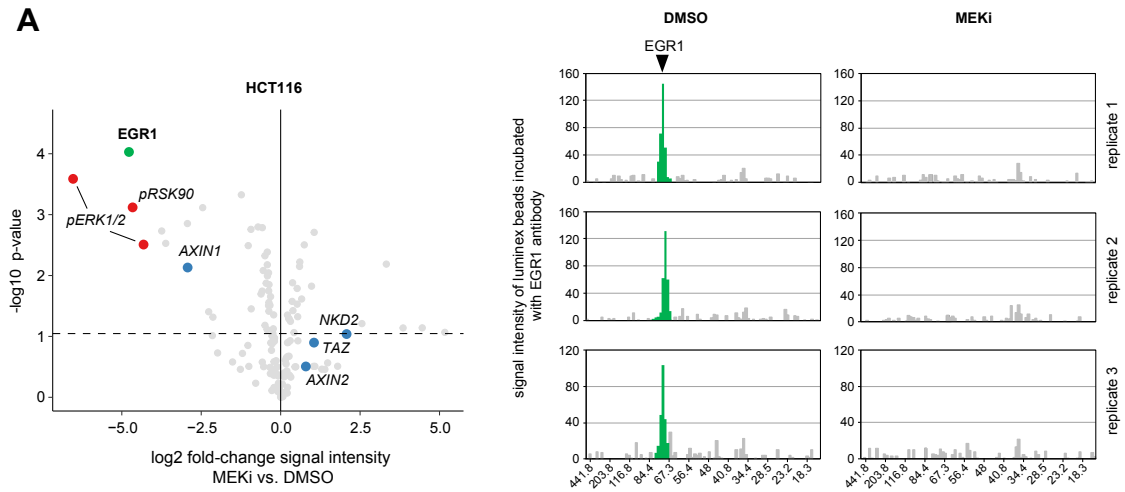
(C) Trametinib causes a reduction of *AXIN1* expression. SW480 and HCT116 cells were treated for 24 h with increasing concentrations of trametinib and expression of *AXIN1* and *GAPDH* were determined by qPCR.

(D) Western Blot showing downregulation of AXIN1 protein levels in HCT116 and SW480 cells upon ERK inhibition. Colorectal cancer cell lines were treated for 24 h with the indicated compounds and then lysed for Western Blot analysis. A representative image of three independent biological replicates is shown.

(E) Stabilization of AXIN1 by treatment with tankyrase inhibitor XAV939 abolishes MEK inhibitor induced Wnt activation. HCT116 cells were co-treated with different concentrations of trametinib and XAV939. Western Blot analysis shows that protein levels of *AXIN1* was reduced by trametinib treatment, but restored by co-treatment with XAV939. Treatment with trametinib alone increased *AXIN2* expression, but co-treatment with XAV939 abolished this effect. A representative image of three independent Western Blot replicates is shown.

(F) CRISPRi mediated knockdown of *AXIN1* mildly stimulates *AXIN2* expression in SW480. SW480 cells stably expressing dCas9-KRAB and sgRNAs targeting *AXIN1* were generated by lentiviral infection and antibiotic selection. Expression levels of *AXIN1* and *AXIN2* were determined by qPCR. Efficiency of knockdown was additionally measured by Western Blot.

(B-D) Data from three (C, F) or four (E) independent experiments are presented as mean $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-sided Student's t-test.



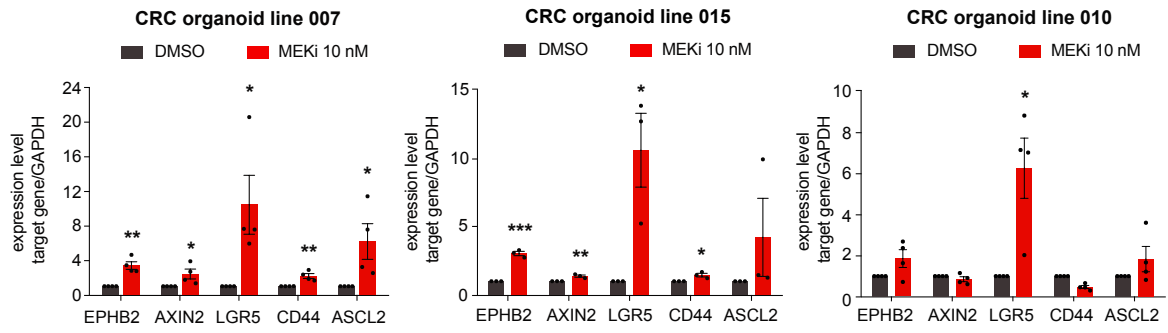
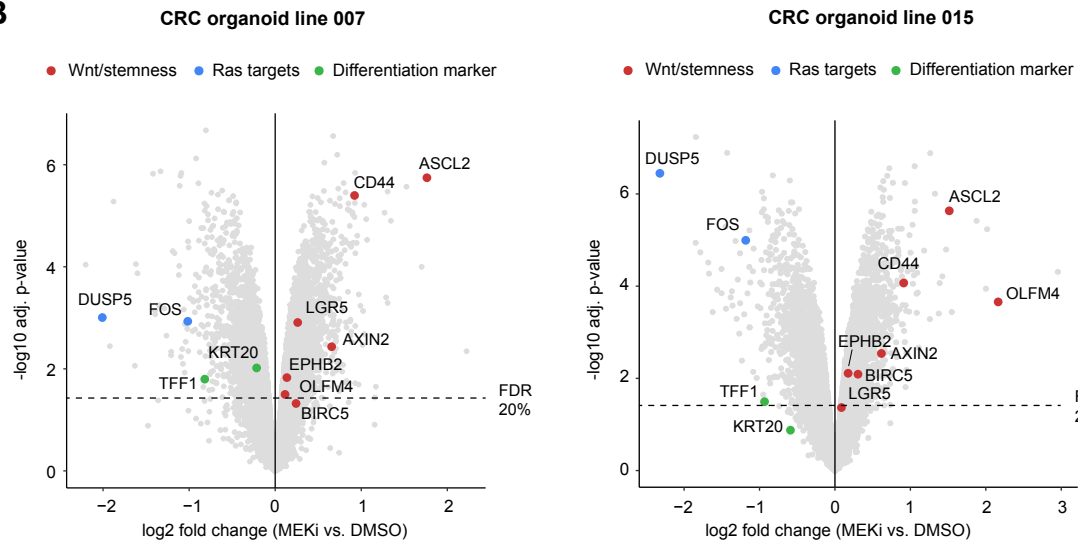
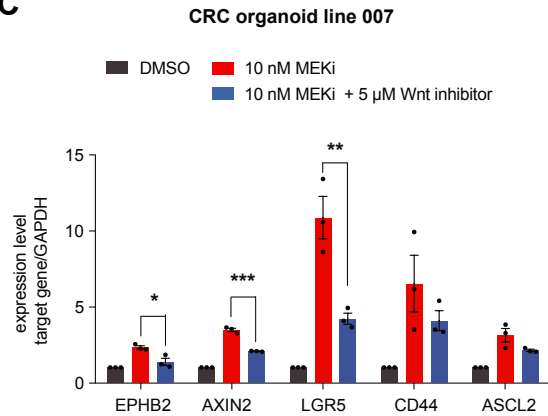
Supplementary Figure 7: Trametinib causes rapid downregulation of EGR1 levels in CRC.

(A) Volcano plot showing signal intensity of EGR1 (green) and selected Wnt and Ras pathway members in trametinib versus DMSO treated HCT116 cells as determined by DigiWest analysis. Mean values of three independent replicates are shown. Statistical significance was determined by a moderated t-test. (B) The signal intensity of Luminex beads incubated with an anti-EGR1 antibody (green) is shown for all three biological replicates.

(C-D) MEK inhibition induces a rapid depletion of EGR1 protein levels and expression. Trametinib (100 nM), XAX939 (10 μ M) and DMSO were added to cells, followed by parallel determination of protein (C) and transcript levels (D) at different time points (1, 2 and 4 h). Representative image of three biological replicates is presented in (C). Data from four independent experiments are presented as mean $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-sided Student's t-test.

(E) MEK inhibition downregulates expression of *EGR1* in colorectal cancer organoids. Four colorectal cancer organoid lines were treated for 72 h with the 10 nM trametinib or DMSO and the relative expression of *EGR1* was measured using qPCR. Data from at three (organoid line 004) or four (organoid line 007, 015, 019) independent experiments are presented as mean $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-sided Student's t-test.

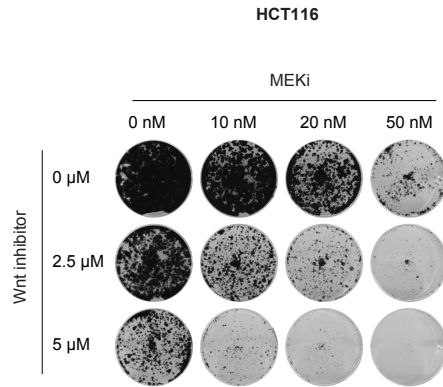
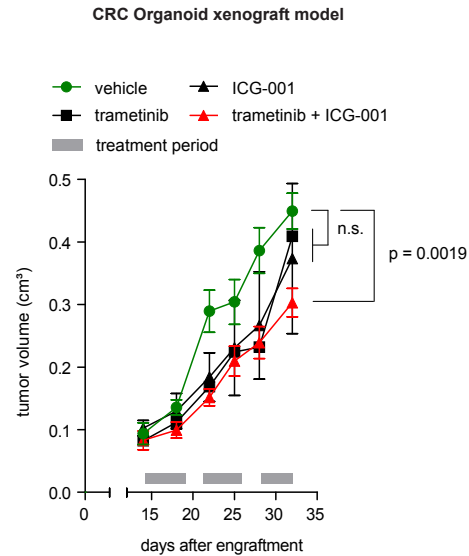
(F) Overexpression of *EGR1* rescues downregulation of *AXIN1* transcript levels by MEK inhibition. HCT116 cells were either mock transfected or transfected with plasmids encoding mCherry or EGR1. Twenty-four hours after transfection, cells were treated with either DMSO or 100 nM trametinib for 4 h. Cells were then harvested and expression levels of *AXIN1* and *EGR1* determined by qPCR. Data from three independent experiments are presented as mean $\pm$ s.e.m. ** $p < 0.01$ and *** $p < 0.001$, two-sided Student's t-test.

A**B****C**

Supplementary Figure 8: Trametinib induces Wnt activity in CRC organoids.

(A-B) MEK inhibition induces expression of Wnt target genes and intestinal stemness markers. (A) Three CRC organoid lines were treated for 72 h with the 10 nM trametinib or DMSO and the expression of selected genes was measured using qPCR. Data from three (organoid line 007, 010) or four (organoid line 015) independent experiments are presented as mean $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-sided Student's t-test. (B) Expression analysis with microarray of two colon cancer organoid lines treated with trametinib shows increased expression of Wnt target genes and intestinal stemness markers, and a reduction of RAS target genes and differentiation markers.

(C) Human colorectal cancer organoids were treated with 10 nM of trametinib and 5 μ M of the Wnt inhibitor PRI-724. Expression of selected Wnt target and stemness genes was analysed by qPCR. Data from three independent experiments are presented as mean $\pm$ s.e.m. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, two-sided Student's t-test.

A**B**

Supplementary Figure 9: Co-inhibition of Wnt and Ras signalling reduces proliferation of CRC.

(A) Co-treatment of trametinib with PRI-724 synergistically reduces proliferation of colorectal cancer cells. HCT116 were seeded at low density and then treated for six days with combinations of trametinib and PRI-724. Cells were then allowed to recover for four days in media without drugs before fixation and staining with crystal violet solution. A representative image of three independent experiments is shown.

(B) Combined MEK and Wnt inhibition is effective in reducing cancer cell proliferation in a PDX model. Colorectal cancer organoids were engrafted into NOD/SCID gamma mice by subcutaneous injections. Two weeks after engraftment, tumors were treated with trametinib (1 mg kg⁻¹ body weight), ICG-001 (200 mg kg⁻¹ body weight), a combination of both substances or vehicle solution once daily, for a total of 14 d. Seven mice were treated in each group and indicated levels of significance were determined by a two-sided Student's t-test.

Supplementary Tables

Supplementary Table 1: Antibodies used for the DigiWest assays. For antibodies that produce two characteristic signal peaks, both peaks are separately enlisted.

Antibody	Company	Catalogue #	Species
Actin	Santa Cruz	sc-1616	goat
A-Raf	Cell Signaling Technology	4432	rabbit
A-Raf - phospho - Ser299	Cell Signaling Technology	4431	rabbit
A-Raf - phospho - Tyr301/Tyr302	Biorbyt	orb5910	rabbit
ASCL2/Mash2	R&D	AF6539	sheep
Axin1	Cell Signaling Technology	3323	rabbit
Axin2 (conduction)	Cell Signaling Technology	2151	rabbit
Bcl9	ABNOVA	H00000607-M01	mouse
beta-catenin	Cell Signaling Technology	8480	rabbit
beta-Catenin - phospho - Ser552	Cell Signaling Technology	9566	rabbit
beta-Catenin - phospho - Ser675	Cell Signaling Technology	9567	rabbit
beta-Catenin (non-pospho Ser33/37/Thr41; active)	Cell Signaling Technology	8814	rabbit
Bmi1	Cell Signaling Technology	6964	rabbit
BMP4	Epitomics	5163-1	rabbit
b-Raf - phospho - Ser445	Cell Signaling Technology	2696	rabbit
C/EBP alpha - Peak 1	Cell Signaling Technology	8178	rabbit
C/EBP alpha - Peak 2	Cell Signaling Technology	8178	rabbit
C/EBP beta	Cell Signaling Technology	3087	rabbit
C/EBP beta - phospho - Thr235	Cell Signaling Technology	3084	rabbit
Casein kinase 1 alpha	Cell Signaling Technology	2655	rabbit
Casein kinase 1 delta	Abcam	ab85320	mouse
Caseinkinase 1 epsilon	Nanotools	0172-100	mouse
Caseinkinase 2 alpha	Cell Signaling Technology	2656	rabbit
CBP	Cell Signaling Technology	7389	rabbit

CD133	Cell Signaling Technology	3663	rabbit
Cerberus	Santa Cruz	sc-15132	goat
cJun	Cell Signaling Technology	9165S	rabbit
cJun – phospho- Ser63	Cell Signaling Technology	2361S	rabbit
c-Raf	Cell Signaling Technology	9422	rabbit
c-Raf - phospho - Ser259	Cell Signaling Technology	9421	rabbit
c-Raf - phospho - Ser338	Cell Signaling Technology	9427	rabbit
DKK2	Cell Signaling Technology	4683	rabbit
DUSP10 (MKP-5)	Cell Signaling Technology	3483	rabbit
DUSP14 (MKP6, MKP-6, MKP-L)	ProteinTech Group	10172-2-AP	rabbit
DUSP16 (MKP-7)	Abcam	ab65151	rabbit
DUSP4	Abcam	ab72593	rabbit
DUSP6 (MKP3, MKP-3, PYST1)	ProteinTech Group	10433-1-AP	rabbit
Dvl2	Cell Signaling Technology	3224	rabbit
Dvl3	Cell Signaling Technology	3218	rabbit
DVL3 (4D3)	Santa Cruz	sc-8027	mouse
EGFR (ErB-1, HER1) - phospho - Tyr1173	Cell Signaling Technology	4407	rabbit
EGFR (ErB-1, HER1) - phospho - Tyr845	Cell Signaling Technology	2231	rabbit
EGR1	Cell Signaling Technology	4153	rabbit
Elk-1	Cell Signaling Technology	9182	rabbit
Erk1/2 (MAPK p44/42) - Peak 2	Cell Signaling Technology	4695	rabbit
Erk1/2 (MAPK p44/42) - phospho - Thr202/Tyr204 - Peak 1	Cell Signaling Technology	4370B	rabbit
Erk1/2 (MAPK p44/42) - phospho - Thr202/Tyr204 - Peak 2	Cell Signaling Technology	4370B	rabbit
Erk2 (MAPK p42)	Cell Signaling Technology	9108	rabbit
Evi - Peak 1	Protein Tech	17950-1-AP	rabbit
Evi - Peak 2	Protein Tech	17950-1-AP	rabbit
FABP4	Cell Signaling Technology	3544	rabbit
FRA1	Cell Signaling Technology	5281	rabbit

FRA1 - phospho - Ser265	Cell Signaling Technology	5841	rabbit
FRA2	Abcam	ab124830	rabbit
Frizzled 1	Abcam	ab71342	rabbit
Frizzled 7	Abgent	AB64636	rabbit
GAPDH	Abcam	ab9485	rabbit
GSK3 alpha/beta - phospho - Ser21/Ser9 - Peak 1	Cell Signaling Technology	9331	rabbit
GSK3 alpha/beta - phospho - Ser21/Ser9 - Peak 2	Cell Signaling Technology	9331	rabbit
GSK3 alpha/beta - phospho - Tyr279/Tyr216 - Peak 1	Abcam	ab68476	rabbit
GSK3 alpha/beta - phospho - Tyr279/Tyr216 - Peak 2	Abcam	ab68476	rabbit
GSK3 beta	Cell Signaling Technology	9315	rabbit
GSK3 beta - phospho - Ser9	Cell Signaling Technology	9336	rabbit
HRAS	Santa Cruz	sc-29	mouse
Her2	Dako	A0485	rabbit
HIF1 beta (ARNT)	Cell Signaling Technology	3718	rabbit
Ki-67	USBiological	K1700-05D	rabbit
KLF2	Sigma	AV37859	rabbit
KLF4	Cell Signaling Technology	12173S	rabbit
KSR1 - phospho - Ser392	Cell Signaling Technology	4951	rabbit
LRP6	Cell Signaling Technology	3395	rabbit
LRP6 – phospho – Ser1490	Cell Signaling Technology	2568S	rabbit
MEK1	Cell Signaling Technology	2352	mouse
MEK1 - phospho - Thr286	Epitomics	2336-1	rabbit
MEK1 - phospho - Thr292	Epitomics	2233-1	rabbit
MEK1/2 - phospho - Ser217/Ser221	Cell Signaling Technology	9154	rabbit
MEK2	Cell Signaling Technology	9125	rabbit
MEKK3	Transduction Laboratories	M79820	mouse
MKK3/6– phospho- Ser189/Ser207	Cell Signaling Technology	9236	rabbit
Mnk1	Cell Signaling Technology	2195	rabbit

Mnk1 - phospho - Thr197/Thr202	Cell Signaling Technology	2111	rabbit
MSK1	Cell Signaling Technology	3489	rabbit
MSK1 - phospho – Thr589	Cell Signaling Technology	9595P	rabbit
Mst1	Cell Signaling Technology	3682	rabbit
MVP (LRP)	Transduction Laboratories	L44820	mouse
Naked 1	Cell Signaling Technology	2262	rabbit
Nanog	Cell Signaling Technology	4893	rabbit
PCNA	Abcam	ab29	mouse
PTPRR (PTP-SL, PTP13)	Sigma	AV45387	rabbit
Ras	Cell Signaling Technology	8955	rabbit
RKIP (PBP, PEBP, PEBP1)	Cell Signaling Technology	4742	rabbit
RSK 1 (p90RSK)	Epitomics	2004-1	rabbit
RSK 1 (p90RSK) - phospho - Ser380	Cell Signaling Technology	9341	rabbit
RSK 1 (p90RSK) - phospho - Thr573	Abcam	ab62324	rabbit
RSK 1/2/3	Cell Signaling Technology	9347	rabbit
RSK 3 - phospho - Thr353/Thr356	Epitomics	2012-1	rabbit
RSK 4 - phospho - Ser235	Epitomics	2151-1	rabbit
SFRP2	Abcam	ab111874	rabbit
Smad3	Cell Signaling Technology	9523	rabbit
Smad3 - phospho - Ser423/Ser425	Cell Signaling Technology	9520	rabbit
Smad4	Cell Signaling Technology	9515	rabbit
SOX17	Sigma	SAB3300093	rabbit
SOX9	Sigma	HPA001758	rabbit

Supplementary Table 2: Mutations of selected Wnt/Ras pathway component as determined by amplicon sequencing of frequently mutated loci in CRC

Gene	Line 004	Line 007	Line 010	Line 015	Line 019
KRAS		G12D	G13D	G13D	A146T
NRAS	Q61L				
PIK3CA	Q546P				
AKT1		E17K			
PTEN				T160I	
ARID1A					W1844*
APC		R564*/ R60*			Q1378*
CTNNB1				G34V	
FBXW7				R465C	
TCF7L2				R465X	

Supplementary Table 3: Primer for quantitative PCR

Target gene	Species	Forward Primer Sequence	Reverse Primer Sequence
GAPDH	human	agccacatcgctcagacac	gccaatacgaccaaatcc
AXIN1	human	atggagctctccgagacaga	tagtacgccacaacgatgct
AXIN2	human	agagcagctcagcaaaaagg	ccttcatacatcgggagcac
LGR5	human	ttcccaggagtggtattctat	accagactatgcctttggaac
ASCL2	human	gcaccaacacttgagatttt	aatggattctctgtgcccttag
CD44	human	caagcaggaagaaggatggat	aacctgtgtttggatttcag
EPHB2	human	agcggcaagatgtactcca	ggagccgatgatgagtgg
MAP2K1	human	tttaggaaaagttagcattgctgt	agggcttgacatctctgtgc
MAP2K2	human	ttgaactcctggactatatgtgaac	aagtcgggggtgaacaca
MAPK3	human	ccctagcccagacagacatc	Gcacagtgtccattttctaacagt
MAPK1	human	caaagaactaattttgaagagactgc	tcctctgagccctgtcct
APC	human	gcatggaccaggacaaaaat	gaacacacacagcaggacagat
CTNNB1	human	gctttcagttgagctgacca	caagtccaagatcagcagtctc
EGR1	human	agccctacgagcacctgac	ggtttgctggggttaactg
SDHA	mouse	tggtcagttccaccccaca	tctccacgacacccttctgt
AXIN2	mouse	aggatgctgaaggctcaaag	tcgccttctgaaataataacctg
LGR5	mouse	cttcactcgggtgcagtgt	gatcagccagctaccaaattagg
ASCL2	mouse	gagagctaagcccgatgga	aggtccaccaggagtcacc
BIRC5	mouse	cccgatgacaacccgata	catctgcttctgacagtgagg
EPHB2	mouse	tagacatgccttgacacaacc	gtctcgttgacgtggagat
CCDN1	mouse	tttcttccagagtcacaaagtgt	tgactccagaagggttcaa

Supplementary Table 4: List of antibodies

Antibody	Company	Catalogue #	Species	Dilution
AXIN2 (76G6)	Cell Signaling Technology	#2151	rabbit	1 : 1000
Vinculin	Millipore	AB6039	rabbit	1 : 1000
Phospho-p90RSK (Ser380)	Cell Signaling Technology	#9341	rabbit	1 : 1000
RSK1/RSK2/RSK3 (D7A2H)	Cell Signaling Technology	#14813	rabbit	1 : 1000
p44/42 MAPK (Erk1/2)	Cell Signaling Technology	#9102	rabbit	1 : 1000
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	Cell Signaling Technology	#4370	rabbit	1 : 1000
AXIN1 (C76H11)	Cell Signaling Technology	#2087	rabbit	1 : 1000
beta-actin	Santa Cruz Biotechnology	47778	rabbit	1 : 40.000
beta-actin HRP	Santa Cruz Biotechnology	47778	rabbit	1 : 250.000
beta-catenin	BD Biosciences	610154	mouse	1 : 2000
GSK-3beta	BD Biosciences	610201	mouse	1 : 1000
APC (ALI 12-28)	Santa Cruz	53165	mouse	1 : 1000
EGR1	Cell Signaling Technology	15F7	rabbit	1 : 1000
IgG	Cell Signaling Technology	2729	rabbit	
IgG1 K	eBioscience	16-4714-81	mouse	

Supplementary Table 5: Sequences of siRNA reagents (related to Methods). siRNA reagents used for combinatorial knockdowns are highlighted by bold letters.

Target	Company and catalogue #	siRNA #	Sequence
MAPK1	MU-003555-04-0002 SMARTpool single sequences, GE health science	#1	CCAAAGCUCUCGGACUUAUU
		#2	AAACAGAUUUUACAAGCU
		#3	CAAGAGGAUUGAAGUAGAA
		#4	GUACAGGGCUCCAGAAAUU
MAPK3	MU-003592-01-0002 SMARTpool single sequences GE health science	#1	GACCGGAUGUUAACCUUUA
		#2	AGACUGACCUGUACAAGUU
		#3	GCUACACGCAGUUGCAGUA
		#4	CCGGCUUCCUGACGGAGUA
MAP2K1	MU-003571-01-0002 SMARTpool single sequences GE health science	#1	GCACAUGGAUGGAGGUUCU
		#2	GCAGAGAGAGCAGAUUUGA
		#3	GAGCAGAUUUGAAGCAACU
		#4	CCAGAAAGCUAAUUCAUCU
MAP2K2	MU-003573-03-0002 SMARTpool single sequences GE health science	#1	CAAAGACGAUGACUUCGAA
		#2	GAUCAGCAUUUGCAUGGAA
		#3	GGAAGCUGAUCCACCUUGA
		#4	GAAAGUCAGCAUCGCGGUU
AXIN1	MU-009625-01-0002 SMARTpool single sequences GE health science	#2	GAAAGUGAGCGACGAGUUU
		#3	GAGCAAGUUUCACCGAAGA
		#5	CGAGAGCCAUCUACCGAAA
CTNNB1	s438 (Ambion)		CUGUUGGAUUGAUUCGAAAtt
APC	s1433 (Ambion)		GGAUCUGUAUCAAGCCGUUtt
Scrambled	s29712 Silencer Select Negative Control (Ambion)		