Supplementary Tables

Suppl. Table S1. WNT target gene sets used for GSEA

Herbst et al. WNT target gene set				Nusse et al. WNT target gene set
KIAA1199	SLC7A2	SEMA3F	SLC5A6	MYC
NAV2	C12orf24	RNF44	RCL1	MYCN
ASCL2	FGF18	TMEM97	KLHL29	CCND1
EDAR	FGF9	KATNB1	MDN1	HNF1A
ABCB1	GJA3	WFDC9	NHP2L1	TCF7
ADAMTS19	PIK3AP1	HSD11B2	PAICS	LEF1
AXIN2	ADCK3	CR2	DCAF4	PPARD
KCNJ8	ABHD6	LDLRAD3	SORD	JUN
TNFRSF19	ECE2	C5orf13	WDR74	FOSL1
DEPDC7	NPTX2	CDC25A	PTDSS1	PLAUR
SLC4A8	GAS5	AIF1L	NOP2	MMP7
LOC100134361	PPP2R2C	LOC100505644	DDX20	AXIN2
RNF43	BCL11A	RSL1D1	ADCY3	NRCAM
GLS2	SMPDL3B	TMEM80	CD83	TCF4
SLC9B2	MEX3A	QSOX2	SFXN4	GAST
SUSD4	MAOB	TEX10	NME1	CD44
LOC100287482	PDCD2L	TFB2M	MCAM	EPHB2
CTNNB1	PLD6	LOC202181	ADSL	EPHB3
MYC	ACTR3B	GEMIN5	NLE1	BMP4
PXK	TXLNG	KIF9	IMP3	CLDN1
B7H6	NSUN5P1	RFC3		BIRC5
APCDD1	MCOLN2	CCNO		VEGFA
GAD1	RABEPK	SCLY		FGF18
DPYSL3	UBE2CBP	HPCAL4		MET
RORA	POLR3G	KCNJ5		EDN1
TMEM177	C10orf2	LOC100506469		MYC
ISM1	GALNT6	CD44		L1CAM
ZNRF3	SLC16A10	MKI67IP		ID2
VSNL1	SNHG1	SKP2		JAG1
BCL2L15	CSTF3	HAS2		MSL1
LOC100507303	RPIA	MATR3		DKK1
CTDSPL	C7orf40	BCS1L		FGF20
BDNF	C1QTNF9B-AS1	BCL2L11		SOX17
CCNB1IP1	BEGAIN	FXN		SOX9
MIR17HG	SPTLC3	NAV3		TIAM1
SLC2A3	SLC6A16	KIAA1804		LGR5
FKBP7	DKC1	DHX33		WNT

Suppl. Table S2. Gene Expression (real-time RT-PCR) primers used in this study. Primers were selected from the Universal Probe Library (Roche).

Gene	Forward Primer	Reverse Primer
β-ACTIN	CCAACCGCGAGAAGATGA	CCAGAGGCGTACAGGGATAG
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
ADNP #1	CCCATCACTTACGAAAAACCA	GGACATTGCGGAAATGACTT
ADNP #2	GGACCACATTGTCAATTCACACC	GGACAAGCGCTGCAGCAGAAAGG
ADNP #3	GTGACATCGCTTCCCATTTTAG	CCACTCAGCATCAAAATCCATC
AXIN2	AGGCCAGTGAGTTGGTTGTC	CATCCTCCCAGATCTCCTCA

Suppl. Table S3. Antibodies used for immunoblotting and immunohistochemistry.

Antibody	**Species**	**Manufacturer**
ADNP	Rabbit	Proteintech
AXIN2	Rabbit	Cell Signaling
active-β-Catenin	Mouse	Merck Millipore
β-Catenin	Mouse	Merck Millipore
DNMT1	Rabbit	Santa Cruz
TLN1	Mouse	Santa Cruz
LEF1	Rabbit	Cell Signaling
Tubulin	Mouse	Sigma-Aldrich

Suppl. Table S4. Fold change (F.C.) of consistently deregulated genes in three gene expression data sets of colon cancer cells with high vs. low WNT activity, and F.C. in differential expression of these genes in TCGA data in colon cancer vs. normal mucosa. *P* values are t test results.

	WNT high vs. low dataset		**TCGA dataset**	
Gene symbol	**F.C. WNT high vs. low**	***P* value WNT high vs. low**	**F.C. tumor vs. normal mucosa**	***P* value tumor vs. normal mucosa**
INHBB	4.19	0.00005	0.42	0.03477
DPEP1	4.01	0.00039	0.6	0.00344
FN1	3.11	0.00794	0.67	0.19989
ITPR2	2.76	0.00003	1.16	0.43835
DRAM1	2.75	0.0009	1.82	<0.00001
KIF3C	2.48	0.00013	1.32	0.18091
KRT23	2.38	0.00021	0.6	0.00018
PROM1	2.04	0.0002	1.01	0.82154
RGS19	2	0.00064	1.37	0.60719
APOBEC3B	1.95	0.00226	1.5	<0.00001
TCTN1	1.91	0.00284	2.34	<0.00001
ANXA9	1.9	0.00001	1.31	<0.00001
VSNL1	1.86	0.00135	1.14	<0.00001
EML1	1.79	0.04144	0.97	<0.00001
FLNA	1.78	0.00012	0.93	<0.00001
SORBS1	1.77	0.01099	0.87	<0.00001
BCAS3	1.76	0.00219	2.13	0.16157
CEP68	1.71	0.00561	2.35	0.07352
SESN1	1.61	0.00002	1.04	0.00226
SEPT6	1.59	0.00061	1.72	<0.00001
SEZ6L2	1.56	0.00294	1.35	<0.00001
H2AFJ	1.53	0.00221	1.47	0.25736
TTLL5	1.43	0.00167	3.34	0.00099
MSRB2	1.42	0.01328	2.19	0.46093
HYAL2	1.36	0.00463	1.62	<0.00001
CCNF	1.35	0.00203	2.35	<0.00001
CDC45	1.33	0.00322	2.51	<0.00001
SUPT7L	1.29	0.00539	4.2	<0.00001
GINS1	1.29	0.00009	1.75	<0.00001
VPS54	1.24	0.00001	2.91	0.1922
VPS13B	1.22	0.00259	2.3	0.02021
ADNP	1.1	0.0015	2.73	<0.00001
PLCE1	0.7	0.00017	1.61	<0.00001
FA2H	0.67	0.00024	1.79	0.00141
SULT1B1	0.6	0.00004	0.96	<0.00001
RARRES1	0.58	0.00007	0.57	0.01459
SPINK5	0.45	0.01115	0.7	<0.00001
GCNT3	0.43	0.00179	0.71	<0.00001
FCGBP	0.41	0.00084	0.52	<0.00001

Suppl. Table S5. Proteome analysis results upon ADNP depletion. Proteins with significant up- or downregulation are listed (*P* < 0.05; abs. fold change >1.5)

Gene symbol	fold change	*P* value
SCAMP1	17.3	0.04499
RBP1	3.87	0.00969
PFDN1	3.65	0.02272
CBFB	3.29	0.04149
PPP1R14B	3.18	0.00034
ATP5I	2.18	0.02753
IPO11	2.06	0.00486
CLIC4	1.98	0.01883
MOV10	1.95	0.00502
HMGCS1	1.93	0.01151
MSI1	1.89	0.00327
TLN1	1.81	6.1E-06
TBCD	1.75	0.04971
YWHAH	1.73911	0.00148
DSP	1.71421	0.00017
DNMT1	1.65193	0.00004
MTPN	1.59213	0.00055
DCBLD2	1.57902	0.04529
CRABP2	1.56989	0.01137
KIF2A	1.52558	0.04091
ALDH9A1	1.51196	0.00036
GLRX5	0.64654	0.01303
POLR2G	0.55182	0.00224
SMAP	0.54448	0.04437
ASF1B	0.52641	0.01293
HINT2	0.49374	0.02004
DYNC1I2	0.48191	0.02935
ADNP	0.45216	0.00144
UBQLN4	0.44124	0.00544
NAPRT1	0.39740	0.04786
THRAP3	0.20366	0.02930

Suppl. Table S6. Clinical/pathological data and ADNP expression in colorectal cancer.

Characteristics	Total	ADNP expression				*P(score)*	*P(low/high)*
		low		high			
		0	1	2	3		
All patients	221 (100)	33 (14.9)	99 (44.8)	78 (35.3)	11 (5.0)		
Age (y, median 69)							
≤ 68	110 (49.8)	16 (14.8)	45 (41.7)	41 (38.0)	6 (5.6)	0.794	0.336
≥ 69	111 (50.2)	17 (15.0)	54 (47.8)	37 (32.7)	5 (4.4)		
Gender							
Male	121 (54.8)	14 (11.5)	59 (48.4)	42 (34.4)	7 (5.7)	0.344	0.971
Female	100 (45.2)	19 (19.2)	40 (40.4)	36 (36.4)	4 (4.0)		
T-stage (UICC)							
T1	1 (0.5)	0 (0)	1 (100)	0 (0)	0 (0)	0.010	0.067
T2	36 (16.3)	1 (2.7)	18 (48.6)	15 (40.5)	3 (8.1)		
T3	176 (79.6)	27 (15.4)	77 (44.0)	63 (36.0)	8 (4.6)		
T4	8 (3.6)	5 (62.5)	3 (37.5)	0 (0)	0 (0)		
Tumor grade (WHO)							
low grade	200 (90.5)	29 (14.6)	86 (43.2)	73 (36.7)	3 (5.5)	0.308	0.077
high grade	21 (9.5)	4 (18.2)	13 (59.1)	5 (22.7)	0 (0)		
β-Catenin expression							
low	118 (53.4)	22 (18.6)	55 (46.6)	35 (29.7)	6 (5.1)	0.187	0.073
High	103 (46.6)	11 (10.7)	44 (42.7)	43 (41.7)	5 (4.9)		

Column percent values for totals and row percent values for ADNP expression subgroups are given in parentheses. *P(score)* and *P(low/high)* indicate Chi-squared test significance levels when testing characteristics against ADNP expression scores and ADNP low/high categories, respectively.

Suppl. Table S7. Multivariate analysis of cancer specific survival.

Variables	Cancer specific survival		
	HR	(95% confidence interval)	*P*
Age (≥ vs < median)	2.0	(1.03-3.74)	0.040
Gender (F vs M)	0.9	(0.48-1.73)	0.766
T-stage	2.4	(1.04-5.29)	0.039
Tumor grade	1.4	(0.58-3.16)	0.489
β-Catenin (high vs low)	1.5	(0.77-2.75)	0.243
ADNP expression score	0.6	(0.37-0.86)	0.008

Supplementary Materials and Methods

siRNAs, plasmids, transfections, transductions and luciferase assays

For transient knockdown, pre-designed siRNAs targeting *β-Catenin*, *ADNP* (Thermo Fisher), *DNMT1*, or *TLN1* (Dharmacon), or scrambled (siCtrl) were transfected into HCT116 or SW1222 cells at 10 nM final concentration using HiPerFect (Qiagen). 48 h after transfection, cells were harvested for further analysis.

For stable *ADNP* knockdown, oligonucleotides containing specific targeting sequences (sh1ADNP GCCATGATTGGGCACACAAAT, sh2ADNP GCCCGAGAAGAGAGTAGTATT, shCtrl GGCTACGTCCAGGAGCGCACC) were selected from The RNAi Consortium shRNA Library (Broad Institute) and inserted between BamHI and EcoRI restriction sites of the lentiviral pGreenPuro shRNA vector (System Bioscience). Lentivirus was produced in HEK293 cells and used for transduction of HCT116 and SW1222 cells, as previously described (1).

Transient ADNP overexpression was achieved by transfecting HCT116 and SW1222 cells with 1 μg p4.2-hADNP or, as control, empty p4.2 plasmid (a gift from Vivien Bubb), in 6-well plates in the presence of 6 μl FuGENE 6 (Promega).

For luciferase reporter assays, treated cells or cells with stable or transient ADNP knockdown or overexpression were transfected or co-transfected, respectively, in 24-well plates with 10 ng Renilla luciferase control vector and 100 ng TOPflash or FOPflash luciferase reporter constructs carrying either wild-type or mutant TCF-bindings sites (2). Firefly luciferase activity was measured with dual-luciferase Reporter Assays (Promega) after 24 h and normalized to Renilla luciferase activity.

CRISPR/Cas9 genome editing

Synthetic oligonucleotide pairs of two guide sequences targeting the coding region of ADNP (caccgCTACTTGGTGCGCTGGCGTT/ aaacAACGCCAGCGCACCAAGTAGc and caccgCCTGATAGCCTATACGTTCA/ aaacAACGCCAGCGCACCAAGTAGc) were selected using the Zhang Laboratory MIT CRISPR Design Tool. Each pair was annealed and inserted into pSpCas9(bb)-2A-GFP (PX458, a gift from Feng Zhang, Addgene plasmid 48138), resulting in two different ADNP targeting vectors. Both vectors then were co-transfected into HCT116 and SW1222 colon cancer cells in 24-well plates with FuGENE 6. After 2 days, GFP positive single cells were sorted into 96-well plates on a FACSAria III instrument (BD Biosciences) and expanded. Single cell clones with loss of ADNP expression were selected by immunoblotting.

RNA-Seq

Libraries were constructed using the Encore Complete RNA-Seq library system (NuGEN) according to manufacturer's protocol. In brief, 150 ng of total RNA were used for first-strand cDNA synthesis and fragmented. cDNA was end repaired, ligated with barcoded adaptors, and the strand selected library was amplified with AMPure XP beads (Beckman Coulter). Barcoded libraries then were quantified, pooled at 10 nM concentration, and sequenced in multiplex on a HiSeq 1500 as 100 b single reads. Data then were demultiplexed, adaptor sequences and polyA tails were removed, and mapped to the hg19 human reference genome. Sequence reads for annotated genes were counted and comparative analyses of gene expression were done with the DEseq2 package with a <5 % false discovery rate (FDR). GSEA on fold change ranked gene lists were done and heat maps were drawn as described in main materials and methods. Significantly upregulated genes were characterized according to signaling pathways using PANTHER version 10.0 (www.pantherdb.org).

Mass spectrometry (MS)

For mass spectrometry (MS) proteome analysis, cells were lysed, sonicated, and centrifuged through QIA-Shredder devices (Qiagen). 10 µg of total protein was reduced with 4.5 mM dithiothreitol for 30 min, alkylated with 10 mM iodoacetamide for 20 min, and incubated overnight at 37 °C with 200 ng porcine trypsin (Promega). For separation an EASY-nLC 1000 chromatography system connected to an Orbitrap XL instrument (Thermo Scientific) was used. 2.5 µg of peptides in 10 µl 0.1 % formic acid were transferred to PepMap100 C18 trap columns and separated at flow rates of 200 nl/min on analytical PepMap RSLC C18 columns (Thermo Scientific) using consecutive linear gradients from 2 % to 25 % solvent B (0.1 % formic acid, 100 % acetonitrile) in 260 min and 25 % to 50 % solvent B in 60 min. For data acquisition, a top five data dependent collision-induced dissociation method was used at a needle voltage of 1.9 kV. MS raw data files were processed with the Homo sapiens subset of the UniProt database and MaxQuant V1.5.1 with the following parameters: (i) enzyme, trypsin; (ii) mass tolerance precursor, 10 ppm; (iii) mass tolerance MS/MS, 0.8 Da; (iv) fixed modification, carbamidomethylation of cysteine; (v) variable modifications, acetylation of protein N-terminus and oxidized methionine. FDRs at peptide and protein levels were set to 1 %. Missing values for proteins detected in at least three replicates per group were handled by MaxQuant imputation. Proteins with log2 fold changes of ± 0.6 at P values < 0.05 were considered relevant.

Supplementary References

1. Horst D, Chen J, Morikawa T, Ogino S, Kirchner T, Shivdasani RA. Differential WNT activity in colorectal cancer confers limited tumorigenic potential and is regulated by MAPK signaling. Cancer Res. 2012;72:1547-56.

2. Veeman MT, Slusarski DC, Kaykas A, Louie SH, Moon RT. Zebrafish prickle, a modulator of noncanonical Wnt/Fz signaling, regulates gastrulation movements. Curr Biol. 2003;13:680-5.