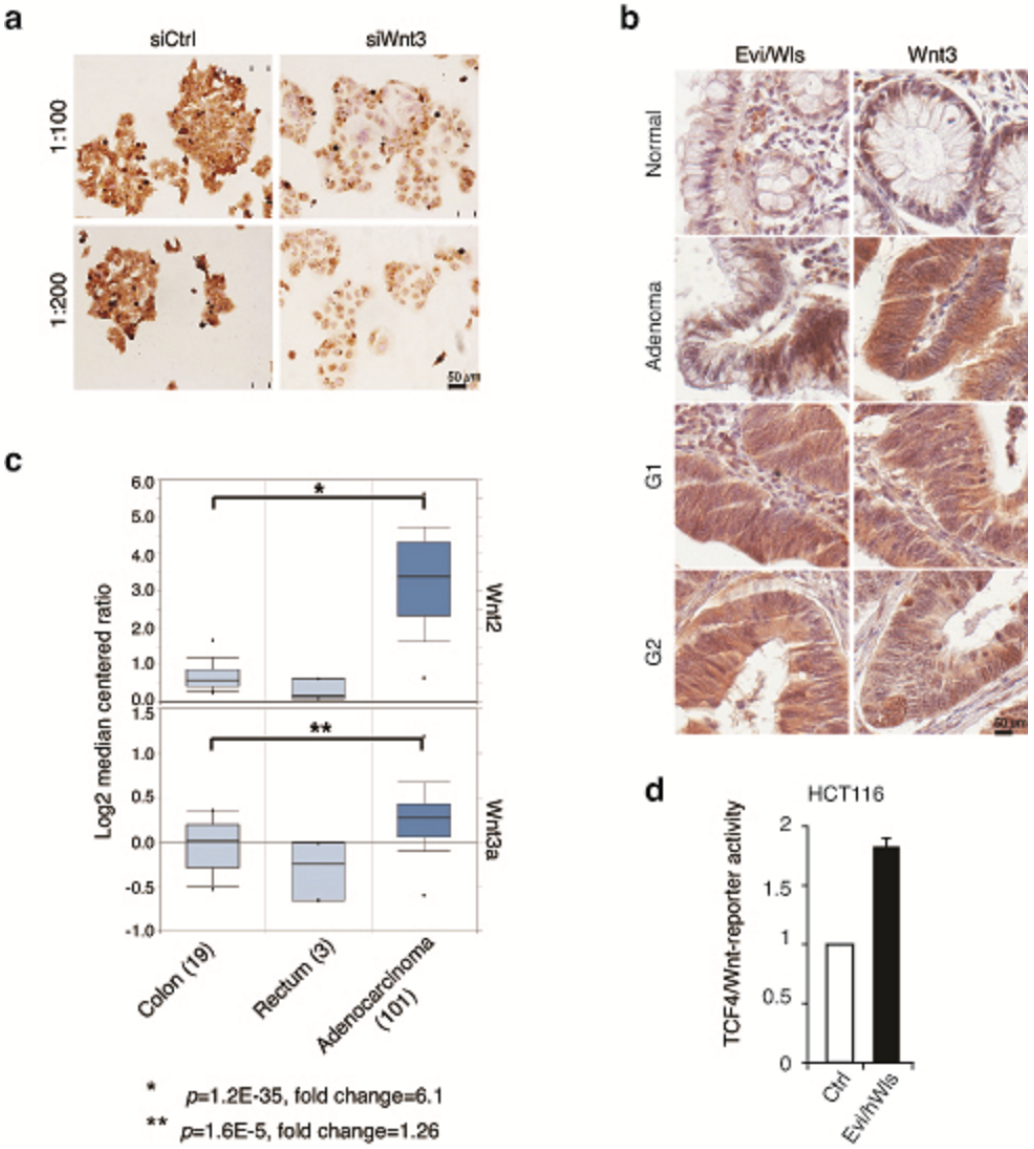
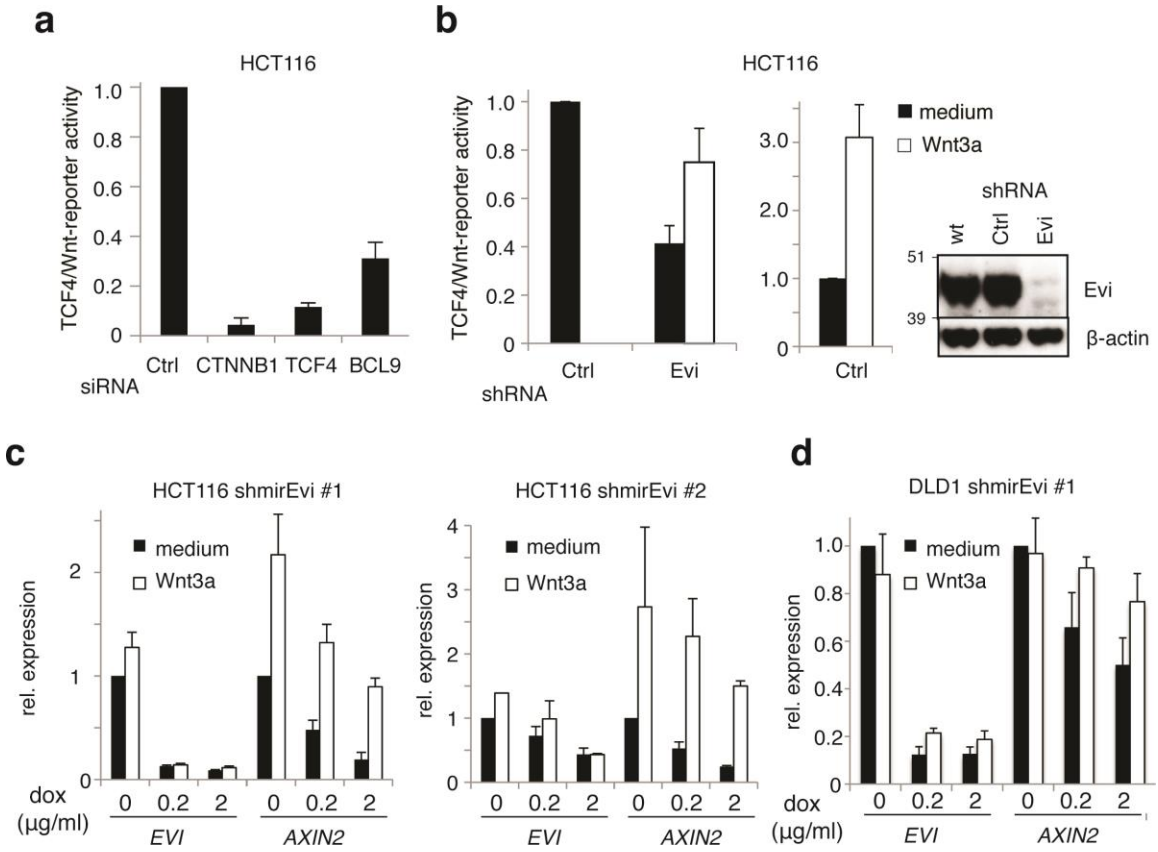
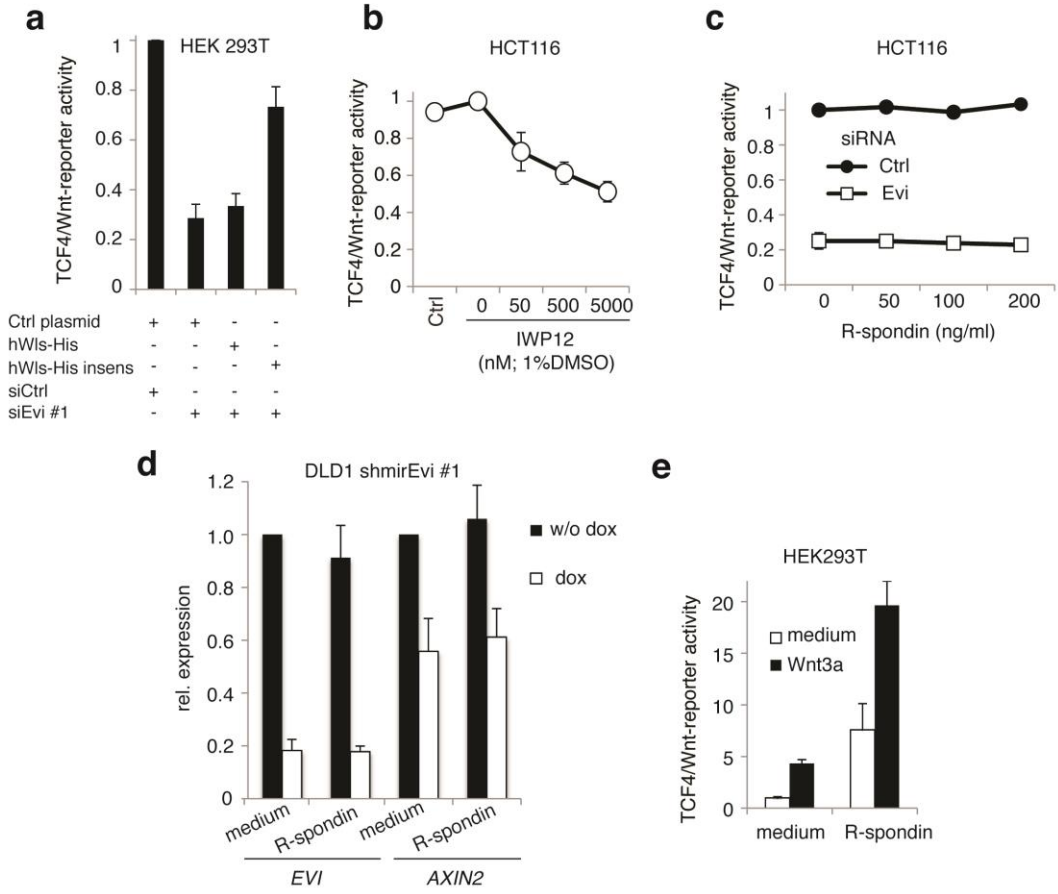


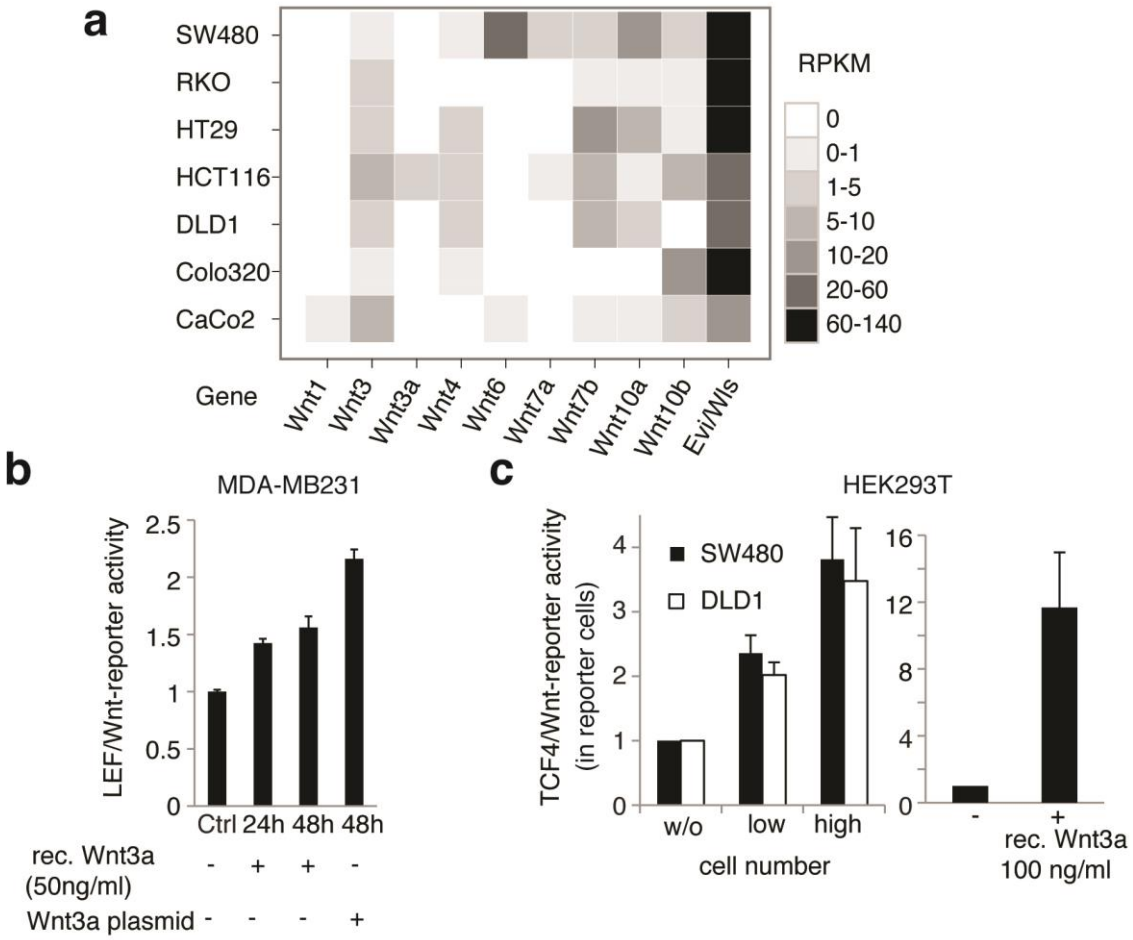
Supplementary Figure S1 Evi/Wls and Wnt3 are overexpressed in epithelial cells in colon cancers. (**a**) Validation of the specificity of the Wnt3 antibody. HCT116 cells were reverse transfected with siControl or siWnt3 #1 for 72 hours, to test for reduction of the antibody signal in the absence of Wnt3; cells were stained according to the protocol for tissue slices. (**b**) Representative immunohistochemical staining for Evi and Wnt3 in normal colon, adenocarcinoma and G1 and G2 carcinoma samples from a colon cancer tumour array. (a,b) Scale bar 50 µm. (**c**) RNA expression of Wnts from TCGA data set was analysed using Oncomine (www.oncomine.org). Pre-processed data from array is log2 transformed and median-centered. The box plots show medians as line within the box, 25th and 75th percentiles as sides of the box, 10th and 90th percentiles as error bars and outliers as circles. Significance of the expression differences was calculated using the Student's *t*-test. Fold change is the magnitude of the difference in normal colon *versus* colon adenocarcinoma samples. (**d**) HCT116 TCF4/Wnt cells were transfected with Wls/Evi-plasmid for 48 hours, and then luciferase activity was measured. Data from 3 independent experiments are presented as mean ± s.e.m.



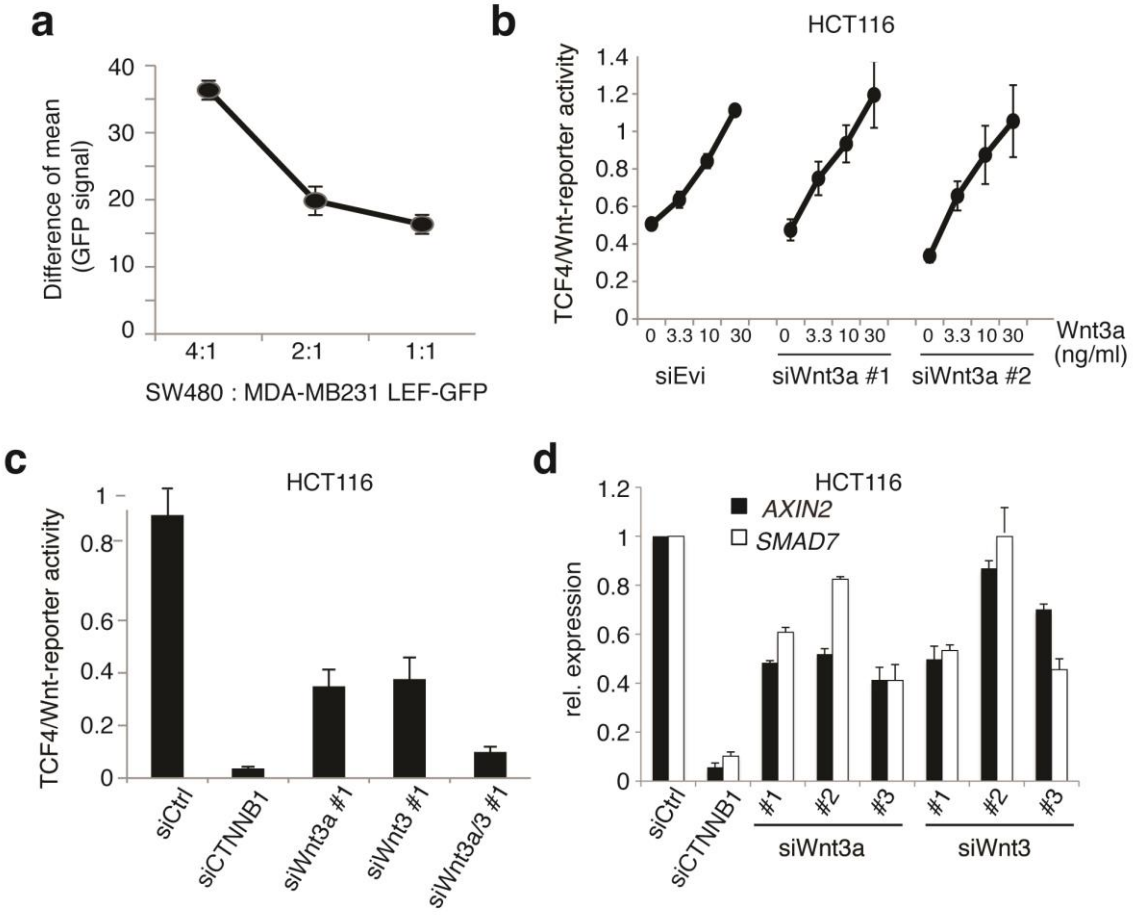
Supplementary Figure S2 Evi/Wls required for canonical Wnt signalling in colon cancer cells. (**a**) The specificity and sensitivity of the TCF4/Wnt-reporter in HCT116, as demonstrated by downregulation of the key components of the canonical Wnt pathway. HCT116 TCF4/Wnt cells were reverse transfected with the indicated siRNAs, and luciferase activity was measured 72 hours later. (**b**) Evi/Wls silencing reduces canonical Wnt signaling. HCT116 TCF4/Wnt cells were stably transduced with a construct expressing an Evi or a control (Ctrl) shRNA. Equal numbers of cells were seeded on 384-well plates and luciferase reporter activity was measured 48 hours later. Recombinant Wnt3a was added 16 hours prior to read-out. Reporter activity was normalised to cell viability (CellTiter-Glo assay). (Right) Western blot assessing Evi knockdown. β-actin served as loading control. (**c,d**) Induction of shmirEvi leads to Evi/Wls knockdown and to reduction of expression of the Wnt target *AXIN2*. HCT116/DLD1 shmirEvi cells were treated with the indicated amounts of dox, and with or without Wnt3a. Relative expression of *AXIN2* mRNA was measured by qPCR. (a-d) Data from 3(a), 5(b), 3-4(c),4(d) independent experiments are presented as mean ±s.e.m.



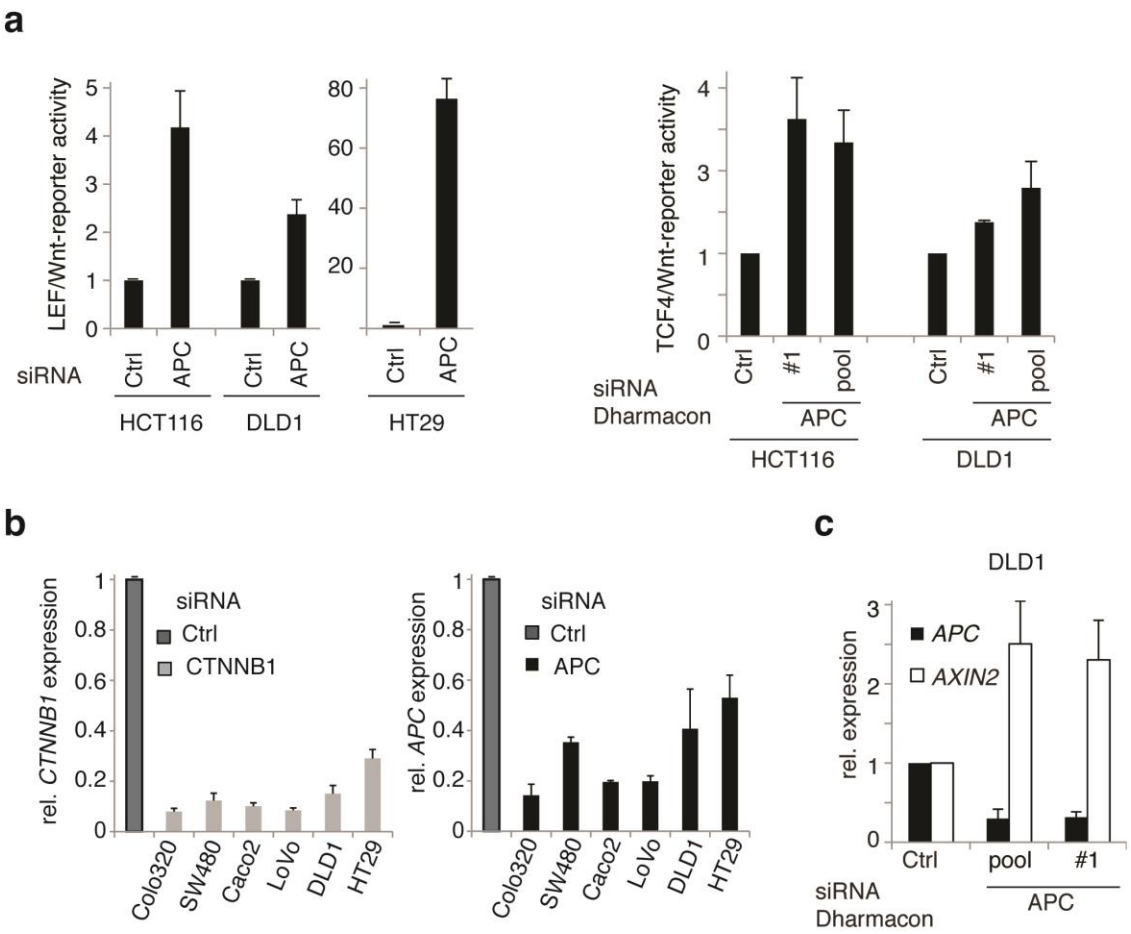
Supplementary Figure S3 The phenotype induced by Evi/Wls silencing is not rescued by R-spondin. (**a**) Evi #1 siRNA-induced phenotype is rescued in HEK293T cells. Cells were reverse transfected with siCtrl or siEvi #1 and, 24 hours later, transfected with TCF4/Wnt-reporter-, actin-renilla reporter-, and Wnt3a-expression and control plasmid or the indicated human Evi expression construct (Evi/hWls-V5-His(Evi) mutated in siEvi #1 binding site). The final concentration of plasmid per well was adjusted to equal levels. Luciferase and renilla signals were measured 48 hours after transfection, and luciferase measurements were normalized to the respective renilla values. Data from 3 independent experiments are presented as mean ±s.e.m. (b) HCT116 TCF4/Wnt-reporter cells were incubated with the porcupine inhibitor IWP12, and luciferase activity was measured 48 hours later. Activity was normalised to cell viability, as assessed by CellTiter-Glo assay. (**c-e**) The effect of downregulated Wnt signalling in HCT116/DLD1 cells cannot be rescued by applying mouse recombinant R-spondin1. (b) HCT116 TCF4/Wnt cells were reverse transfected with the Evi #1 siRNA. 24 hours later the indicated amounts of recombinant R-spondin were added. 48 hours later, luciferase readout was performed. (c) DLD shimirEvi#1 cells were treated with dox for 96 hrs and with 100 ng/ml of R-spondin1 for 48 hrs. (b-e) Data from 4 independent experiments are presented as mean ±s.e.m. (e) HEK293T cells were used as controls for R-spondin1 activity. These cells were transfected with the TCF4/Wnt luciferase- and actin-renilla reporter- constructs, with or without Wnt3a expression plasmid, and treated with 100 ng/ml of recombinant R-spondin1 for 48 hours. Representative example of 3 control experiments is shown.



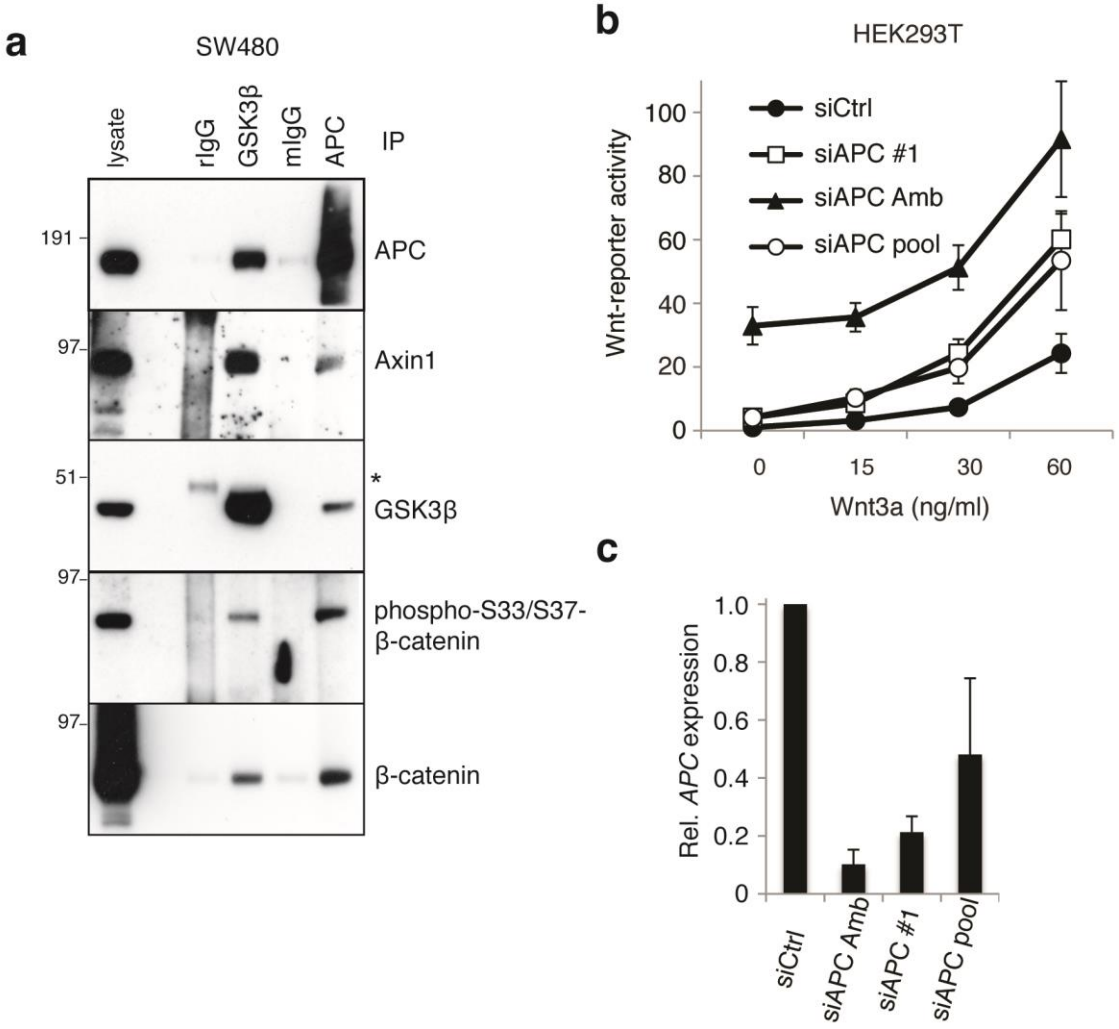
Supplementary Figure S4 The expression of canonical Wnt ligands is required for pathway activation in colon cancer cells. (**a**) The expression of canonical Wnt proteins in the indicated colon cancer cell lines, based on whole transcriptome sequencing. (**b**) MDA-MB231 LEF/TCF reporter cells were treated with recombinant Wnt3a or transfected with the Wnt3a-expression plasmid as control experiments for Figure 3 a,d. (**c**) DLD1 and SW480 colon cancer cells activate the Wnt pathway in co-cultured HEK293-reporter cells (experiment similar to that described in Figure 3a). HEK293T cells were transiently transfected with TCF4/Wnt reporter for 24 hours, after which SW480 or DLD1 cells were seeded into the wells. Reporter activity was measured 48 hours later. (b,c) Data from 3-4 independent experiments are presented as mean ±s.e.m.



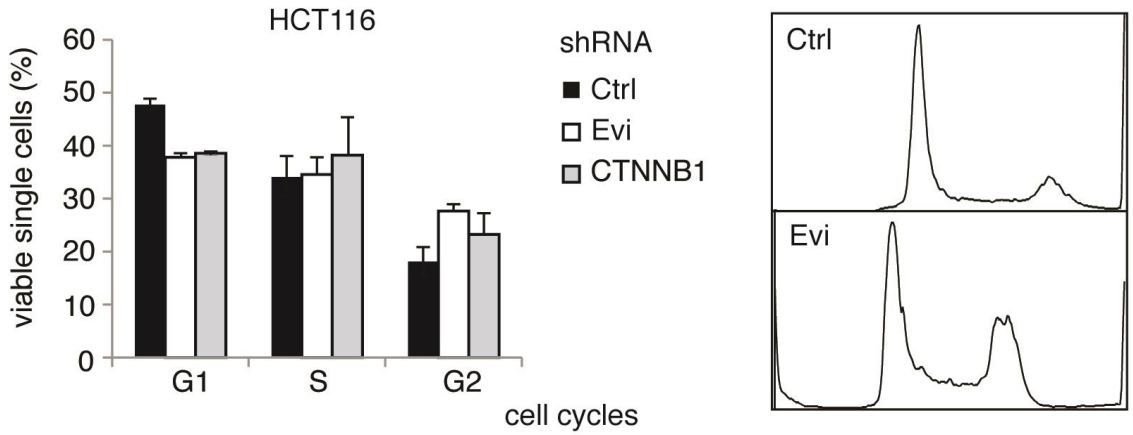
Supplementary Figure S5 Silencing of Wnt3 and Wnt3a regulates canonical Wnt signalling in HCT116 cells. (**a**) SW480 cells induce TCF/LEF-GFP signal in the MDA-MB231 reporter cell line (constitutively expresses mCherry). SW480 cells were co-cultured with $1x10^5$ MDA-MB231 TCF/LEF-GFP cells at the indicated ratio for 48 hours. mCherry-positive cells were gated and analysed for GFP signal. Difference of the mean reporter GFP-signal of cells co-cultured with and without SW480 was determined and plotted according to the cultivation ratio of SW480 cells *versus* reporter MDA-MB231 cells. (**b**) Effects of Wnt3a silencing can be rescued by applying recombinant Wnt3a. HCT116 TCF4/Wnt cells were reverse transfected with the indicated siRNA for 72 hours. The indicated amounts of recombinant Wnt3a were added 16 hours prior to luciferase measurement. (**c**) Combinatorial knockdown of both Wnt3a and Wnt3 leads to stronger reduction of canonical Wnt signalling than silencing of either gene individually. HCT116 TCF4/Wnt cells were reverse transfected with the indicated siRNAs, using the same total concentration of siRNA per well. (**d**) Silencing of Wnt3a or Wnt3 reduces the *AXIN2* and *SMAD7* mRNA levels. HCT116 cells were transfected with the indicated siRNAs for 72 hours, and RNA was isolated for qPCR. (a-d) Mean ±s.e.m. of 3-4 independent experiments are shown.



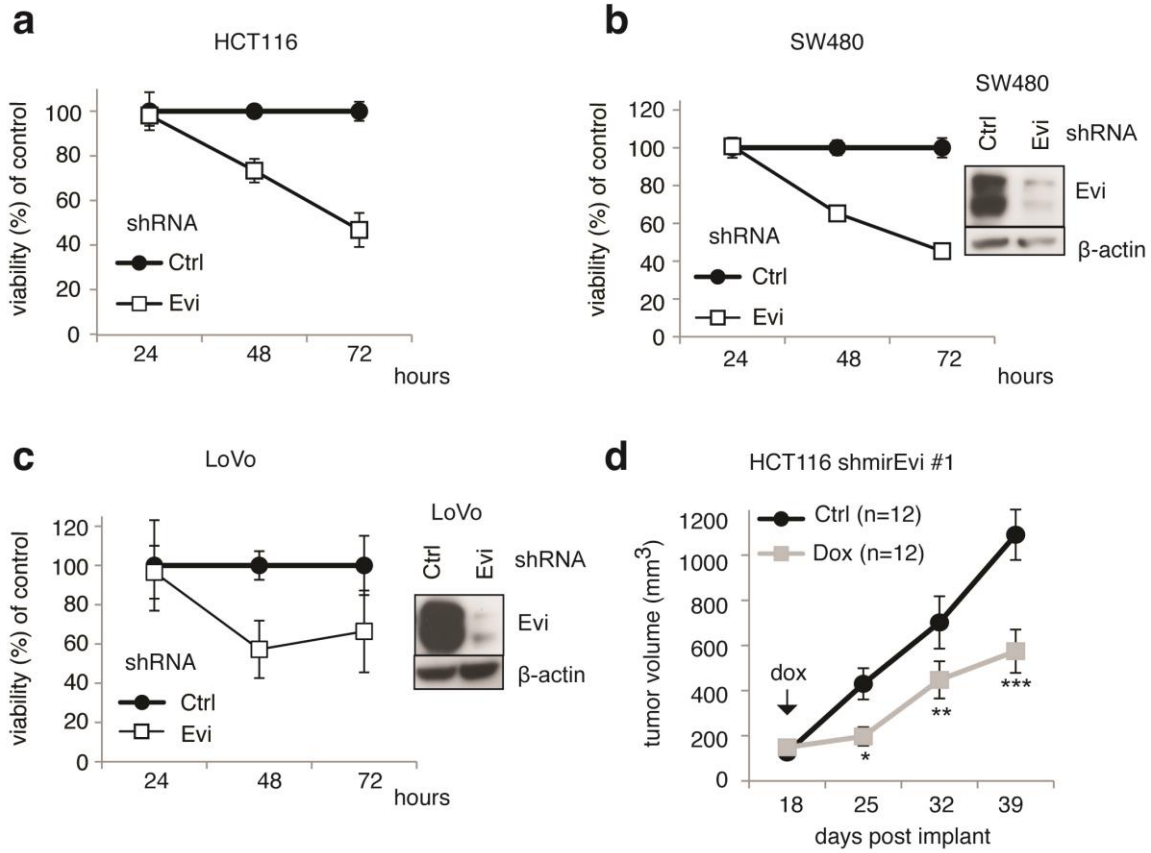
Supplementary Figure S6 APC silencing regulates Wnt signalling in colon cancer cell lines. (**a**) APC silencing induces Wnt-reporter activity in colon cancer cells in which APC is truncated. APC knockdown in HCT116, DLD1 and HT29 cells leads to enhanced Wnt-reporter activity. HCT116, DLD1, HT29 cells stably transduced/transfected with TCF/LEF- or TCF4/Wnt-reporter were transfected with the indicated APC siRNAs and luciferase activity was measured 72 hours later. (**b**) Relative expression of *CTNNB1* and *APC* after silencing in the indicated cell lines. Control of knockdown efficiencies for the different cell lines used in Figure 5A. (**c**) Knockdown of APC by independent siRNAs (distinct from those in b) leads to upregulation of *AXIN2* mRNA. (a-c) Average ±s.e.m. of 3-4 independent experiments are shown.



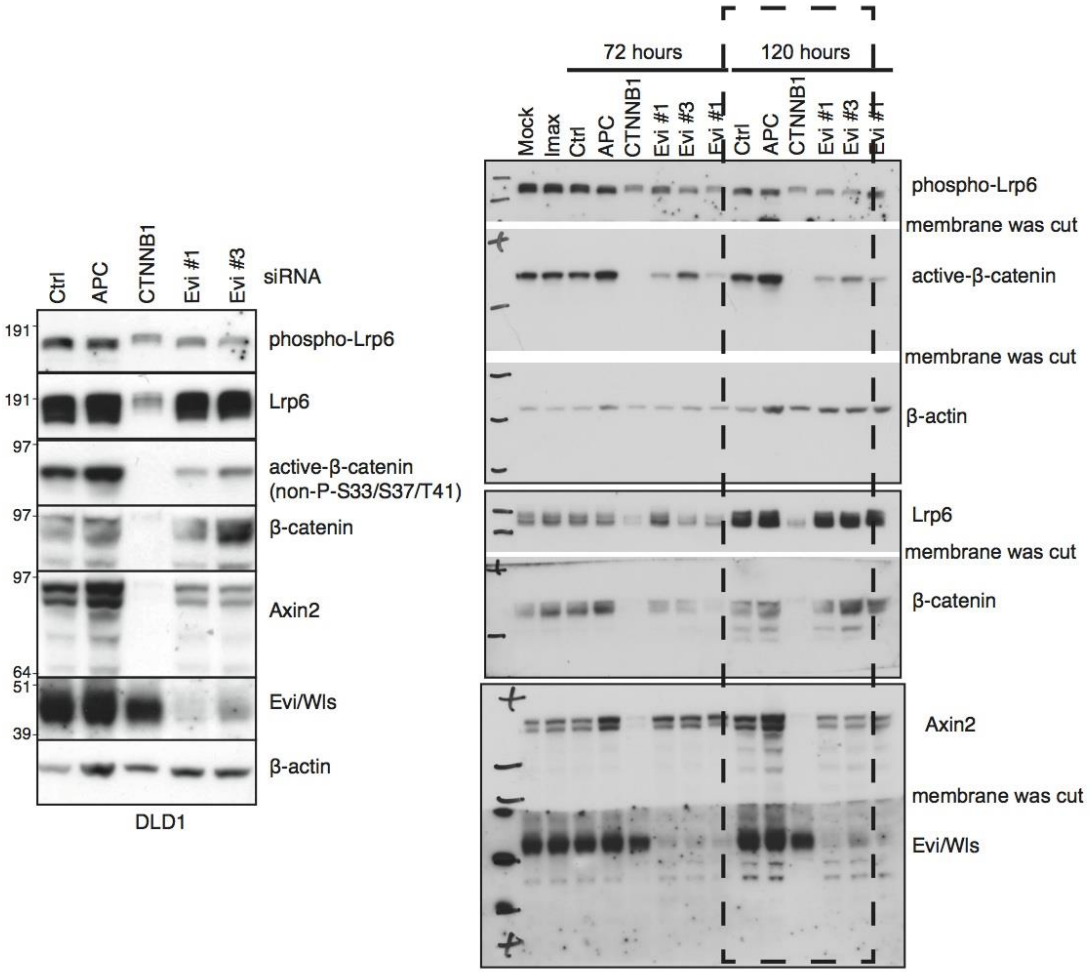
Supplementary Figure S7 Molecular mechanism underlying Wnt pathway regulation in the context of truncated APC. (**a**) The destruction complex in colon cancer cells expressing a truncated form of APC. In SW480 cells, APC and GSK3β antibodies pull down other key components of the destruction complex as well as phosphorylated β-catenin. Western blots are representative of 3 independent experiments. (**b**) Wnt reporter activity in APC-depleted cells. HEK293T cells were reverse transfected with different siRNAs targeting APC and transfected with TCF4/Wnt-luciferase and renilla-actin reporters at 24 hours post-transfection. Cells were also treated with the indicated amount of recombinant Wnt3a at this time point. Luciferase signal was measured 48 hours later. The firefly signal was normalised to renilla signal. (**c**) Relative expression of APC upon silencing of this gene in (b). (b,c) Results of 3-4 independent experiments are presented as mean ±s.e.m.



Supplementary Figure S8 HCT116 cells show increased G2 arrest in the context of Evi/Wls downregulation. HCT116 cells were stably transduced with a control (Ctrl) or an Evi shRNA and FACS analysis was used to assess cell cycle distribution. Results of 3 independent experiments are shown and presented as mean ±s.e.m.

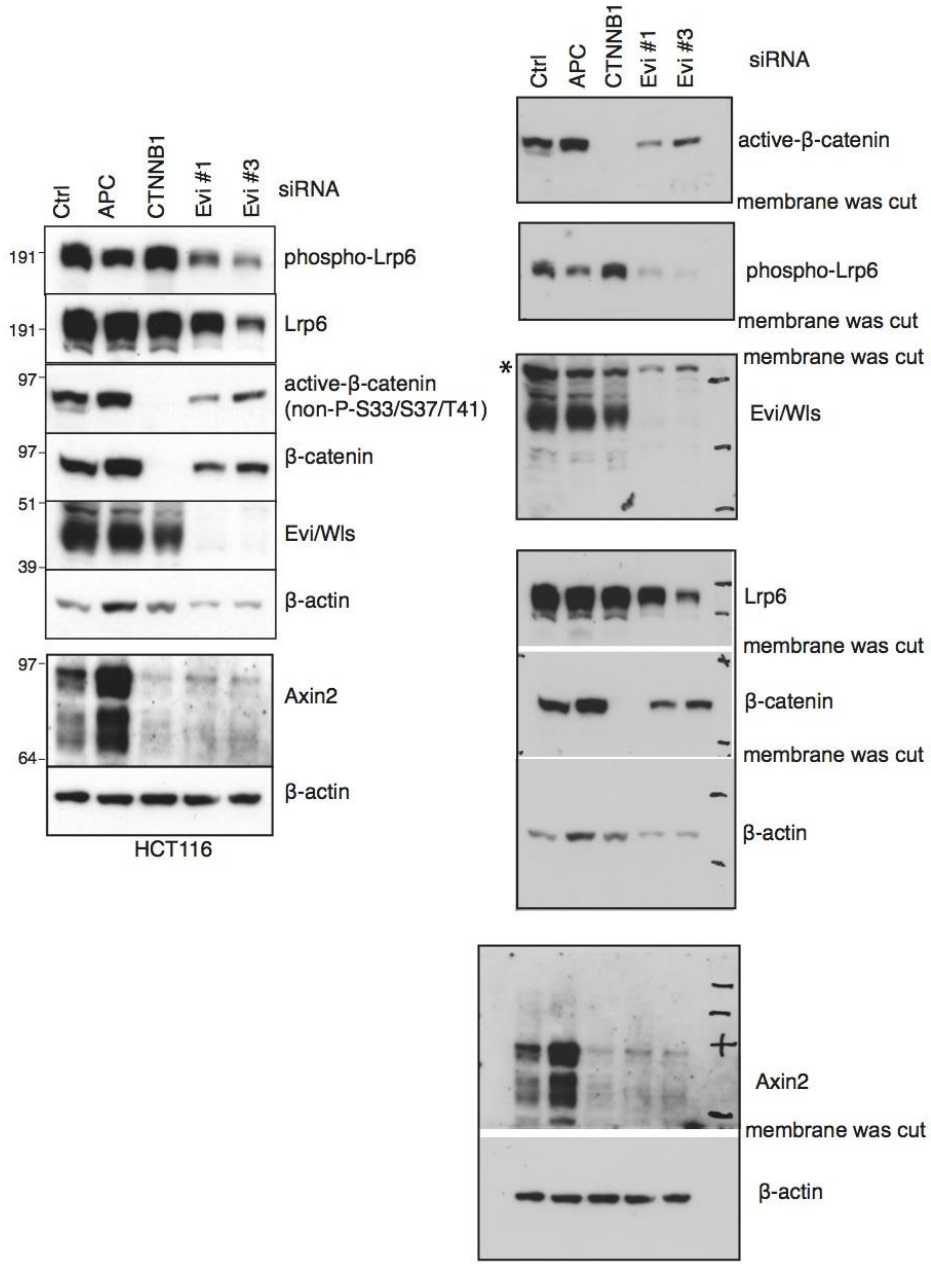


Supplementary Figure S9 Silencing of Evi/Wls reduces the cell survival capacity of colon carcinoma cell lines. (**a-c**) Evi/Wls silencing reduces the viability of HCT116, SW480 and LoVo cells. Cells were stably transfected with a control (Ctrl) or an Evi shRNA and then analysed for viability by CellTiter-Glo assay. Representative examples of 3 independent experiments are shown ±s.d. (**d**) Evi/Wls downregulation reduces tumour volume *in vivo*. Nod/SCID mice were *s.c.* injected with $2*10^6$ HCT116 shmirEvi#1 cells. 18 days later, when tumour volumes reached about 150 mm^3, the mice were randomly separated into two groups: one of which was treated with dox (added to the drinking water). Every week, tumour volume was measured and significance for each time point was calculated using the Student's *t*-test (p=*0,008;**0,087;***0,002). The experiment was terminated when tumours in the control group reached about 1200 mm^3. Mean ±s.e.m. per group (12 tumors) is shown.



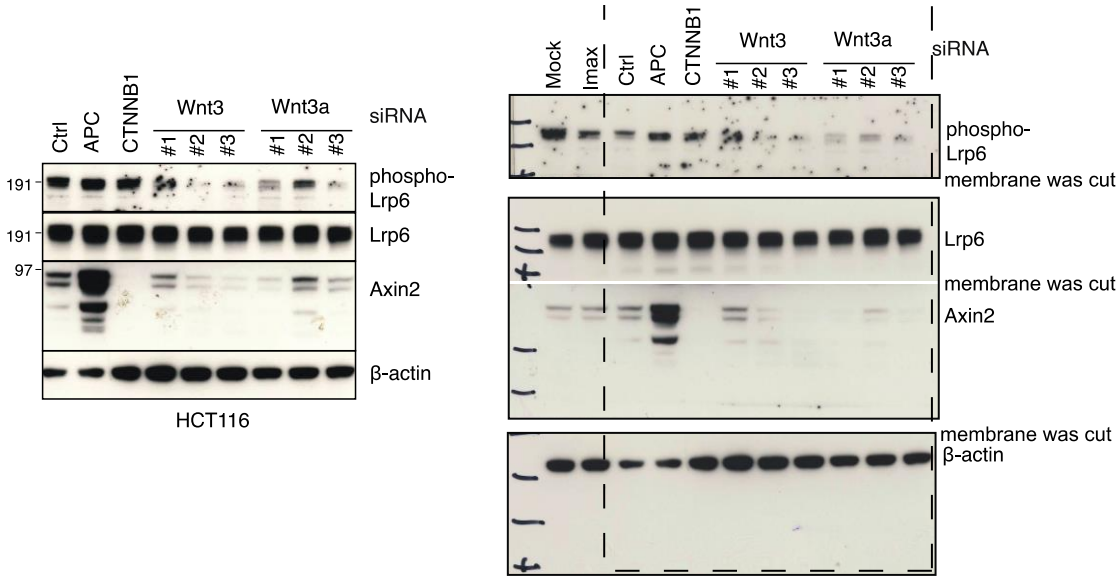
Suppelementary Figure S10 Full scans of western blots.

Figure 2c Silencing of Evi/Wls reduces the level of active-β-catenin in DLD1, which express mutated APC. DLD1 cells were transfected with the indicated siRNAs for 120 hours. Subsequently, the cells were lysed and Western blot was performed with the indicated antibodies. β-actin served as the loading control. The reduction of total Lrp6 in β-catenin knockdown cells, which is consistently observed in DLD-1 cells, may be a consequence of reduced β-catenin levels at the adherent junctions[60].



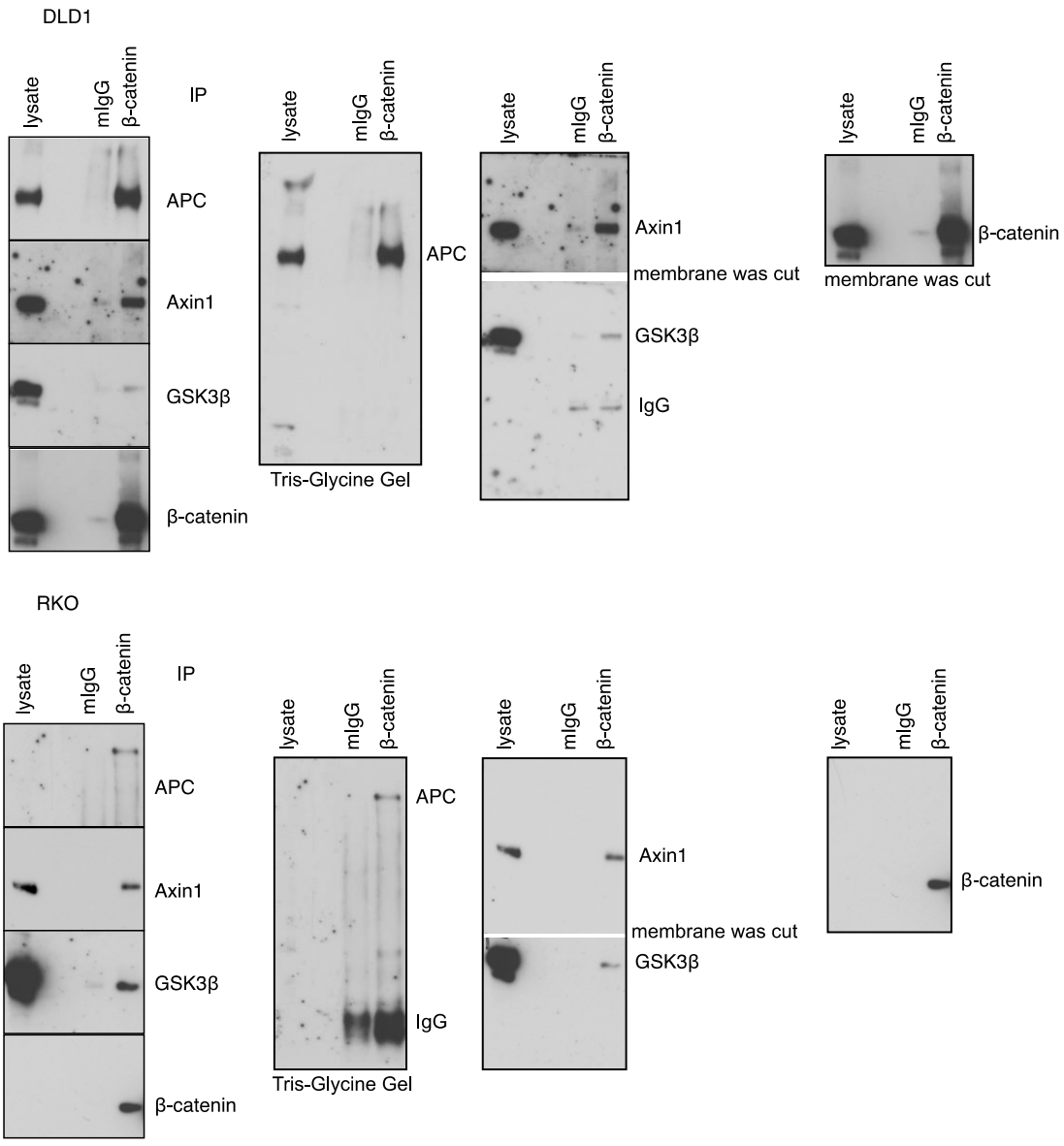
Suppelementary Figure S10 (cont.)

Figure 2c Silencing of Evi/Wls reduces the level of active-β-catenin in HCT116 cells, which express mutated β-catenin. HCT116 cells were transfected with indicated siRNAs for 72 hours. Subsequently, the cells were lysed and Western blot was performed with the indicated antibodies. β-actin served as the loading control.

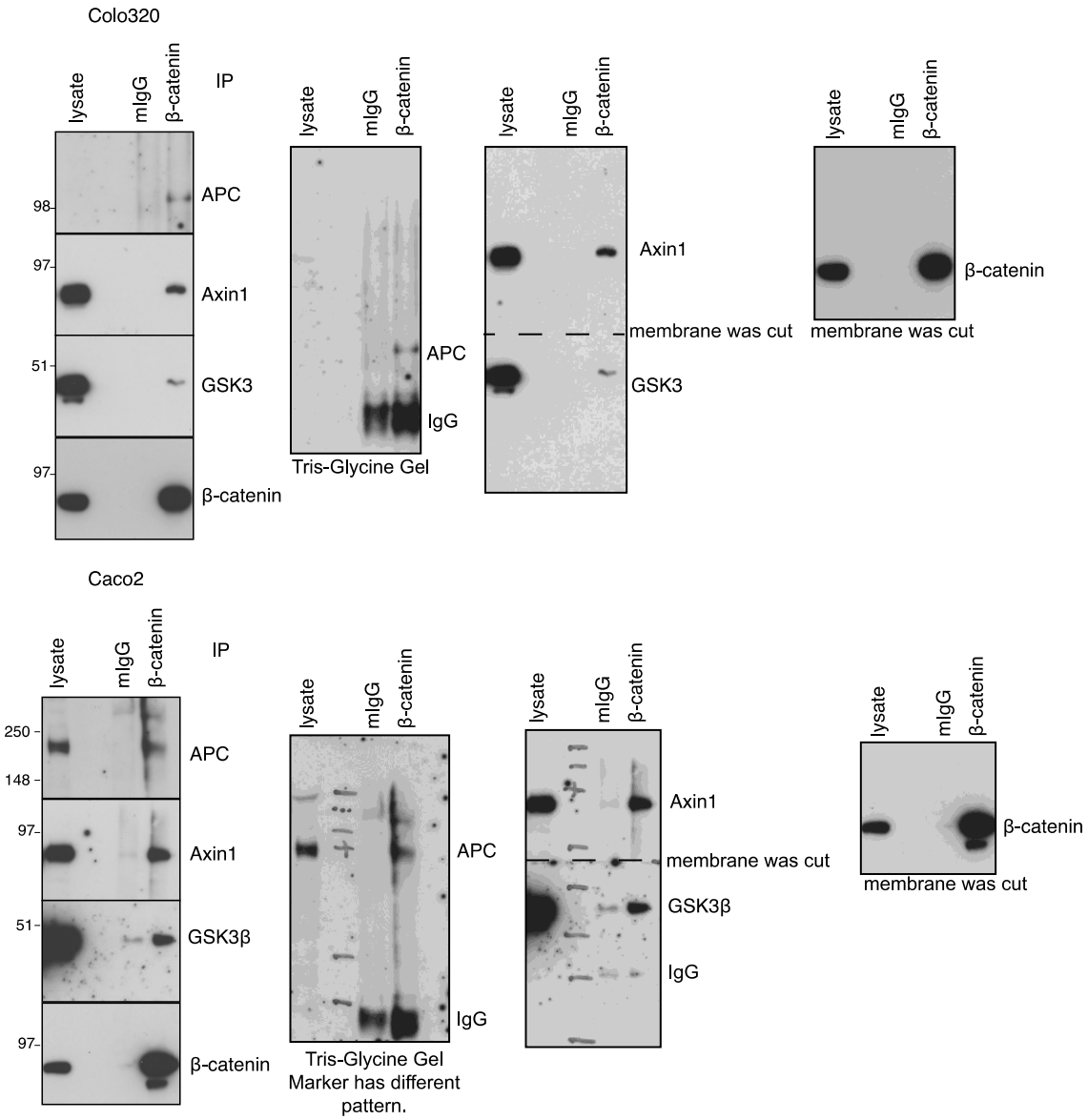


Suppelementary Figure S10 (cont.)

Figure 3c Silencing of Wnt3a or Wnt3 reduces Axin2 protein expression. HCT116 cells were transfected with the indicated siRNAs for 72 hours. Cell lysates were used for Western blot with indicated antibodies. β-actin served as loading control.

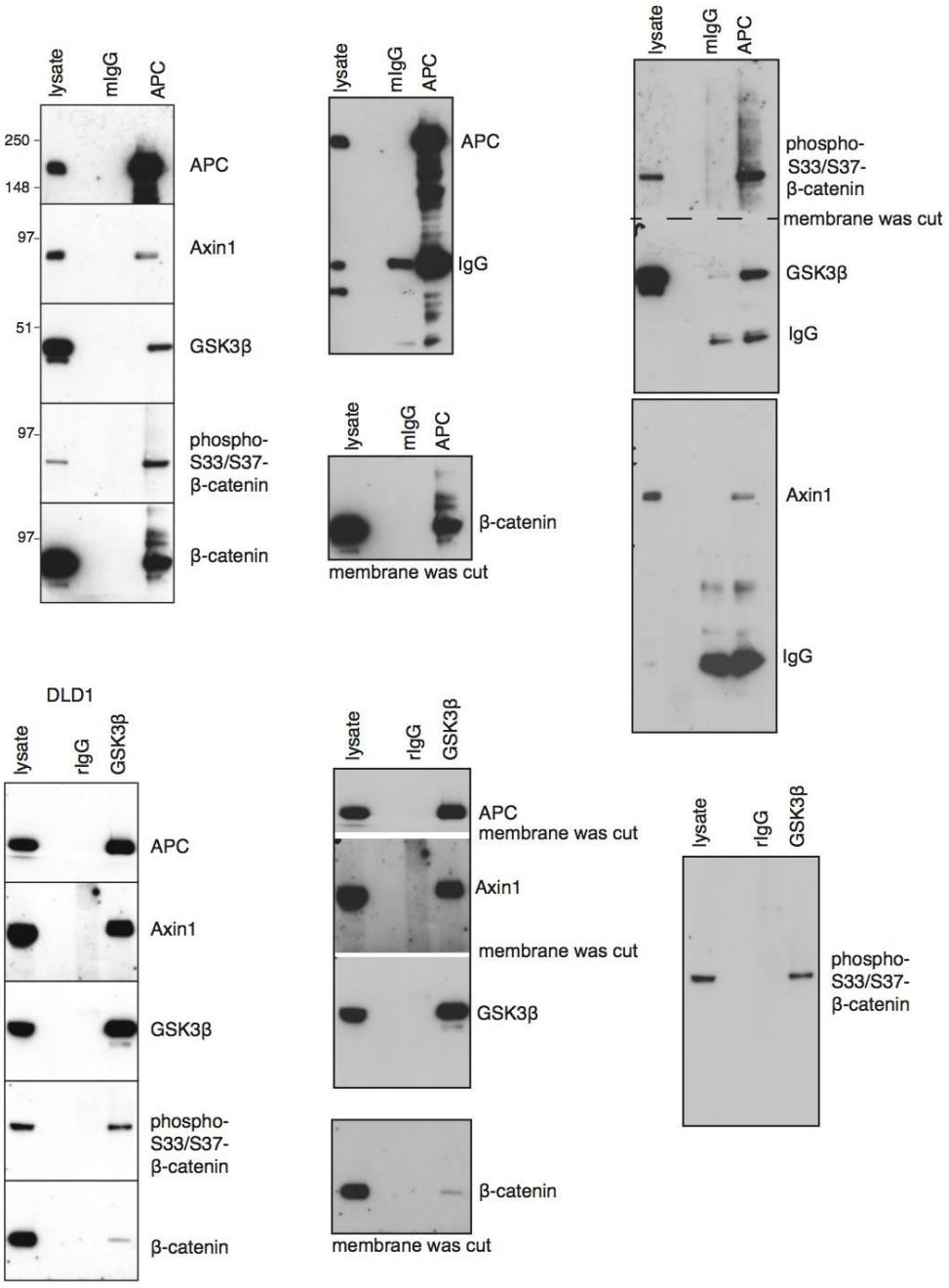


Suppelementary Figure S10 (cont.)

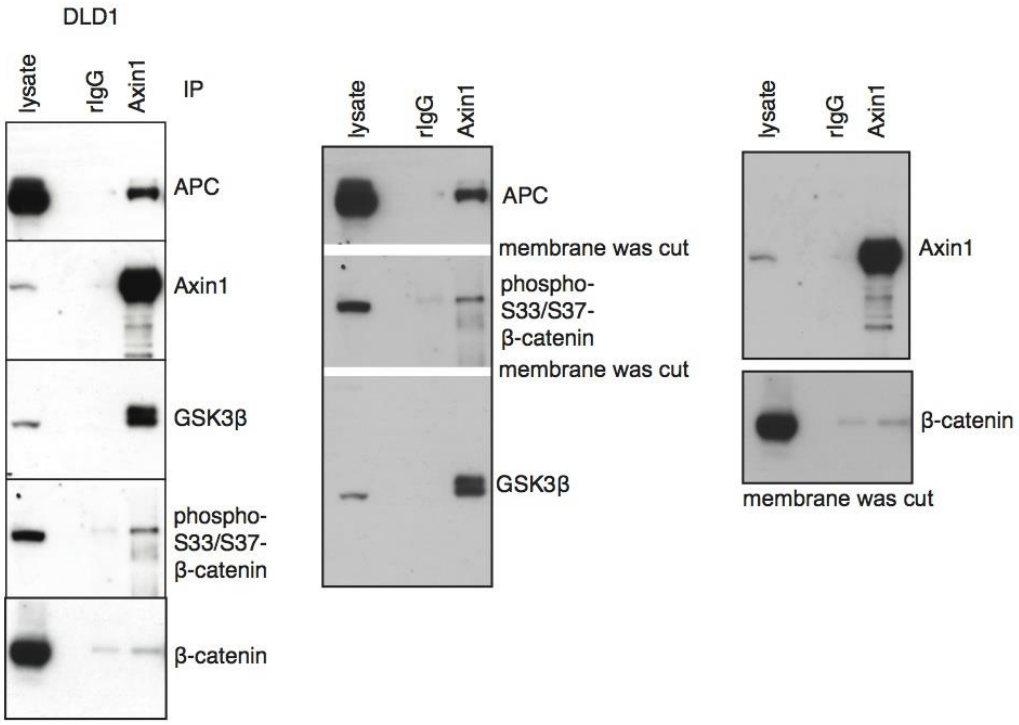


Suppelementary Figure S10 (cont.)

Figure 5b β-catenin binds to the main components of the destruction complex in colon cancer cells. The indicated cell lines were subjected to immunoprecipitation with anti-β-catenin antibody. Regardless of the extent of APC truncation, β-catenin co-immunoprecipitated APC, Axin1 and GSK3β. Western blots are shown as representative of 3 independent experiments using the indicated antibodies.

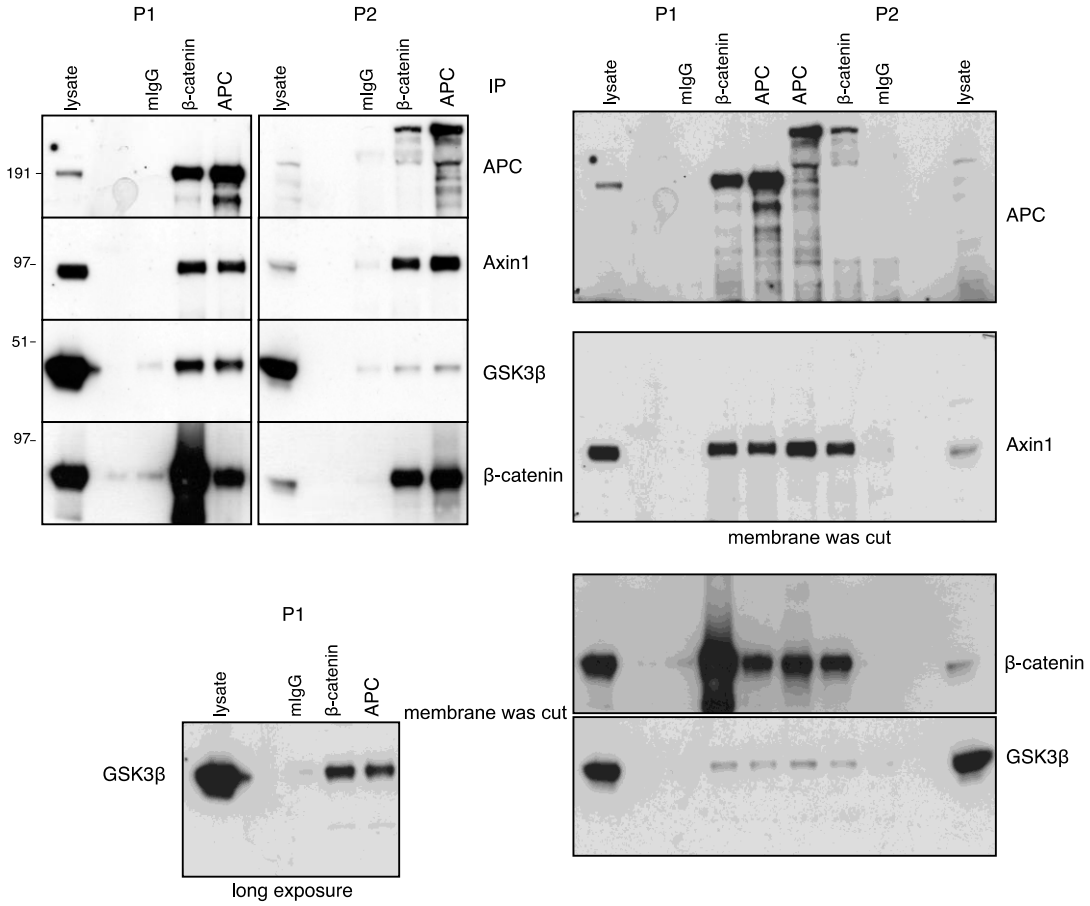


Suppelementary Figure S10 (cont.)

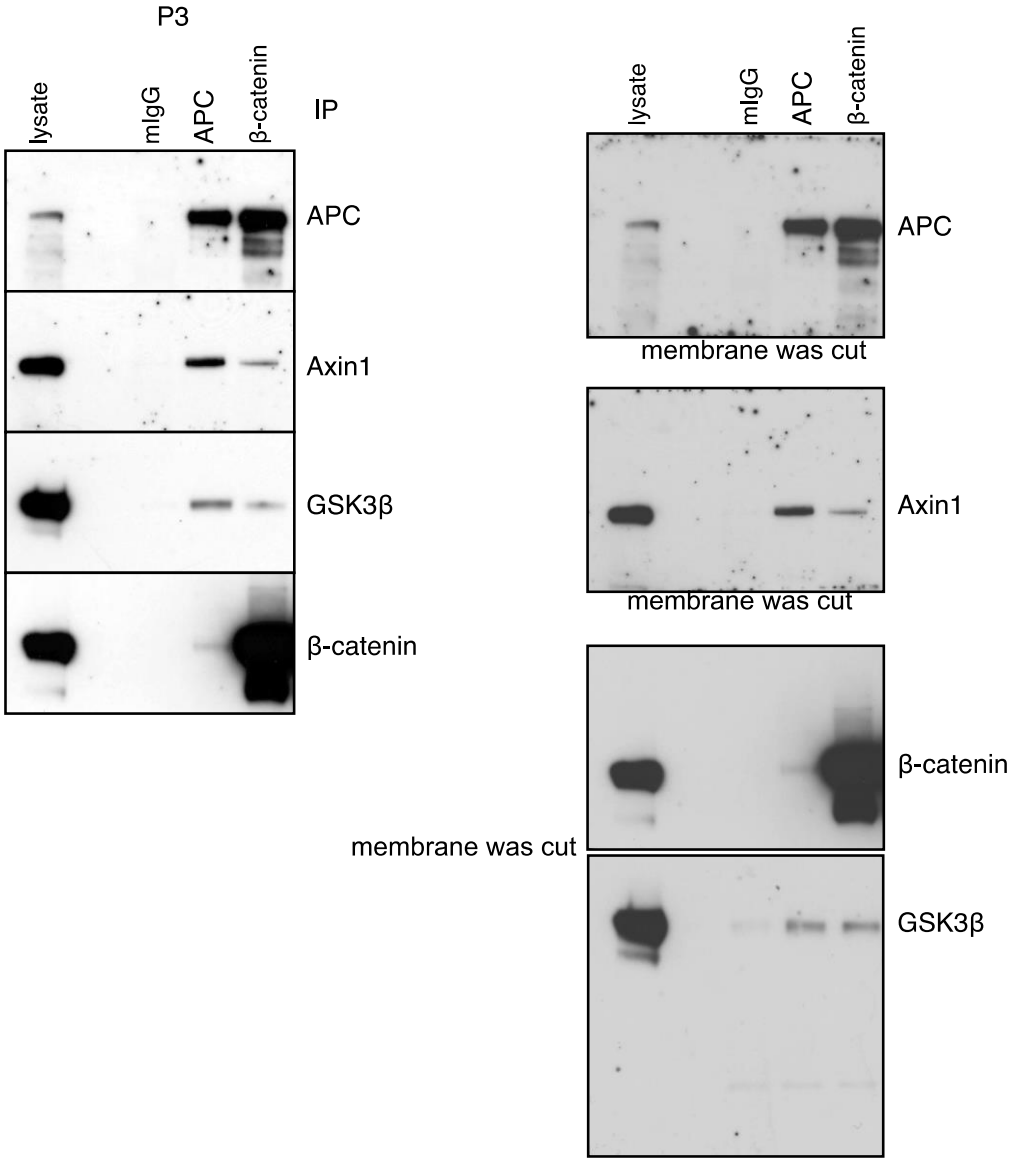


Suppelementary Figure S10 (cont.)

Figure 6a β-catenin bound to the main components of the destruction complex in DLD1 cells is phosphorylated. DLD1 cells were subjected to immunoprecipitation with anti-APC, -GSK3β or -Axin1 antibody. Western blots are representative of 3 independent experiments.

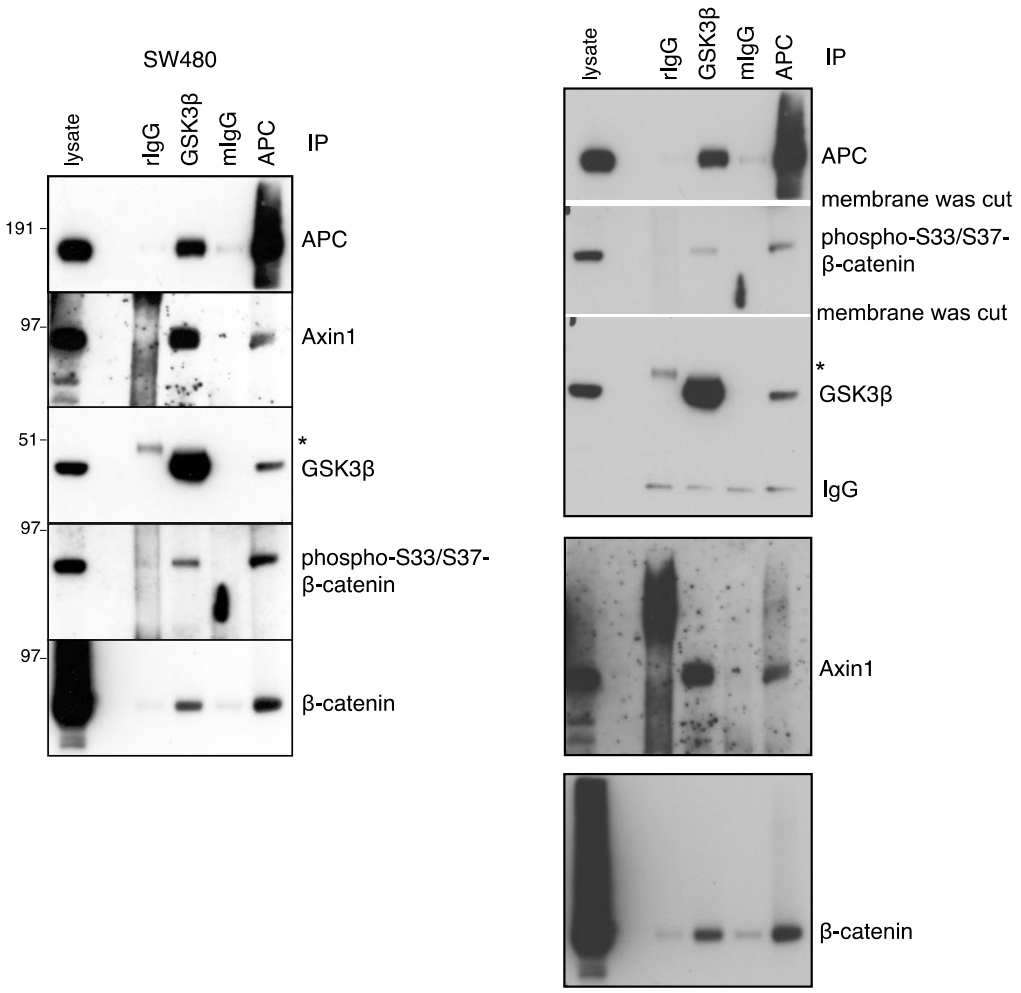


Suppelementary Figure S10 (cont.)



Suppelementary Figure S10 (cont.)

Figure 8a Destruction complex is functional in primary colon cancer spheroids independent of the mutation status of APC. Indicated cells were lysed and the main components of the destruction complex were co-immunoprecipitated with anti-β-catenin/APC antibody. P1 and P3 show APC truncation, while in P2 wild type APC was detected. Representative examples of 3 independent experiments are shown.



Suppelementary Figure S10 (cont.)

Supplementary Figure 6a The destruction complex in colon cancer cells expressing a truncated form of APC. In SW480 cells, APC and GSK3β antibodies pull down other key components of the destruction complex as well as phosphorylated β-catenin. Western blots are representative of 3 independent experiments.

Supplementary Table S1 Quantitative PCR primers

Gene	Forward Primer	Reverse Primer	UPL Probe
APC	GCATGGACCAGGACAAAAAT	GAACACACACAGCAGGACAGAT	17
AXIN1	AGCGAAGGCAGAGAGTTCAG	CCGGCATTGACATAATAGGG	36
AXIN2	AGAGCAGCTCAGCAAAAAGG	CCTTCATACATCGGGAGCAC	88
BCL9	TCTCCCAACTTGCCATCAA	GACCTGAAATTCGAGGATTCTG	72
Evi/Wls	TCATGGTATTTCAGGTGTTTCG	GCATGAGGAACTTGAACCTAAAA	38
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC	60
SMAD7	CGATGGATTTTCTCAAACCAA	AGGGGCCAGATAATTCGTTC	69
TCF7L2	TGAACACAGCGAATGTTTCC	TTAGGAGCGCTCAGGTCTGT	69
UBC	GGCAAAGATCCAAGATAAGGAA	GGACCAAGTGCAGAGTGGAC	11
CTNNB1	GCTTTCAGTTGAGCTGACCA	CAAGTCCAAGATCAGCAGTCTC	21
LRP6	ATCCGAAAGGCACAAGAAGA	GACTCGGAACTGAGCTCACAA	71
DVL1	ATCGTCACTGTCACGCTCAA	TCATGATGGAGCCAATGTAGA	43
DVL3	CCCTGAGCACCATCACCT	GGATGGACAAGTGGAAGTCG	17

Target gene	Cat #	Sequence (sense)
Ctrl/control	s29712 - Silencer Select Negative Control (Ambion)	
APC/APC#A	s1433 (Ambion)	GGAUCUGUAUCAAGCCGUUtt
APC pool	D-003869 (Dharmacon)	GCACAAAGCTGTTGAATTT CGAAATAGCTCCTCAAGTA GACAAGAGCTAGAAGATAA GAAATAGGATGTAATCAGA
CTNNB1	s438 (Ambion)	CUGUUGGAUUGAUUCGAAAtt
Evi#1	s36745 (Ambion)	GGACAUUGCCUUCAAGCUAtt
Evi#3	s36747 (Ambion)	GGAUUUCCAUGACCUUUAUtt
Wnt3a#1	s195523 (Ambion)	GGAAGGUUCCAUGAAGCGAtt
Wnt3a#2	s195524 (Ambion)	GCCAUGAACCGCCACAACAtt
Wnt3a#3	s195525 (Ambion)	GAACCGCCCUCCUGAUUAAtt
Wnt3#1	s14868 (Ambion)	CGAUAUCCUGGACCACAUtt
Wnt3#2	s14869 (Ambion)	GCAAUUACAUCGAGAUCAUtt
Wnt3#3	s14870 (Ambion)	AGAUGGUAGUAGAGAAGCAtt
TCF7L2	s13880 (Ambion)	GAUGGAAGCUUACUAGAUUtt
TCF7L2	s13881 (Ambion)	GGUCAACCAGUGUACCCAAtt
Bcl9	s1937 (Ambion)	CGUUAUACCAUGAUGCUAUtt
Lrp6	MU-003845 SMARTpool single sequences (Dharmacon)	CAGATGAACTGGATTGTTA GGACAGACCTCGAGCCATT GCAGATATCAGACGAATTT GAACTATGATTCAGAACCT
Dvl1	MU-004068 SMARTpool single sequences (Dharmacon)	GCGAGTTCTTCGTGGACAT CGACCAAGGCCTATACAGT CGGCACACGGTCAACAAGA GGGAGTCAGCAGAGTGAAG
Dvl3	MU-004070 SMARTpool single sequences (Dharmacon)	GAGGAGATCTCGGATGACA CAGGAGATATGTTGTTACA GGTAAACGAGATCAACTTT GAACCTGGACAATGACACA

Supplementary Table S2 siRNAs

Plasmid	**Target**	**Sense sequence**	**Source**
pLKO	shRNA Evi	GATCTACAAGTTGACCCGCAA	Sigma
pLKO	shRNA CTNNB1	CCTTGTAAACACCAATAGTAA	Sigma
pLKO	shRNA APC#1	CCCAGTTTGTTTCTCAAGAAA	Sigma
pLKO	shRNA APC#2	GCTGTGAAATTCACAGTAATA	Sigma
pLKO	shRNA Ctrl	CCCGTGTAAATATGTACATTT	Sigma
pTRIPZ	shmirEvi#1	GGCGTCACAGTCCAAGTGA	Thermo
pTRIPZ	shmirEvi#2	GGACATTGCCTTCAAGCTA	Thermo
pTRIPZ	shmirCtrl	Cat# RHS4743	Thermo

Supplementary Table S3 sh/shmirRNAs

Short name	**Name**	**Source**
LEF-GFP reporter	pCF778:pLenti 7xTcf-eGFP/SV40-mCherry	Fuerer et al.[26] / #24304 Addgene
TCF/LEF luciferase reporter	pCF826:pLenti 7xTcf-Firefly luciferase//SV40-PuroR	Fuerer et al.[26] /#24308 Addgene
Evi/Wls	pcDNA-hWls V5-His	Belenkaya et al.[29]
Evi/Wls	pcDNA-V5-hWls	Belenkaya et al.[29]
TCF4/Wnt luciferase reporter	6xKD; pGL4.26 6xTcf-Firefly luciferase	K. Demir (Boutros lab)
Renilla reporter	pAct-RL (Renilla luciferase)	D. Nickles (Boutros lab)

Supplementary Table S4 Constructs

Protein	Cat. Number	Species	Dilution	Source
APC	sc-53165	mouse	1:1000	Santa Cruz
Axin1	C76H11, 2087	rabbit	1:1000	NEB
Axin2	76G6, 2151	rabbit	1:1000	NEB
Active β-catenin	8E7	mouse	1:1000	Millipore
β-catenin	9F2	mouse	1:1000	BD Pharmingen
phospho- β-catenin (Ser33/37)	2009	rabbit	1:1000	Cell Signalling
GSK3β	27C10, 9351	rabbit	1:1000	NEB
phospho-Lrp6 (Ser1490)	2568	rabbit	1:1000	Cell Signalling
Lrp6	C47E12, 3395	rabbit	1:1000	Cell Signalling
β-actin	AC-40	mouse	1:20000	Abcam
Wnt3	ab32249	rabbit	1:200	Abcam
Cytokeratin20	M7019	mouse	1:50	Dako
Evi	C1	rabbit	1:200(ICH)/1:1000 (WB)	Augustin et al. [10]
EphB2	R&D	rabbit	1:100	R&D

Supplementary Table S5 Antibodies