Supporting Information

Scholer-Dahirel et al. 10.1073/pnas.1104182108

SI Materials and Methods

Cell Culture. All cell lines were maintained in advanced RPMI medium 1640 (Invitrogen) with 10% FBS (Invitrogen). Infected cell lines were maintained under 1 μg/mL of puromycin (MP Biomedicals) for selection.

Short Hairpin RNA Constructs. Control short hairpin RNA (shRNA), GGATAATGGTGATTGAGATGG, *β-catenin* shRNA-35, CCCTAGCCTTGCTTGTTAAAA, and *β-catenin* shRNA-36, GGACAAGCCACAAGTTACAA, were cloned into the inducible pLKO-Tet-On puromycin vector as previously described (1).

Lentivirus and Infection. Lentiviral supernatants were generated according to our previously established protocol (1) and concentrated by ultracentrifugation followed by cell infection. A total of 100 μL of lentivirus was used to infect 300,000 colorectal cancer cells in a six-well plate, in 8 μg/mL polybrene (Chemicon). Medium was replaced and after 24 h, cells were selected by puromycin (MP Biomedicals) and expanded. Induction of shRNA was obtained by addition of 100 ng/mL doxycycline (Clontech) to the medium.

RNA Extraction and Quantitative Reverse Transcription–PCR. Total RNA was isolated using the RNeasy Mini kit (Qiagen). RNA levels were measured using the ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, ABI). ABI taqman gene expression assays include *AXIN2* Hs00610344_31, *β-catenin* Hs00170025_m1, *c-MYC* Hs00153408_m1, *BMP4* Hs00370078_m1, *EPHB2* Hs01031827_m1, *LGR5* Hs00969418_m1, *CA2* Hs00163869_m1, *MUC2* Hs00159374_m1, and *TM4SF4* Hs00270335_m1. VIC-MGB primers/probe sets (Applied Biosystems) were used in each reaction to coamplify the *GAPDH* or *18S* transcripts. All experiments were performed in either duplicate or triplicate and normalized to *GAPDH* or *18S* levels as indicated.

Immunohistochemistry, Immunofluorescence, and Image Analysis. Primary antibodies used are β-catenin from Santa Cruz (sc-1496), CA2 from Rockland Immunochemicals, (100-401-136), c-MYC from AbCAM (clone Y69; ab32072), EPHB2 from R&D System (clone 512001; MAB4671), Ki67 from Vector Laboratories (clone SP6; VP-RM04), p21 from Cell Signaling (clone 12D1; 2947), Vimentin from Santa Cruz Biotechnologies (sc-7557), E-Cadherin from Cell Signaling (4065), and KLF4 from R&D Systems (AF3640). Secondary antibodies used for immunofluorescence are Alexa Fluor-488 donkey anti-goat IgG (11055) and Alexa Fluor-594 chicken anti-rabbit IgG (21442) from Invitrogen. Xenograft tumor samples and human colorectal adenocarcinoma specimens were fixed and stained as described in *Materials and Methods*. The immunohistochemical images were captured using Aperio Scanscope (Aperio Technologies). Images of whole tumor sections were analyzed with Aperio ImageScope (Aperio Technologies) or ImageJ. The necrotic area and stromal tissue portions were manually excluded using the tools provided by the imaging systems. Immunohistochemical stains with unimodal distribution of signal intensity were quantified as mean signal intensity. Immunohistochemical stains with bimodal or strongly right-skewed distribution of signal intensity were quantified as percentage of positive events (positive nuclei for nuclear proteins or positive pixels for cytoplasmic/membranous proteins). The immunofluorescent images were captured using Aperio Scanscope FL (Aperio Technologies). Depending on the size of the tumor, one or two 20× fields captured on ImageScope, corresponding to at least 1,000 cells, per xenograft, were analyzed by ImageJ.

Transcriptional Profiling. RNA was isolated using the Qiagen RNeasy mini kit. Generation of labeled cDNA and hybridization to HG-U133 Plus2 arrays (Affymetrix) were performed as previously described (1). Expression values were normalized on a per-sample basis using the Affymetrix MAS5 algorithm (2).

Immunoblots. Western blots were performed as follows: Tumor lysates were prepared using Nonidet P-40 Cell Lysis Buffer (Invitrogen) supplemented with 1× Halt Protease and Phosphatase Inhibitor Mixture (Thermo Scientific). Thirty micrograms of total tumor lysates were separated by SDS/PAGE and electrotransferred to nitrocellulose membrane (Invitrogen). Membranes were blocked in PBS and 0.1% (vol/vol) Tween-20 (PBS-T) and 4% (wt/vol) nonfat dry milk (Bio-Rad) for 1 h on a shaker at room temperature. Primary antibodies were added to the blocking solution at 1:1,000 (LC3; Novus Biologicals, NB100-2220), 1:1,000 (SQSTM1/p62; Cell Signaling Technology, 5114S), and 1:10,000 (GAPDH; Cell Signaling Technology, 2118S) dilutions and incubated overnight and a rocker at 4 °C. Immunoblots were washed three times, 5 min each with PBS-T, and secondary antibody was added at 1:10,000 dilution into PBS-T milk for 1 h on a shaker at room temperature. After several washes, enhanced chemiluminescence (ECL) reactions were performed according to manufacturer's recommendations (SuperSignal West Dura Extended Duration Substrate; Thermo Scientific). Band quantification was performed using GeneTools software (Syngene).

Gene Set Analysis. Gene set activity score calculation is a two-step process. The first step in the process of calculating gene set activity scores is to perform *z*-score transformation for each probe expression value across a set of samples:

$$Zi,j = (Xi,j - \mu)/\delta + \varepsilon.$$

Xi,j is the MAS5 expression value [calculated as previously described (2)] for probe i in sample j. ε is the SD constant.

The second step is to calculate gene set activity scores by adding Zi,j scores from genes in a particular gene set and normalizing by the square root of the number genes in the gene set:

$$Sj = \left(\sum_{i=1}^{N} Zi,j\right) \Big/ \sqrt{N}.$$

Sj is the gene set activity score of the given gene set in sample j. N is the number of genes in the gene set.

Gene set score permutation fractions are calculated on the basis of gene set activity scores calculated for 1,000 randomly generated gene sets of a particular size in a particular sample. A permutation fraction of 0.001 corresponds to unusually high activity of the gene set, and a permutation fraction of 1.000 corresponds to unusually low activity of the gene set.

Wnt pathway activity was assessed using the following Wnt custom gene set: *AXIN1* (Entrez Gene ID 8312), *AXIN2* (Entrez Gene ID 8313), *BIRC5* (Entrez Gene ID 332), *CCND1* (Entrez Gene ID 595), *CD44* (Entrez Gene ID 960), *CLDN1* (Entrez Gene ID 9076), *CTNNB1* (Entrez Gene ID 1499), *ENC1* (Entrez Gene ID 8507), *GAD1* (Entrez Gene ID 2571), *LEF1* (Entrez Gene ID 51176), *MMP3* (Entrez Gene ID 4314), *MMP7* (Entrez Gene ID 4316), *MYC* (Entrez Gene ID 4609), *PPARD* (Entrez

Gene ID 5467), *RBP1* (Entrez Gene ID 5947), *SOX9* (Entrez Gene ID 6662), *STRA6* (Entrez Gene ID 64220), *TCF1* (Entrez Gene ID 6927), *TCF7* (Entrez Gene ID 6932), *VEGFA* (Entrez Gene ID 7422), *WISP1* (Entrez Gene ID 8840), and *WNT5A* (Entrez Gene ID 7474).

Mutation Status Analysis. We queried the following databases for mutation status in large intestine cancer cell lines: COSMIC (3) and the Broad–Novartis Cancer Cell Line Encyclopedia (CCLE). Mutation statuses in the CCLE database were generated as previously described (4).

1. Wiederschain D, et al. (2009) Single-vector inducible lentiviral RNAi system for oncology target validation. *Cell Cycle* 8:498–504.
2. Hubbell E, Liu WM, Mei R (2002) Robust estimators for expression analysis. *Bioinformatics* 18:1585–1592.
3. Bamford S, et al. (2004) The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. *Br J Cancer* 91:355–358.
4. Thomas RK, et al. (2007) High-throughput oncogene mutation profiling in human cancer. *Nat Genet* 39:347–351.

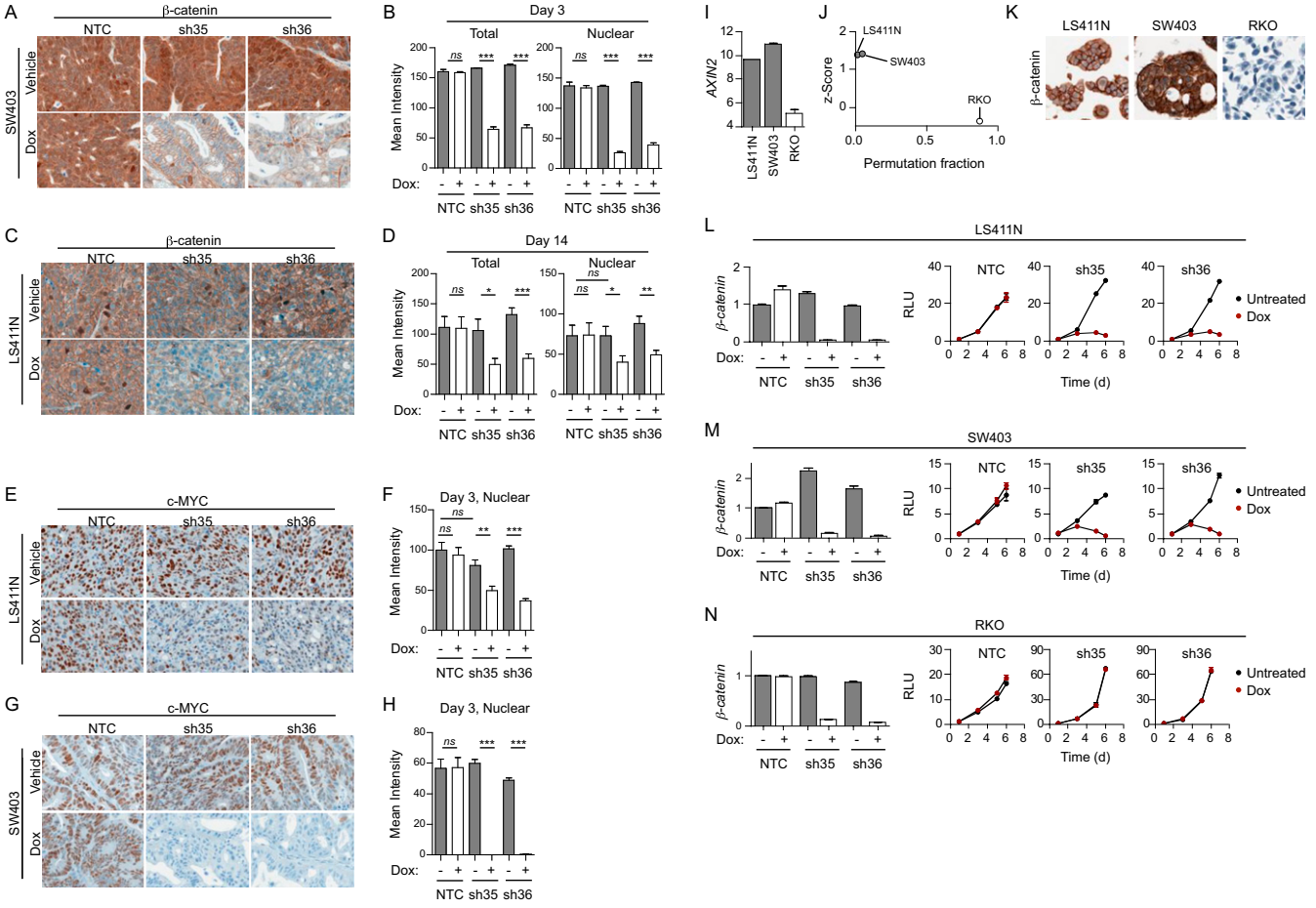

Fig. S1. Efficient and specific inhibition of the Wnt/β-catenin pathway by *β-catenin* shRNA. (*A* and *B*) SW403 cancer cells stably expressing dox-inducible NTC, sh35, or sh36 *β-catenin* shRNA were inoculated into mice. Tumor-bearing mice were treated for 3 d with either vehicle or doxycycline. (*A*) Representative images of β-catenin staining by IHC. (*B*) Mean signal intensity of total β-catenin (*Left*) and nuclear β-catenin (*Right*). Graphs represent mean ± SEM values. $n = 3$ per treatment group. (*C* and *D*) LS411N cancer cells containing NTC, sh35, or sh36 *β-catenin* shRNA were inoculated into mice. Tumor-bearing mice were treated for 14 d with either vehicle or doxycycline. (*C*) Representative images of β-catenin staining by IHC after 14 d of treatment. (*D*) Mean signal intensity of total β-catenin (*Left*) and nuclear β-catenin (*Right*). Graphs represent mean ± SEM values. Two independent experiments are represented ($n = 8$ per treatment group). (*E–H*) LS411N (*E* and *F*) or SW403 (*G* and *H*) cancer cells stably expressing dox-inducible NTC, sh35, or sh36 *β-catenin* shRNA were inoculated into mice. Tumor-bearing mice were treated for 3 d with either vehicle or doxycycline. (*E*) Representative images of c-MYC staining of LS411N xenografts by IHC. (*F*) Mean signal intensity of nuclear c-MYC in LS411N xenografts. Graphs represent mean ± SEM values. Two independent experiments are represented ($n = 3$ per treatment group). (*G*) Representative images of c-MYC staining of SW403 xenografts by IHC. (*H*) Mean signal intensity of nuclear c-MYC in SW403 xenografts. Graphs represent mean ± SEM values. $n = 3$ per treatment group. (*I*) *AXIN2* mRNA expression in indicated colorectal tumor xenografts (log2 scale). (*J*) Wnt pathway activation was assessed using gene set analysis (Wnt custom gene set) as described in *SI Materials and Methods*. The graph represents the *z*-score as a function of the permutation fraction in indicated colorectal tumor xenografts. (*K*) Representative images of β-catenin staining by IHC in indicated cell lines. (*L–N*) LS411N (*L*), SW403 (*M*), or RKO (*N*) cancer cells containing NTC, sh35, or sh36 *β-catenin* shRNA were grown in vitro in the absence or presence of doxycycline. (*Left*) Quantitative RT-qPCR of the remaining *β-catenin* after 3 d of treatment. Expression was normalized to 18S mRNA. Graphs represent mean ± SEM values. Arbitrary units are shown. (*Right*) In vitro cell growth of cancer cells containing indicated shRNA. The number of viable cells was assessed at indicated time points. Graphs represent mean ± SEM Relative Luminescence Unit (RLU).

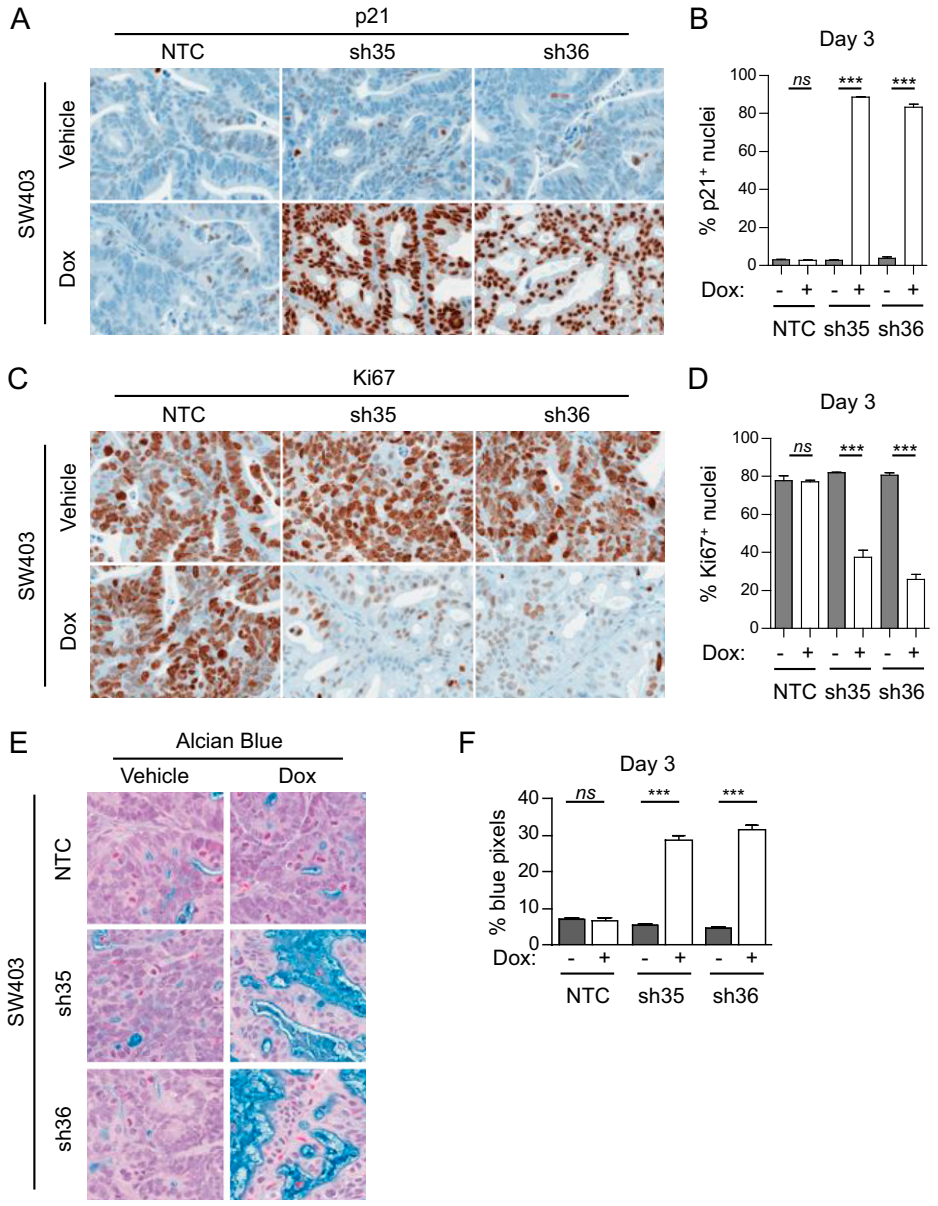

Fig. S2. *β-Catenin* shRNA induces cell cycle arrest and differentiation in SW403 cells in vivo. (*A–F*) SW403 cancer cells containing NTC, sh35, or sh36 *β-catenin* shRNA were inoculated into mice. Tumor-bearing mice were treated for 3 d with either vehicle or doxycycline. (*A*) Representative images of p21 staining by IHC. (*B*) Percentage of nuclei positive for p21. Graphs represent mean ± SEM values. (*C*) Representative images of Ki67 staining by IHC after 3 d of treatment. (*D*) Percentage of nuclei positive for Ki67. (*E*) Representative images of Alcian blue staining after 3 d of treatment. (*F*) Percentage of blue pixels. Graphs represent mean ± SEM values. *n* = 3 per treatment group.

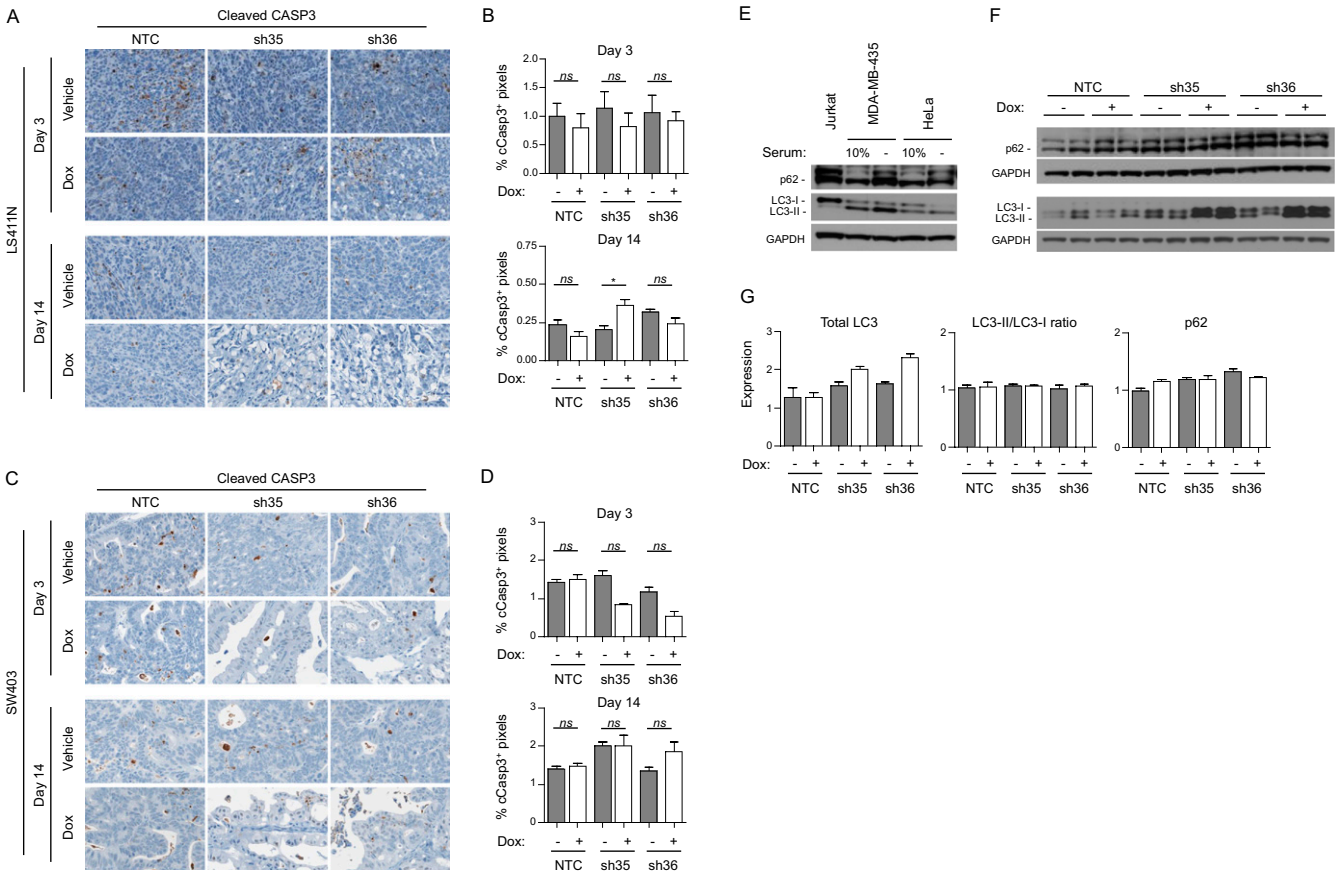

Fig. S3. Expression pattern of apoptosis and autophagy markers following Wnt pathway inhibition. (*A* and *B*) LS411N cancer cells containing NTC, sh35, or sh36 *β-catenin* shRNA were inoculated into mice. Tumor-bearing mice were treated with either vehicle or doxycycline. (*A*) Representative image of cleaved caspase 3 staining by IHC after 3 d or 14 d of treatment. (*B*) Percentage of positive pixels. Graphs represent mean ± SEM values. *n* = 3–6 per treatment group. (*C* and *D*) SW403 cancer cells containing NTC, sh35, or sh36 *β-catenin* shRNA were inoculated into mice. Tumor-bearing mice were treated with either vehicle or doxycycline. (*C*) Representative image of cleaved caspase 3 staining by IHC after 3 d or 14 d of treatment. (*D*) Percentage of positive pixels. Graphs represent mean ± SEM values. *n* = 3–8 per treatment group. (*E*) Jurkat cells, MDA-MB-435 cells, and HeLa cells were cultured in presence or absence of serum. Immunoblot for LC3, p62, and GAPDH is shown. (*F* and *G*) SW403 cancer cells containing NTC, sh35, or sh36 *β-catenin* shRNA were inoculated into mice. Tumor-bearing mice were treated for 3 d with either vehicle or doxycycline. Tumor fragments were collected at day 3 for protein extraction. (*F*) Immunoblot for LC3, p62, and GAPDH. (*G*) Quantification of LC3-I, LC3-II, and p62 expression normalized to GAPDH expression. Graphs represent mean ± SEM values.

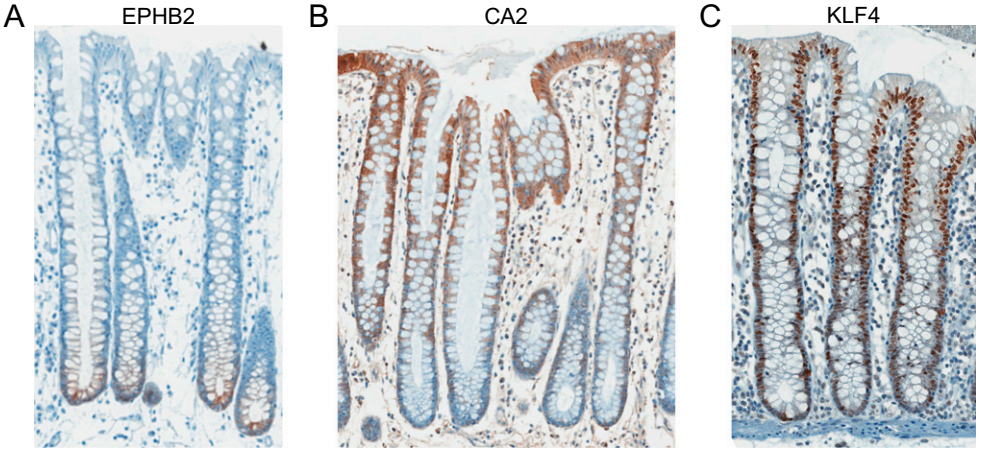

Fig. S4. EPHB2, CA2, and KLF4 staining in normal human colon. (*A*) Representative image of EPHB2 staining by IHC in normal human colon. (*B*) Representative image of CA2 staining by IHC in normal human colon. (*C*) Representative image of KLF4 staining by IHC in normal human colon.

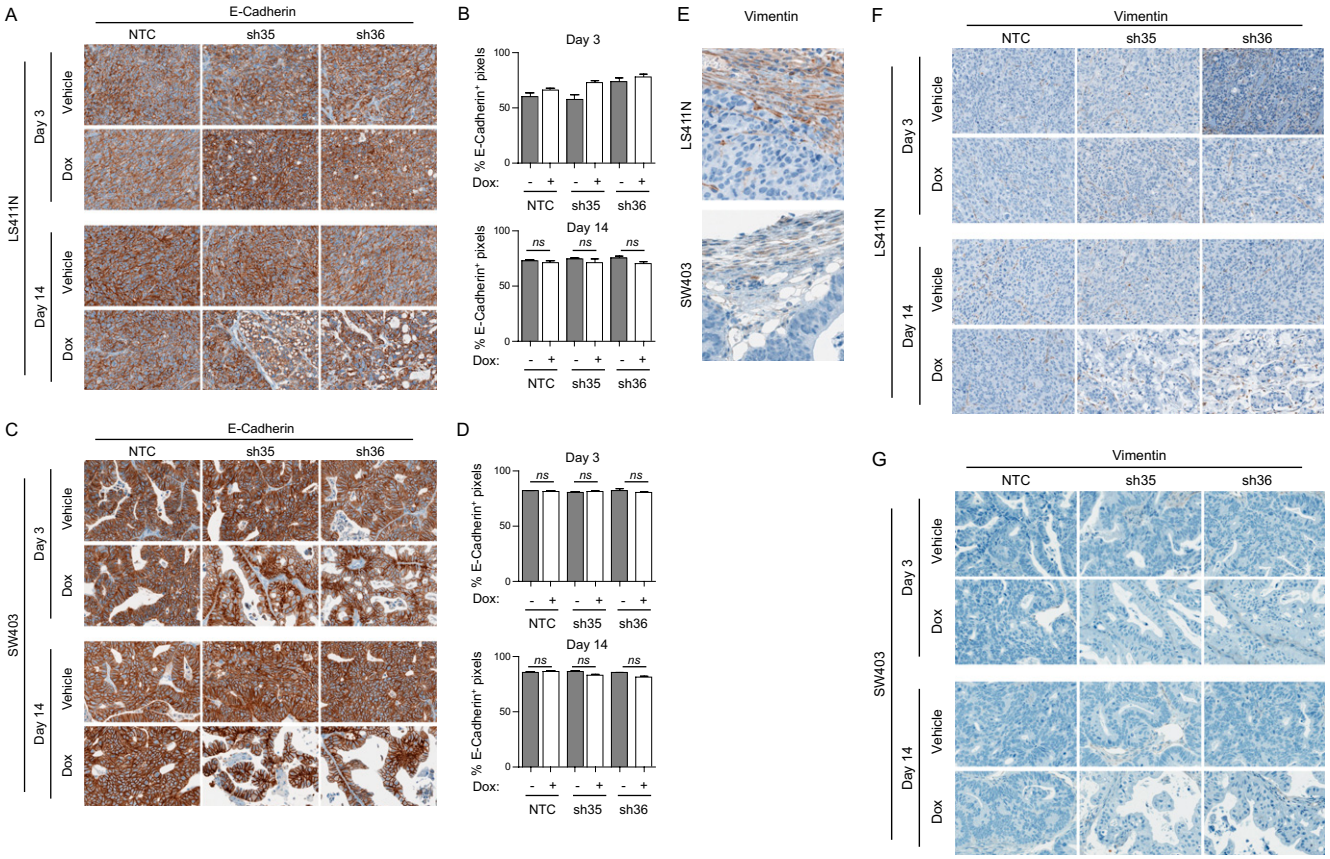

Fig. S5. E-Cadherin and Vimentin expression patterns are not altered after Wnt pathway inhibition. (*A* and *B*) LS411N cancer cells containing NTC, sh35, or sh36 *β-catenin* shRNA were inoculated into mice. Tumor-bearing mice were treated with either vehicle or doxycycline. (*A*) Representative image of E-Cadherin staining by IHC after 3 d or 14 d of treatment. (*B*) Percentage of positive pixels. Graphs represent mean ± SEM values. n = 2–4 per treatment group. (*C* and *D*) SW430 cancer cells containing NTC, sh35, or sh36 *β-catenin* shRNA were inoculated into mice. Tumor-bearing mice were treated with either vehicle or doxycycline. (*C*) Representative image of E-Cadherin staining by IHC after 3 d or 14 d of treatment. (*D*) Percentage of positive pixels. Graphs represent mean ± SEM values. n = 3–8 per treatment group. (*E*) Representative images of Vimentin staining in stroma adjacent to LS411N tumor xenograft (*Upper*) and stroma adjacent to SW403 tumor xenograft (*Lower*). (*F*) LS411N cancer cells containing NTC, sh35, or sh36 *β-catenin* shRNA were inoculated into mice. Tumor-bearing mice were treated with either vehicle or doxycycline. Representative image of Vimentin staining by IHC after 3 d or 14 d of treatment is shown. (*G*) SW403 cancer cells containing NTC, sh35, or sh36 *β-catenin* shRNA were inoculated into mice. Tumor-bearing mice were treated with either vehicle or doxycycline. Representative image of Vimentin staining by IHC after 3 d or 14 d of treatment is shown. n = 2–8 per treatment group.

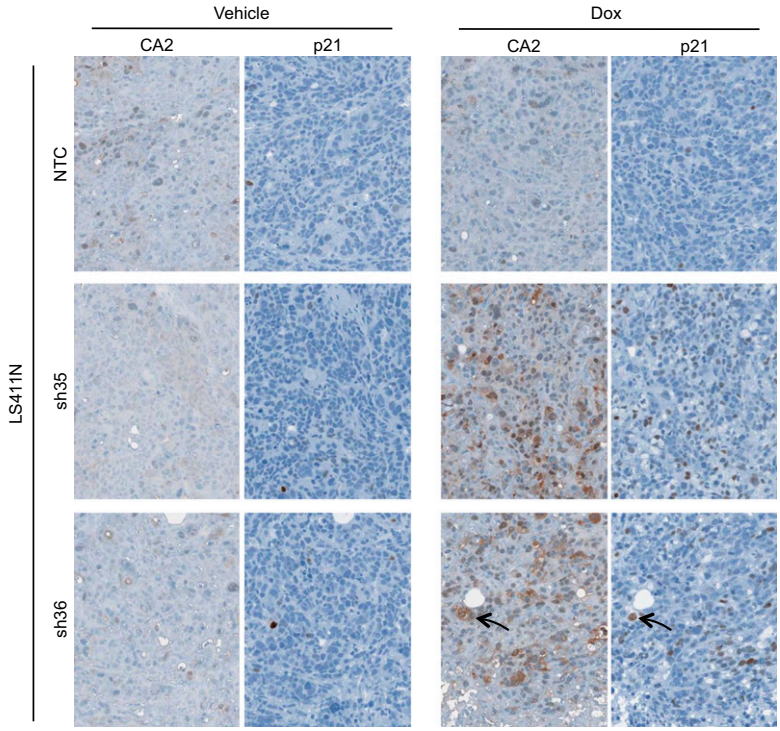

Fig. S6. Complementary p21 and CA2 expression patterns following Wnt pathway inhibition. LS411N cancer cells containing NTC, sh35, or sh36 *β-catenin* shRNA were inoculated into mice. Tumor-bearing mice were treated for 3 d with either vehicle (*Left*) or doxycycline (*Right*). Representative images of CA2 and p21 staining by IHC on serial sections are shown. Arrows mark a cell that was positive for both CA2 and p21.

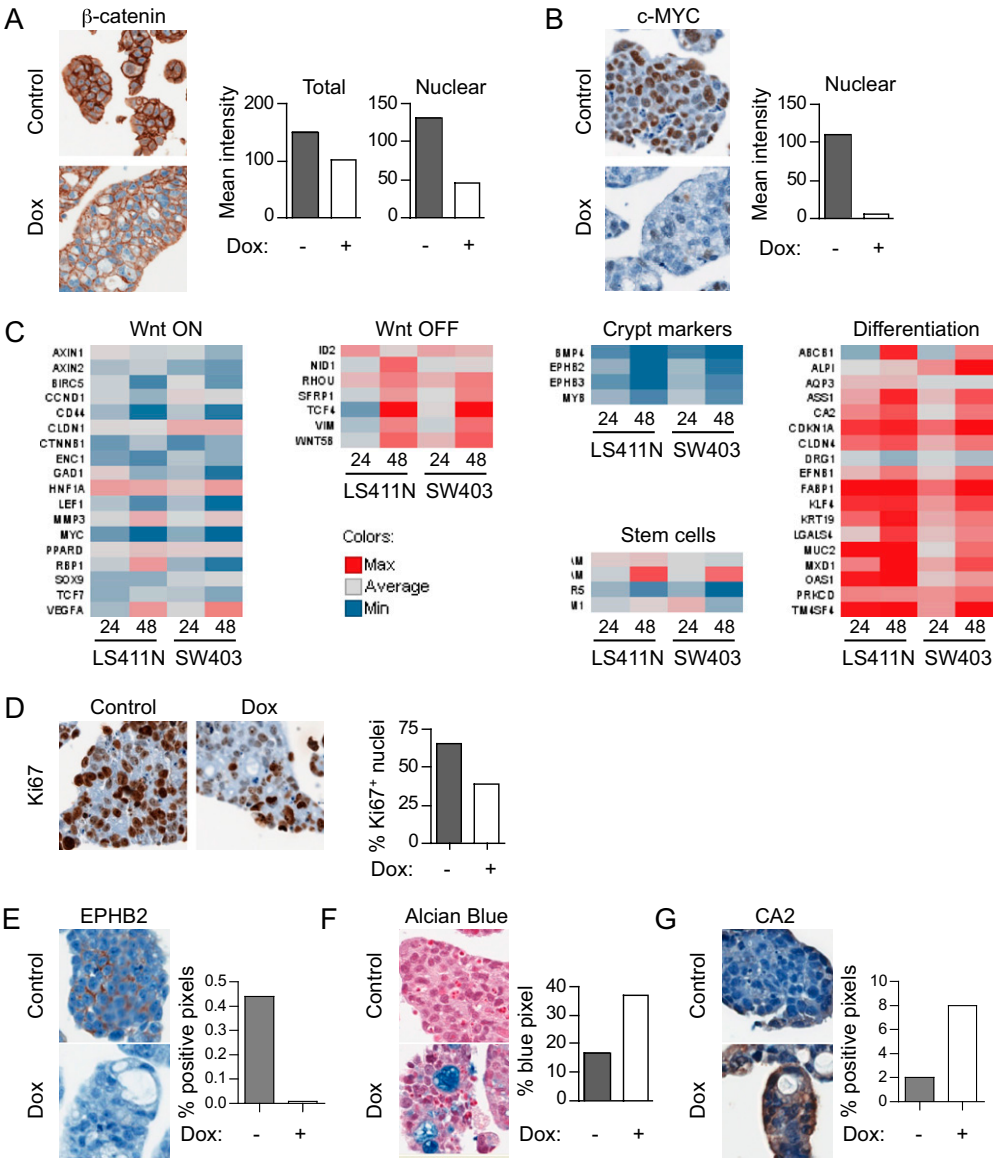

Fig. S7. Colorectal cancer cell differentiation is induced by *β-catenin* shRNA in vitro. (*A* and *B*) LS411N cancer cells containing NTC, sh35, or sh36 *β-catenin* shRNA were grown in vitro in the absence or presence of doxycycline for 3 d. (*A*, *Left*) Representative images of β-catenin staining by IHC. (*Center* and *Right*) Mean signal intensity of total β-catenin (*Center*) and nuclear β-catenin (*Right*). (*B*, *Left*) Representative images of c-MYC staining by IHC. (*Right*) Mean signal intensity of nuclear c-MYC. (*C*) Heat maps representing mRNA expression of selected genes in LS411N and SW403 cancer cells containing NTC, sh35, or sh36 *β-catenin* shRNA after 24 and 48 h of doxycycline treatment. Expression values were normalized to untreated samples. Red and blue: increased and decreased expression following doxycycline treatment, respectively. The "Wnt ON" and "Wnt OFF" gene sets refer to genes that are upregulated or down-regulated by Wnt signaling, respectively. (*D*–*G*) LS411N cancer cells containing sh36 *β-catenin* shRNA were grown in vitro in the absence or presence of doxycycline for 3 d. (*D*, *Left*) Representative images of Ki67 staining by IHC. (*Right*) Percentage of positive nuclei. (*E*, *Left*) Representative images of EPHB2 staining by IHC. (*Right*) Percentage of positive pixels. (*F*, *Left*) Representative images of Alcian blue staining. (*Right*) Percentage of blue pixels. (*G*, *Left*) Representative images of CA2 staining by IHC. (*Right*) Percentage of positive pixels.

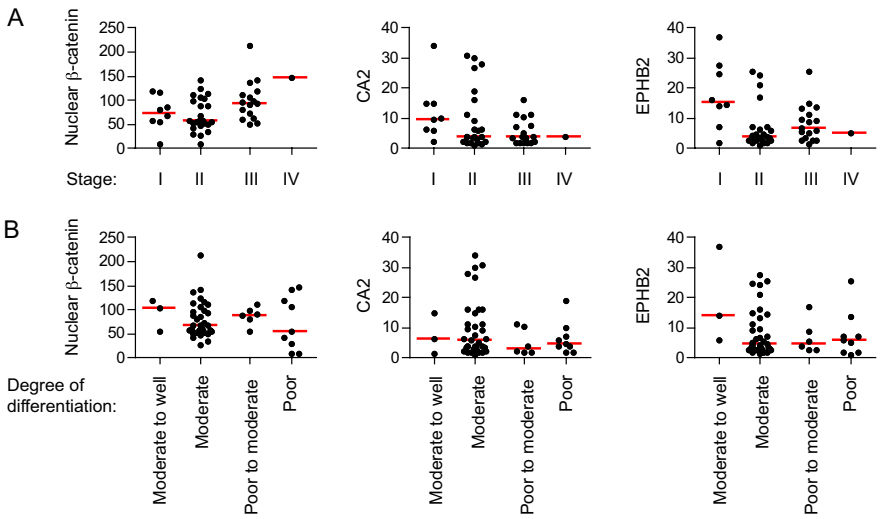

Fig. S8. Nuclear β-catenin, CA2, and EPHB2 expression do not correlate with tumor stage or histologic grade. (*A* and *B*) Mean signal intensity of nuclear β-catenin staining (*Left*), CA2 staining (*Center*), and EPHB2 staining (*Right*) on serial sections from human colonic adenocarcinomas of the indicated tumor stage (*A*) and histologic grade (degree of differentiation) (*B*). Tumor stage and histologic grade (degree of differentiation) were obtained from pathology reports. Each dot represents one patient, and the red line indicates the median. n = 52 patients.

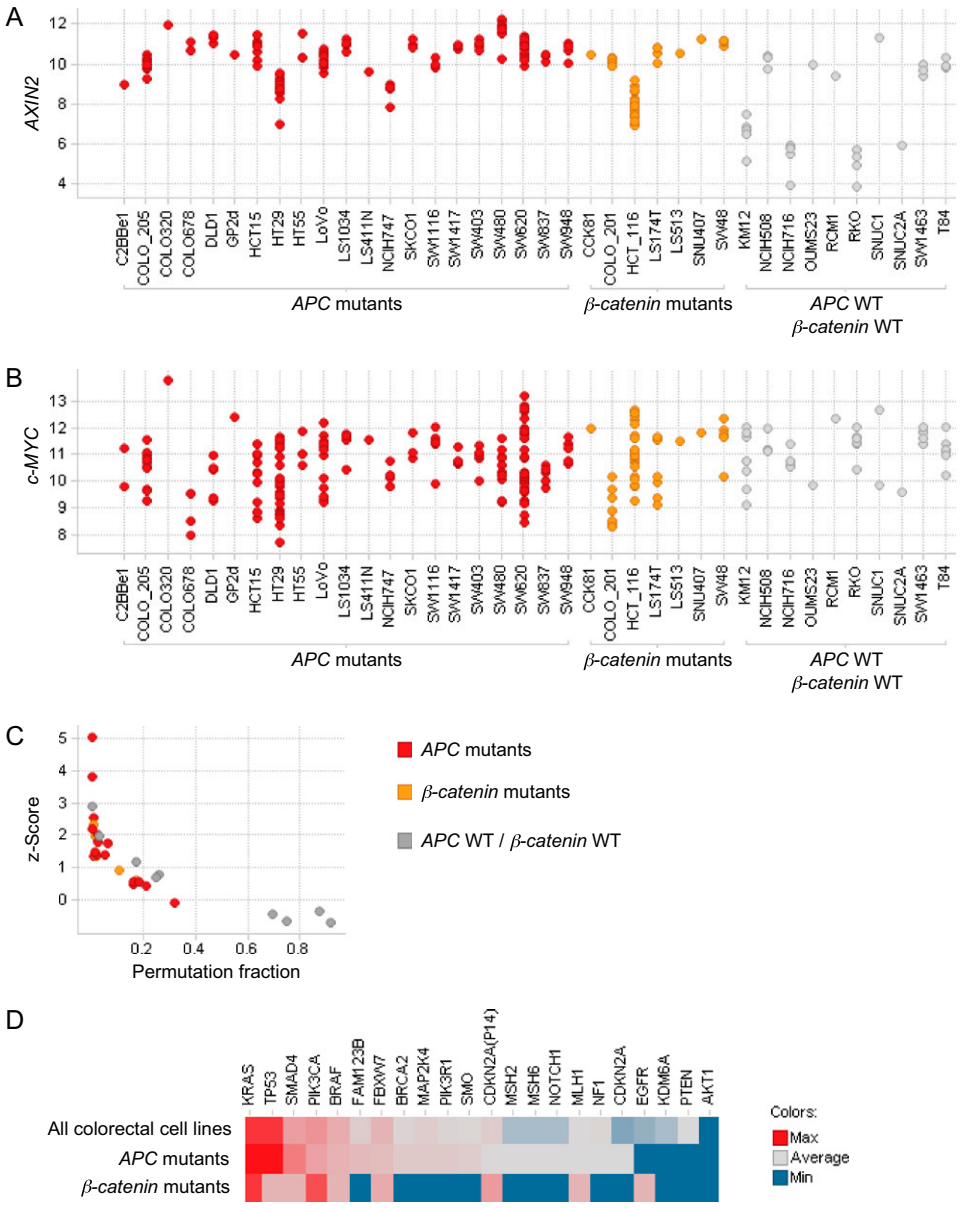

Fig. S9. Magnitude of aberrant Wnt pathway activity and cooperating mutations in colorectal cancer cells containing *APC* versus *β-catenin* mutations. (*A* and *B*) *AXIN2* (*A*) and *c-MYC* (*B*) mRNA expression in indicated colorectal tumor xenografts (log2 scale). (*C*) Wnt pathway activation was assessed using gene set analysis (Wnt custom gene set) as described in *SI Materials and Methods*. The graph represents the *z*-score as a function of the permutation fraction in colorectal tumor xenografts harboring mutations for *APC* (red) or *β-catenin* (yellow) or wild type for both (gray). (*D*) Heat maps representing the frequency of mutation for the indicated genes in colorectal cancer cell lines harboring mutations for *APC* or *β-catenin* as indicated. A total of 44 cell lines were analyzed.