Supplementary Figure Legends

**Supplementary Figure 1.** Determination of IC\textsubscript{50}-values using XLfit. The IC\textsubscript{100} value is defined as the level of the positive control (30% Wnt3a-CM (A), 25 mM LiCl (B) or sample with mixture and PARP enzyme (C and D)) subtracted by the negative control (no stimulation). The IC\textsubscript{0}-value is defined as the level of the negative control. The grey rectangles depict the IC\textsubscript{50}-value (Y-axis, relative activities in percentage %) and the concentration in µmol/L (X-axis). A, the mean ST-Luc activity values from figure 1A are plotted in the graph and the calculated IC\textsubscript{50}-value is 470 nM. B, the mean ST-Luc activity values from figure 2A are plotted and the calculated IC\textsubscript{50}-value is 360 nM. C, the mean TNKS1 or TNKS2 activity values after JW55 exposure from figure 3B (upper panel) are plotted and the calculated IC\textsubscript{50}-values are 1.9 µM and 830 nM, respectively. D, the mean TNKS1, TNKS2 and PARP1 activity values (logarithmic scale) after XAV939 exposure from figure 3B (lower panel) are plotted and the calculated IC\textsubscript{50}-values are 15 nM, 78 nM and 640 nM, respectively. The results in the reporter assays (A and B) show the mean values of at least three independent experiments and the PARP assays show the mean values from two independent setups.

**Supplementary Figure 2.** The figure shows full-length and uncropped Western blots of analyzed lysates from SW480 cells exposed to different concentrations of JW55 for 24 hours (see Fig. 3A). All controls were incubated with 0.1% DMSO. The molecular weights of the proteins are indicated by a line and the sizes in kilo Daltons (kD) are showed. The AXIN2 steady-state level (upper left panel) was dose-dependently elevated by the presence of JW55, while the cytoplasmic and nuclear β-catenin levels were decreased (lower left and mid panels). The anti ABC antibody (active β-catenin) recognizes β-catenin that is not phosphorylated at the N-terminus. Right panel: Active degradation of β-catenin, mediated by JW55 exposure, was detected by using an antibody that recognizes β-catenin phosphorylated at the N-terminus (pβ-catenin). Lower right panels: TNKS1/2 Western blot after JW55 and XAV939 exposures of SW480 cells (48 hours). Antibodies against ACTIN (cytoplasmic) or LAMIN B1 (nuclear) documented equal protein loading. The figures show representative data taken from multiple experiments.

**Supplementary Figure 3.** The distribution and steady-state level of β-catenin as visualized by immunofluorescence. SW480 CRC cells were exposed to 5 or 1 µmol/L JW55 for 48 hours and
analyzed with an antibody against total β-catenin and secondary antibody coupled to Alexa488. Control cell were cultured in 0.05% DMSO. The images were captured with identical shutter speed and 40 times magnification.

Supplementary Figure 4. A, Superposition of JW55 (docking) and IWR-1 (X-ray, PDB code 3UA9) in the PARP domain of TNKS2. The dashed lines depict hydrogen bonds. As in the case of IWR-1 (yellow), the amido groups of JW55 (green) form hydrogen bonds with the main chains of the amino acids Asp1045 and Tyr1050 in our model. The furan ring of JW55 stacks to His1048. The benzene spacer lies in the hydrophobic region formed by the side chains of Phe1044, Ile1039 and Phe1035. The hydrophobic region, formed by the side chains Tyr1050, Tyr1060 and Tyr1071 is occupied by the norbornyl fragment of IWR-1. In the case of JW55 it is occupied by the pyrane ring and partially by the adjacent benzene ring. B, Solvent-excluded surface of the TNKS2 PARP domain is shown and colored by atom types: red (oxygen), blue (nitrogen), white (carbon) and yellow (sulphur).

Supplementary Figure 5. A fluorescence polarization competition assay was performed using a fluorescein-tagged analog of XAV939 (XAV939-Fluorescein) versus JW55 and a structurally similar but inactive analog of JW55. A, the structures of JW55, the inactive analog of JW55 and XAV939-fluorescein. The inactive analog showed no reduction of ST-Luc activity in HEK293 cells at doses up to 10 µmol/L. B, the fluorescence polarization signal (mP) of 200 µL 1 µM XAV939-fluorescein was defined as 0%. TNKS2 was added to a concentration of 1 nM. The elevated mP signal due to XAV939-fluorescein binding was defined as 100%. JW55 was added in various molar ratios (1/100, 1/10, 1/1, 10/1, 100/1) relative to XAV939-fluorescein (black). The same ratios were tested with the inactive analog of JW55 (grey). The figure shows that JW55 dose-dependently releases TNKS2 and a complete release is observed at the molar ratio 100/1. In contrast, the inactive analogue releases less than 50% of TNKS2 with a molar ratio 100/1.

Supplementary Figure 6. In both experimental groups, flat adenomas (Fig. 7A) were identified almost exclusively in the ileum of the small intestine, and most frequent distally. Pedunculated or semi-sessile adenomas were not observed. A, all adenomas were microscopically similarly delineated with straight tubular glands with no branching. B, crowding of immature enterocytes with elongated nuclei were identified in the epithelium from the bottom of the crypts along the glands to the surface, intermingled with numerous Paneth cells and a few goblet cells (arrow), corresponding to low-grade dysplasia. C, a sharp transition between adjacent normal surface
epithelium and neoplastic glands (arrow) could be observed. D, in areas with no obvious adenoma formation aberrant crypt foci (arrows) could be observed at the lower part of single crypts or within a few crypts. Infiltration of neoplastic glands beneath the muscularis propria was not observed in any lesions.

**Supplementary Figure 7.** A, human liver microsome (HLM) test of JW55. Half-life (t½) = 10.1 min. The level of JW55 in the plasma, from mice treated (p.o.) with 100 mg/kg JW55 24 hours prior to blood collection, was also beneath the detection limit (10 ng/mL) of the AFFL-SPE-LC-UV system. B, weight measurement of $Apc^{CKO/CKO}Lgr5-CreERT2^+$ knockout mice treated with either 100 mg/kg JW55 (black, n = 3) or vehicle (DMSO, grey, n = 4). No substantial weight loss was detected among the injected mice compared to vehicle-treated animals. The dotted lines indicate presumed weight developments as the body weight was not measured at the experiment end (21 days). C, quantification of tumor number and area using Ellipse software. Microscopic greyscale images of the anti-β-catenin-stained colon sections generated from the vehicle (a) and JW55-treated (b) mice. The identical images are presented in color in Fig. 7 (d’) and (e’), respectively. The regions of neoplastic growth were selected according to the intensity of the staining when using the automatic tool integrated in the software. The shape of the preselected regions (along the rostrocaudal axis of the intestine) were adjusted manually (red line) and the numbers and areas of the individual lesions were obtained. Black bar in lower right corners = 0.1 mm.

**Supplementary Table 1.** The table shows the results from an Illumina expression analysis that examined mRNA from SW480 CRC cells exposed to 25 µmol/L JW55 for 72 hours. All genes with a 0.5-fold change in expression are shown in the table. The P-value threshold level (P = 0.03) refined the results into a table with 610 differently expressed genes that are listed ascending. Several Wnt target genes listed at the Wnt homepage were differently regulated (highlighted in yellow). Up-regulated (Log2 ≥ 0.5): WISP3, TCF7, PLAUR, EFNB2 and NOTUM. Down-regulated (Log2 ≤ − 0.5): AXIN2, NKD1, DKK1, MMP7, ID2, GAST, FZD2, EDN1, CYR61, SOX18. Other Wnt-regulated genes have previously been found in Ls174T CRC cells (1) (highlighted in tan). The Illumina analysis also revealed other interesting genes with different expression (highlighted in green): SFRP5, CSNK1G2, ALPL, PPARG, KLF4, KLF5 and KLF6. Some genes from the SPANX gene family (SPANXA1, SPANXB2, SPANXC and SPANXE) were substantially down-regulated (highlighted in pink).
References


Supplementary Materials and Methods

Plasmids, constructs, cell lines and conditioned media

SuperTOP-Flash (ST-Luc) (7 X TCF binding sites promoter) (1) was kindly provided by R. Moon. To create SuperTOP-d1EGFP (ST-d1EGFP), the promoter from ST-Luc was cloned into d1EGFP-N1 (Clontech) after excision of the CMV promoter. For stable transfection experiments, the ST-Luc cassette was subcloned into a pClneo plasmid (Promega) giving rise to the construct ST-Luc-neo (selection: 500-1000 µg/mL Genetecin (G418), Sigma). NF-κB-Luciferase and pRL-TK (Renilla) were purchased from Promega. For stable transfection experiments, the pRL-TK (Renilla) cassette was subcloned into pPUR (Promega) giving rise to the construct pRL-TK-puro (selection: 1-5 µg/ml Puromycin, Sigma). Linearized ST-Luc or pRL-TK-puro were transfected in succession into various colon cancer lines and selected. The constructs for β-catenin and dominant active β-catenin (da-Cat) (S33, 37, 41, 45A mutated) were obtained from V. Korinek and R. Kemler, respectively. To create the Shh expression vector, mouse Shh cDNA was cloned into a pLenti6.2-GW/EmGFP expression control vector. The lentivirus was produced using ViraPower Lentiviral Systems (Invitrogen).

The cell lines SW480, DLD-1, HCT116, HCT-15, RKO, HEK293, HeLa, PANC-1, NIH/3T3 Shh Light II cells and L Wnt3a-expressing cells were purchased from ATCC (American Type Culture Collection) and maintained according to the supplier’s recommendations. All cell lines (authenticated and characterized according to ATCC standard protocols) were always kept in culture for less than six months after the arrival from ATCC. Wnt3a containing conditioned media (Wnt3a-CM) from L Wnt3a-expressing cells was harvested as described by ATCC. Shh-CM was harvested from PANC-1 cells that were infected with Shh-expressing lentivirus. ST-d1EGFP HEK293 cells were created by stable transfection of the ST-d1EGFP construct (FuGENE6, Roche). After selection with G418 (500 µg/ml, Invitrogen)
resistant clones were pooled and stimulated with a conditioned medium (Wnt3a-CM) from L
Wnt3a-expressing cells. d1EGFP-expressing cells were FACS-sorted and pooled.

Transfection and luciferase assays

80,000 HEK293 cells were seeded in 48-well plates coated with poly-L lysine. 24 hours
after seeding, 0.25 µg total plasmid DNA and 0.75 µl FuGENE6 (Roche) was combined in a total
volume of 25 µl Opti-MEM® (Invitrogen) as described by the manufacturer (Roche). The
transfection mixture was added to the plated cells and media were changed after 24 hours. All
luciferase assays contained a minimum of three replicates for each differently treated sample if
not mentioned otherwise. ST-Luc assay in HEK293 cells: Transfected cells (0.23 µg ST-Luc +
0.02 µg Renilla) were incubated for an additional 24 hours with various concentrations of JW55
in 30% Wnt3a-CM or 25mM LiCl. NF-κB-Luciferase assay in HEK293 cells: 24 hours after
transfection (0.23 µg NF-κB-Luciferase + 0.02 µg Renilla) cells were exposed for 24 hours to 1
or 10 µmol/L JW55 in 10 ng/ml rTNF-α (R&D Systems). β-catenin or da-Cat in ST-Luc assay:
transfected HEK293 cells (0.215 µg ST-Luc + 0.02 µg Renilla + 0.015 µg β-catenin or 0.23 µg
ST-Luc + 0.02 µg Renilla + 0.2 ng da-Cat) were exposed to 10 µmol/L JW55 for 24 hours
starting at the time of transfection (a minimum of six replicates for each treatment). Shh Light II
assay: 100,000 Light II cells (stable transfectants with Gli1-Luc and Renilla) were seeded in 48-
well plates and incubated for 48 hours in 50% Shh-CM mixed with 10 or 1 µmol/L JW55. ST-
Luc assay in colorectal cancer cells: 50,000 HCT-15, SW480 or HCT116 cells, stably transfected
with ST-Luc and Renilla (FuGENE6, Roche), were seeded in 48-well plates and cultured
overnight. The medium was changed to 0.05% DMSO or different concentrations of JW55. The
cells were incubated for another 48 hours. At incubation end, all reporter cells were lysed and the
firefly luciferase and Renilla activities were measured in a 20/20n Luminometer (Turner
(Promega).

IC_{50}-value calculation

XLfit (idbs) was used to determine the IC_{50}-values of inhibition experiments (ST-Luc,
TNKS and PARP-assays). The following formula was used to fit the data points:
Langmuir Binding Isotherm:
fit = ((A+(B*x))+(((C-B)*(1-exp((-1*D)*x))))/D))
res = (y-fit)
Statistical analysis

SigmaPlot® 11 (Systat Software Inc.) was used to perform all statistical analyses. For comparisons of two groups, normal distributions of the datasets were first analyzed with the Shapiro-Wilk tests. When the Shapiro-Wilk test passed ($P > 0.05$), a Student’s $t$-test was performed. If the Shapiro-Wilk test failed ($P < 0.05$), a Mann-Whitney rank sum test was applied. When performing Students $t$-test and Mann-Whitney rank sum tests, $P < 0.05$ was regarded as a statistically significant difference.

Xenopus double axis assay

Capped $XWnt8$ mRNA was synthesized from a linearized plasmid template using the mMESSAGE mMACHINE kit (Ambion). 4 nl $XWnt8$ mRNA (10 pg), with 2 pmol JW55 or a corresponding volume of DMSO, was injected into the equatorial regions of the two prospective ventral blastomeres of four-cell stage Xenopus embryos. The embryos were incubated at 19 °C and axis duplication was scored after 36 hours.

Real-time RT-PCR

100,000 DLD-1 or SW480 cells were seeded in 12-well plates. After 24 hours, JW55 was added to a final concentration of 10 or 25 µmol/L. The medium containing JW55 was changed daily for three days. mRNA was harvested using GenElute™ Mammalian Total RNA Miniprep Kit (Sigma). cDNA was synthesized from the purified mRNA with AffinityScript™ QPCR cDNA Synthesis Kit (Stratagene). Real-time RT-PCR (SYBR Green PCR Master mix, Stratagene) was performed in Mx3000P® QPCR System real-time thermal cycler (Stratagene).

Primers (Eurofins MWG Operon):

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\text{AXIN2 forward: } 5'-\text{CCCAAGCCCCCATAGTGCCCAAAG-3'}
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\[
\text{AXIN2 reverse: } 5'-\text{CAGGGGAGGCATCGCAGGGTC- 3'}
\]
\[
\text{SP5 forward: } 5'-\text{GCGGCGAGGGCAAGGGG-3'}
\]
\[
\text{SP5 reverse: } 5'-\text{CGCCGAGGGCATGGACACCCG-3'}
\]
\[
\text{NKD1 forward: } 5'-\text{TCACTCCAAGGCCGGCCG-3'}
\]
\[
\text{NKD1 reverse: } 5'-\text{TCCCGGGTGCTTCGGCCTATG-3'}
\]
\[
\text{GAPDH forward: } 5'-\text{GCCCCCTCTGCTGATGCCCCCA-3'}
\]
\[
\text{GAPDH reverse: } 5'-\text{TGGGTGCGAGTGAGTC-3'}
\]

Illumina gene expression analysis
SW480 mRNA from three independent experiments (exposed to 25 µmol/L JW55, see RT-PCR section) was amplified for hybridization on Illumina® BeadChips using the Illumina® TotalPrep RNA amplification Kit (Ambion) # IL1791, using 400 ng of the total RNA. In vitro transcription reaction was incubated overnight (14 hr). Labeled cRNA was hybridized to the Illumina Human-6 v3 BeadChips (Illumina) at 58ºC overnight, according to the Illumina Whole-Genome Gene Expression Protocol for BeadStation (Illumina). The hybridized BeadChip was stained with streptavidin-Cy3 (Amersham) for visualization and scanned with an Illumina® BeadArray Reader. The scanned images were imported into BeadStudio 3.1.3.0 (Illumina) for extraction and quality control.

**Immunoblotting**

100,000 SW480 cells per well were seeded in 12-well plates and treated with JW55 at various concentrations for 24 hours. Lysates were immunoblotted using the following primary antibodies: monoclonal active-β-catenin (#05-665ABC) (Millipore), β-catenin (610153, BD Transduction Laboratories™), phospho-β-catenin (Ser33/37/Thr41, Cell Signaling Technology), AXIN2 (76G6, Cell Signaling Technology), Tankyrase-1/2 (H350: sc-8337, Santa Cruz ) (after 48 hour incubations with 5 µM JW55 or XAV939), ACTIN (A2066, Sigma) and LAMIN B1 (ab16048-100, Abcam). Primary antibodies were visualized with secondary HRP-conjugated antibodies (sc-2313 or sc-2314, Santa Cruz Biotechnology) and enhanced chemiluminescent substrate (Pierce® ECL Western Blotting Substrate, Thermo Scientific).

**Immunocytochemistry and section staining**

50,000 SW480 cells were seeded in 24-well plates on glass slides and exposed to 1 or 5 µmol/L JW55 for 48 hours. After the incubation, the cells were fixed in 4% PFA in PBS for 10 minutes. Immunostaining was performed as described in standard protocols. Primary antibodies: β-catenin (610153, BD Transduction Laboratories™) or AXIN2 (76G6, Cell Signaling Technology). Secondary antibodies: DyLight549 (555) donkey-anti-mouse and Cy2-donkey-anti-rabbit (both Jackson ImmunoResearch, 1:1000). The samples were imaged by using a Zeiss Axiovert 200M Fluorescence/Live cell Imaging Microscope at 40 times magnification. A Zeiss LSM780 at 63 times magnification was used for confocal microscopy. For immunohistochemical analyses of tissue sections, de-waxed sections were treated with 0.75% H₂O₂ in methanol for 20 min. The antigen retrieval was performed in citrate buffer (10 mM pH 6.0) in a steam bath (20 min at 98 °C). The slides were washed in PBS and incubated with 5% goat non-immune serum (Jackson ImmunoResearch) for 20 min at RT to block non-specific binding. Mouse anti-β-
catenin monoclonal antibody (sc-7963, Santa Cruz) was diluted (1:500) in Tris-buffered saline [(TBS); 50 mM Tris pH 7.6, 150 mM NaCl], 1% BSA (Fraction V; Sigma), 5% goat non-immune serum and incubated with the slides overnight at 4°C. The antibody was visualized with goat anti-mouse biotinylated antiserum (Molecular Probes) and streptavidin/biotin/horseradish peroxidase (HRP) detection system (Vectastain ABC kit; Vector Laboratories). HRP was detected using H₂O₂ and diaminobenzidine (DAB; Fluka) substrate; tissue was counterstained with hematoxylin and mounted in Biomount mounting medium (Electron Microscopy Sciences). The specification and working dilution of additional antibodies: goat anti-EphB2 polyclonal antibody (AF467, R&D Systems, 1:1000); mouse anti-Ki67 monoclonal antibody (Mob 237, Diagnostic BioSystems, 1:50); mouse anti-Krt20 monoclonal antibody (M7019, DAKO, 1:25). Hematoxylin and Eosin staining (H&E): De-waxed and rehydrated sections were stained with hematoxylin (4 min)(0.4%, GHS-2 32, Sigma) and counterstained with hexamine (1 min) (0.25%, H1130-0, Sigma) and Eosin (3 min)(0.05%, E4382, Sigma). After dehydration the sections were mounted.

**TNKS1, TNKS2 and PARP1 in vitro biochemical assays**

JW55 inhibitory activity at various doses (duplicates) was tested against TNKS1 (80564), TNKS2 (80566) and PARP1 (80551) Chemiluminescent Assay Kits (all from BPS Bioscience, Nordic Biosite). The procedures were performed according to the protocols from BPS Bioscience, Nordic Biosite.

**Molecular modeling**

In order to evaluate the positioning of JW55 in the PARP domain of TNKS2 we performed a molecular docking study. The PARP domain structure from human TNKS2 in complex with IWR1 (X-ray, PDB code 3UA9) (2) was used for docking of JW55. The structure was processed before docking by adding hydrogen atoms using Molprobity server (3). Water molecules, sulphate ions, PEG molecules and IWR1 were deleted from PDB structure. Frog2 software was used to generate 3D coordinates for JW55 (4). Gold5.1 software (5) with CHEMPLP scoring function was used for the docking studies. The number of iterations was set to the option “very flexible”. The whole 3ua9 structure was defined as a docking region. Amino acids side chains in the binding site were treated as flexible using rotamer libraries. The structure with top score was used. During the docking the side chain of one amino acid (Tyr1050) changed its position against X-ray structure of IWR1 complex (3ua9). To evaluate this change, the molecular dynamics using AMBER11 package and Amber99SB force field was performed (6).
During a two nanosecond run with TIP3P waters, the Tyr1050 side chain changed its position back towards the same position as in the case of 3ua9.

**Fluorescence polarization competition assay**

2 mg of XAV939 including a primary amino group (TC scientific) was dissolved in 250 µL DMSO, mixed with 4 mg NHS-fluorescein (Thermo Scientific) in 100 µL DMSO, and 100 µL sodium phosphate (20 mM). The solution was incubated at room temperature overnight. The labeled product (XAV939-fluorescein) was isolated from the excess reactants by semi-preparative reverse phase liquid chromatography, using a 10 mm i.d. ×250 mm 5 AQ C18 column (ACE). The mobile phase was ACN/0.1% FA (aq) (43/57, v/v). The eluent was dried with a SpeedVac concentrator overnight. The product was examined by LC-MS (Bruker) and the purity was assessed to be ≥99%. The product was weighed and a stock solution of 1 mM was prepared with DMSO. A working solution of 1 µM was prepared by dilution with water. The inactive analog (TC scientific) was tested in a ST-Luc assay with Wnt3a activation as described in the transfection and luciferase assays section. A F200 PRO fluorescence polarizer (Tecan) with appropriate filters (EX 485/EM 535) was employed for the competition assay.

**Cell cycle analysis**

100,000 SW480 cells per well were seeded in 12-well plates and exposed to 10 µmol/L JW55 or 0.1% DMSO for three days. The cell culture medium was changed daily. After 30 minutes of incubation with 10 µmol/L BrdU, the cells were trypsinized, fixed in 4% PFA and stained with mouse anti-BrdU (Roche, 1:100) followed by binding of anti-mouse Alexa Fluor® 488 (Invitrogen). Counterstaining with 10 µg/mL propidium iodide (PI) was followed by treatment with RNase I (both Sigma). The samples were analyzed in a PAS-PPCS flow cytometer (Partec).

**Long-term culture**

40,000 SW480 cells per well were seeded in 24-well plates as duplicates. A day after seeding, the cell medium was changed to 0.05% DMSO (control) or 5, 2.5 or 1 µmol/L JW55 solutions. The cells were split 1:5 twice a week while cultured in JW55 or control solutions. At each passage a proportion of the discarded cells were counted by a PAS-PPCS flow cytometer to determine the proliferation rate (Partec).

**AFFL-SPE-LC-UV analysis**
For analysis of JW55 in the plasma, 50 µL samples were diluted with 0.3 mL water, protein-precipitated with 0.25 mL 30 % TCA (aq) and 0.125 mL methanol was added to disrupt the drug-protein binding. Samples were centrifuged and the supernatant was injected. 0.1 mL was injected in an AFFL-SPE-LC-UV system with microbore LC settings (7). The samples were chromatographed using an isocratic mobile phase of 0.1% formic acid (aq)/ACN (55/45, v/v). Since JW55 was difficult to detect with electrospray ionization MS, UV detection was employed instead (290 nm).

**HLM analysis**

The human liver microsome analyses (HLM) were performed according to the standard protocols of Cyprotex, United Kingdom.

**References**


