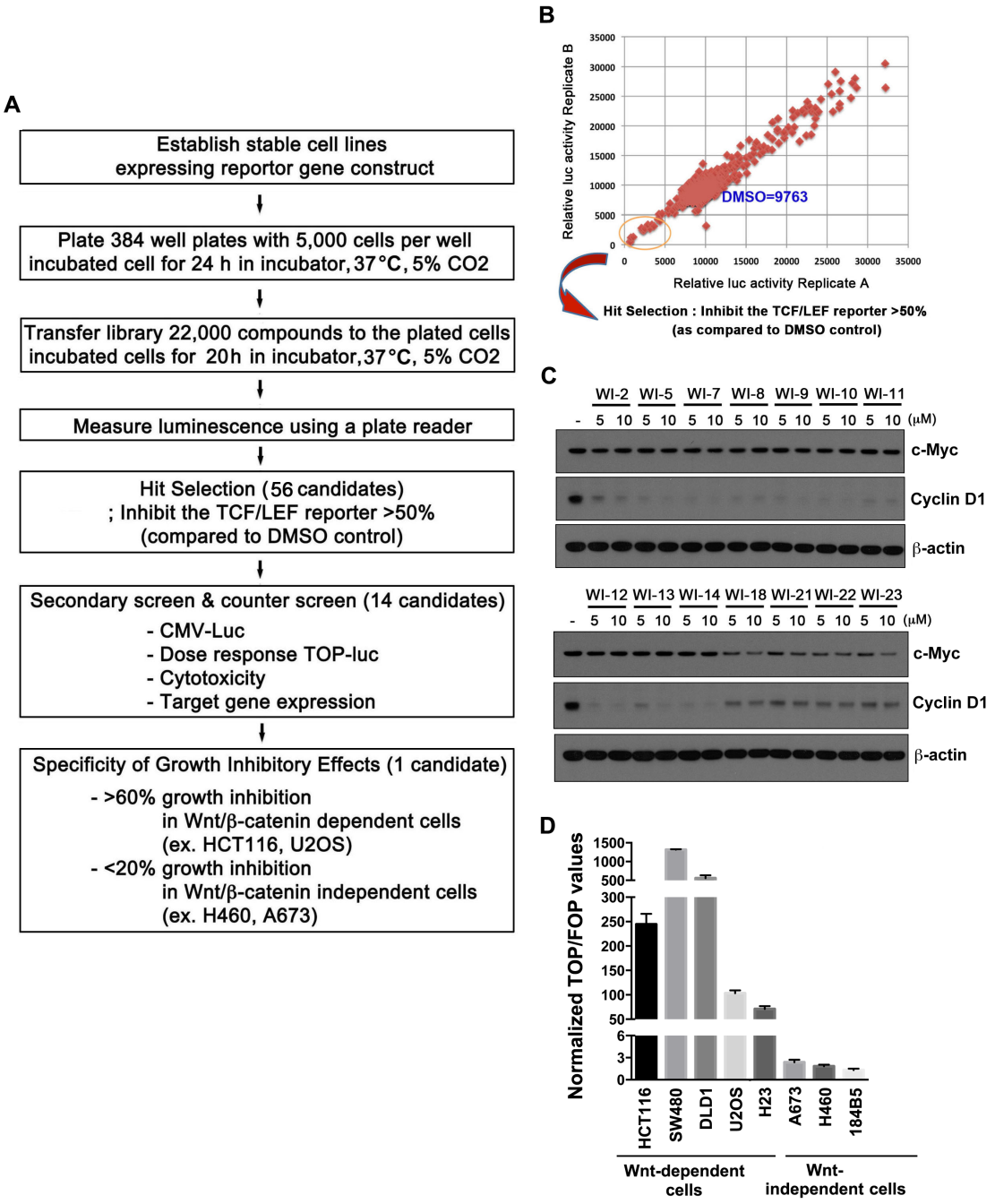


Figure S1, related to Figure 1. Overview of screening method and the identification of Wnt/β-catenin pathway inhibitor compound candidates. (A) Schematic of high-throughput chemical screening strategy for Wnt/β-catenin inhibitors using Wnt-dependent cancer cell line HCT116 that carries a TOP-Luciferase reporter plasmid. HCT116 cell line stably expressing TOP-Luc reporter construct was generated. Five thousand cells in 30 μl of medium were plated into each well of 384 well plates using automated plate filler. Following 24 h of incubation, small molecule library compounds were pin-transferred from stock plates and added to the 384 well plates. After 20 h of incubation with test compounds, 30 μl of Steady-Glo reagent (Promega) was added to each well, followed by 15 min of shaking at room temperature. Lumin-

escence was measured through an automated plate reader. **(B)** High–throughput chemical screening was performed in duplicates, and relative luciferase activity was calculated. Compounds that inhibited TOP-Luc reporter activity more than 50 % compared to DMSO-treated control were selected. Data from two independent replicates of the screen were plotted as a XY-scatter plot with dots corresponding to each compound. **(C)** Western blot analyses were carried out to test the inhibitory effect of candidate compounds on Wnt-target gene expression on the protein level. HCT116 cells were treated with each candidate compound (5 or 10 μM) for 20 h and the effect on Wnt target gene products was examined through western blot analyses. β-actin level was used as a loading control. **(D)** Wnt signaling activity was measured in various cell lines via TCF mediated luciferase reporter activity. Luciferase reporter activity was measured in various cell lines carrying TOP-Luc or FOP-Luc reporter plasmid, and the value of TOP/RL was divided by FOP/RL to calculate the ratio TOP/FOP, which is shown as a bar graph. Error bars indicate SD of mean values obtained from triplicates carried out at the same time.

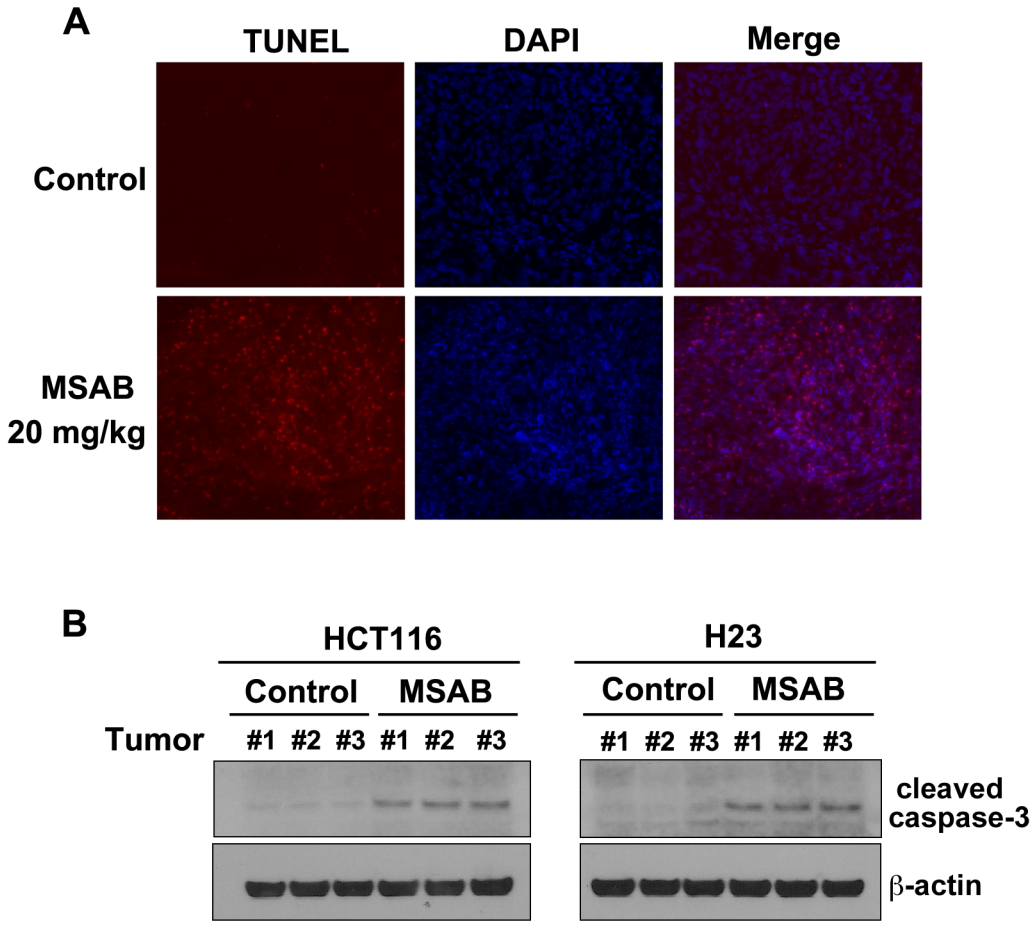


Figure S2, related to Figure 2. MSAB induces apoptosis in Wnt-dependent cancer cells in mouse xenograft models. (A) Apoptosis in tumor xenografts detected by TUNEL staining. Tumors harvested from HCT116 xenograft mice model after 14 days of MSAB treatment (20mg/kg) were subjected to immunohistochemical analysis. Results from TUNEL and DAPI staining of tumor sections from control- and MSAB-treated mice are shown. **(B)** Western blot showing the level of cleaved caspase 3 expression in tumor tissues isolated from mice bearing HCT116 and H23 cells. β-actin was used as a loading control.

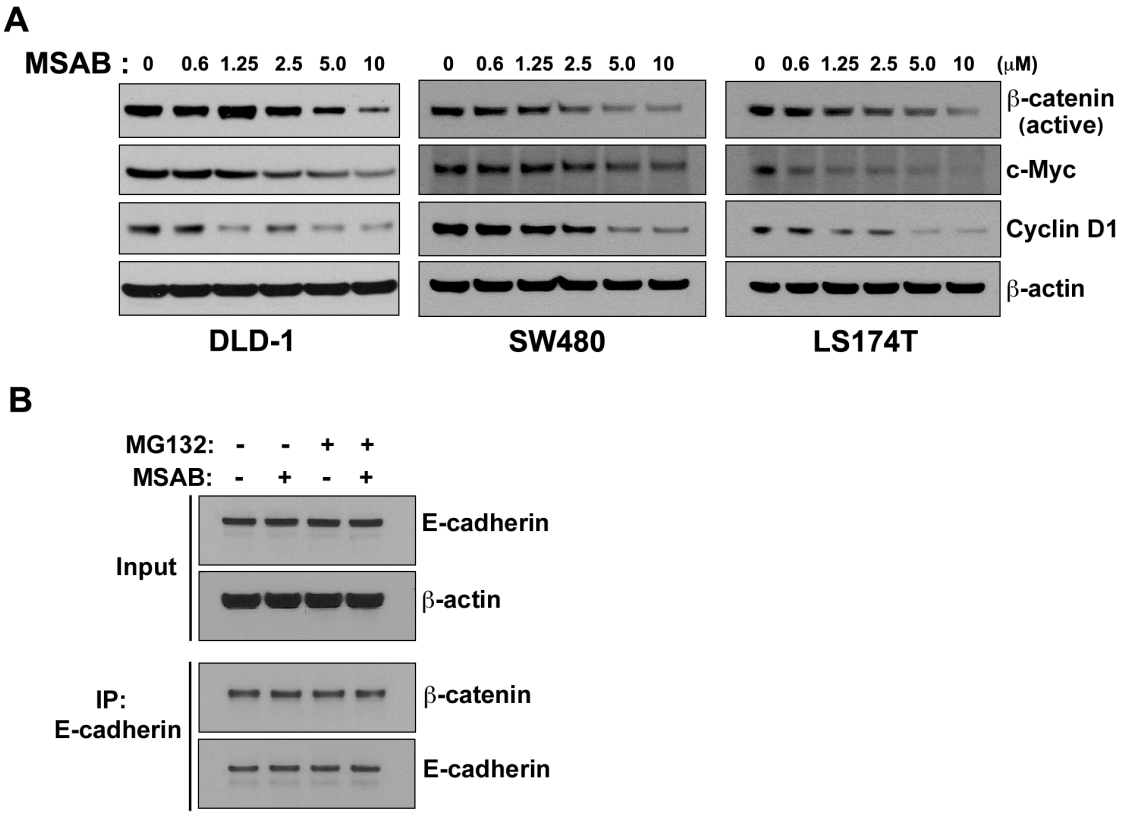


Figure S3, related to Figure 3. MSAB inhibits β-catenin activity and stimulates proteasomal degradation of β-catenin. (A) MSAB inhibits Wnt/β-catenin target genes in DLD-1, SW480 and LS174T cancer cell lines. DLD-1, SW480 and LS174T cells were treated with the indicated concentrations of MSAB for 20 h and the effects on β-catenin and Wnt target gene products, c-Myc and Cyclin D1, were examined by western blot analysis. β-actin level was used as a loading control. **(B)** MSAB shows little effect on the interaction between E-cadherin and β-catenin. Western blot analysis was carried out against the same HCT116 cell lysates used in Figure 3E (Right panels). E-cadherin was immunoprecipitated using anti-E-cadherin antibody. Input and IP fractions were examined by western blot analysis using antibodies as indicated on the right.

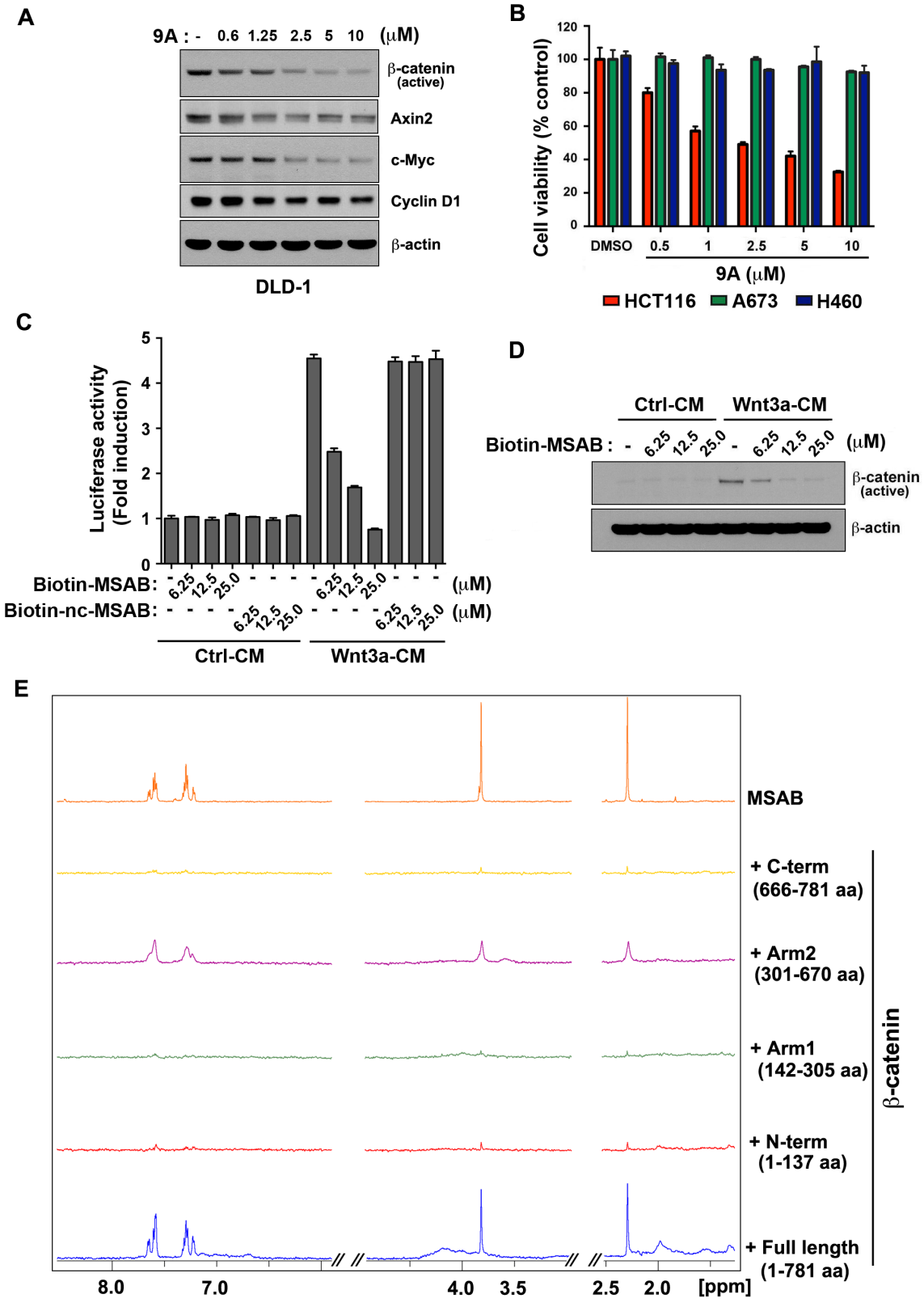
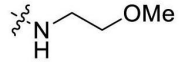
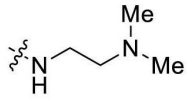
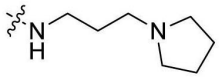
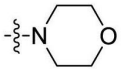

A
9A : - 0.6 1.25 2.5 5 10 (μM)
β-catenin (active)
Axin2
c-Myc
Cyclin D1
β-actin
DLD-1
B
Cell viability (% control)
120
100
80
60
40
20
0
DMSO 0.5 1 2.5 5 10
9A (μM)
HCT116 A673 H460
C
Luciferase activity (Fold induction)
5
4
3
2
1
0
Biotin-MSAB: - 6.25 12.5 25.0 - - - - 6.25 12.5 25.0 - - - (μM)
Biotin-nc-MSAB: - - - - 6.25 12.5 25.0 - - - - 6.25 12.5 25.0 (μM)
Ctrl-CM
Wnt3a-CM
D
Ctrl-CM
Wnt3a-CM
Biotin-MSAB : - 6.25 12.5 25.0 - 6.25 12.5 25.0 (μM)
β-catenin (active)
β-actin
E
MSAB
+ C-term (666-781 aa)
+ Arm2 (301-670 aa)
+ Arm1 (142-305 aa)
+ N-term (1-137 aa)
+ Full length (1-781 aa)
β-catenin
8.0
7.0
4.0
3.5
2.5
2.0
[ppm]

Figure S4, related to Figure 4. MSAB serves as a lead compound for inhibitors of Wnt/β-catenin pathway and can bind to β-catenin. (A) MSAB analog 9A inhibits the activity of Wnt/β-catenin pathway. The effects of 9A on β-catenin and Wnt target gene products in DLD-1 cells were examined by western blot analyses. β-actin level was used as a loading control. **(B)** Cell viability was tested after treating cells with indicated concentrations of compound 9A for 48 h. 9A selectively decreased cell viability of Wnt-dependent cell HCT116, while showing little effect on Wnt-independent cells, A673 and H460. Cell viability was measured by SRB assay. **(C, D)** Biotinylated MSAB (Biotin-MSAB) retains the activity of MSAB and can inhibit Wnt/β-catenin activity. HEK293T cells transiently expressing TOP-Luc reporter were treated with control conditioned medium (control-CM) or Wnt3a conditioned medium (Wnt3a-CM) along with indicated concentrations of Biotin-MSAB for 20 h, followed by (C) luciferase assay and (D) western blot analysis. Wnt3a-induced TOP-Luc activation and increase of active β-catenin levels in HEK293T cells were suppressed specifically by Biotin-MSAB. β-actin level serves as a loading control. Error bars indicate SD of mean values obtained from triplicates conducted at the same time. **(E)** MSAB interacts with β-catenin in vitro, based on saturation transfer differential (STD) ^{1}H NMR spectroscopy analysis. For each experiment, 200 μM MSAB and 5 μM of protein was mixed in deuterated 1x PBS solution. Results from the following experiments are shown: 1D ^{1}H spectrum of MSAB in the absence of protein, orange; STD of MSAB and β-catenin C-terminal region (C-term, 666-781aa), yellow; STD of MSAB and β-catenin Armadillo repeat region 2 (Arm2, 301-670aa), purple; STD of MSAB and β-catenin Armadillo repeat region 1 (Arm1, 142-305aa), green; STD of MSAB and β-catenin N-terminal region (N-term, 1-137aa), red; STD of MSAB and β-catenin full length protein (Full length, 1-781aa), blue.

Route	Dose (mg/kg)	T_{max} (h)	C_{max} (ng/mL)	$AUC_{0-\infty}$ (hr*ng/mL)	$T_{1/2}$	CL (mL/min/Kg)	V_{ss} (L/Kg)	F (%)
IV	1	-	-	400	0.50	41.7	1.13	-
PO	10	1.0	523	1220	-	-	-	30.5

* IV = intravenous injection, PO = oral delivery, T_{max} = time of maximum plasma concentration, C_{max} = maximum plasma concentration, AUC = area under the curve (measure of exposure), $T_{1/2}$ = half-life, CL = plasma clearance, Vss = volume of distribution, F = oral bioavailability.

Table S1, related to Figure 2. The Pharmacokinetic parameters of MSAB in mice. The pharmacokinetics of MSAB was evaluated in Balb/c mice given a single intravenous or oral dose. MSAB was found to have a moderate tissue distribution with a calculated volume distribution of 1.1 L/kg and moderate clearance of 41 mL/min/kg. MSAB also exhibited moderate bioavailability with 30.5% of the dose absorbed.

Cmpd ID	n	R^1	R^2	R^3	IC_{50} (μM)[a]
MSAB	0	-CH$_3$	-OCH$_3$	H	0.583
Analogs					
1A	0	-CH$_3$	-OH	H	>20
2A	0	-CH$_3$	-CH$_3$	H	1.795
3A	0	-CH$_3$	-NHEt	H	>20
4A	0	-CH$_3$		H	>20
5A	0	-CH$_3$		H	>20
6A	0	-CH$_3$		H	>20
7A	0	-CH$_3$		H	>20
8A	0	SCH$_3$	-OCH$_3$	H	2.40
9A	0	OCH$_3$	OCH$_3$	H	0.530
10A	0	-CH$_3$	OCH$_3$	Cl	1.419
11A	0	-CH$_3$	OCH$_3$	CH$_3$	>20
12A	0	-CH$_2$CH$_3$	-OCH$_2$CH$_3$	H	3.318
13A	1	F	OCH$_3$	H	>20
14A	1	Cl	OCH$_3$	H	>20

[a] IC_{50} means inhibition of 50% of TOP-luc activity in HCT116 cells

Table S2, related to Figure 4. IC_{50} values of MSAB analogs for TOP-Luc activity in HCT116 cells.

Rank	Gene Name
1	ATP2A2
2	CTNNB1
3	IPO9
4	KPNB1
5	MACF1
6	DOCK6
7	LLGL2
8	HYOU1
9	MCM3
10	MYBBP1A
11	ACTN4
12	ACTN1
13	AP3D1
14	IARS
15	IPO5
16	XRCC5
17	GPRC5A
18	SUPT16H
19	CSE1L
20	EPRS

Table S3, related to Figure 4. List of proteins that co-precipitated with Biotin-MSAB from HCT116 human colorectal cancer cell lysate. Biotin-MSAB or Biotin-nc-MSAB was mixed with HCT116 cell lysate, incubated at 4°C for 1h, and was precipitated using streptavidin-agarose. Co-precipitated proteins were identified through tandem mass spectrometry. Proteins with at least two unique peptides detected were selected for further semi-quantitative analysis based on spectral counting. Proteins were ranked based on the ratio of peptide-spectrum matches (PSMs) in Biotin-MSAB fraction to PSMs in Biotin-nc-MSAB fraction, and the top 20 proteins are listed above.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell Culture

Cells were cultured in media containing 10% fetal bovine serum (FBS) (Invitrogen), 100 units/ml penicillin, 100 μg/ml streptomycin at 37°C in a humidified incubator containing 5% CO_2. Cell lines, HCT116, DLD-1, SW480, LS174T, HT29 and HT115 human colorectal carcinoma, H460, H23, H2170, SW900, H1975 and H1355 human lung cancer, U2OS human osteosarcoma, A673 sarcoma, MDA-MB-435, MCF7, MDA-MB-231 and T47D human breast cancer cell, HEK293T, Wnt3a-secreting L cells were obtained from the American Type Culture Collection (ATCC) and were cultured according to ATCC's recommended media and conditions. Human HDF, MCF10A, 184B5 and 76N cells were cultured as previously described (Band et al., 1990). Cells were grown in D-MEM/F-12 mixture (1:1, vol/vol) containing 15 mM HEPES buffer and 2.5 mM L-glutamine, supplemented with 1% fetal bovine serum, 12.5 ng/ml epidermal growth factor, 10 nM triiodothyronine, 50 μM freshly made ascorbic acid, 1 μg/ml insulin, 1 μg/ml hydrocortisone, 0.1 mM ethanolamine, 0.1 mM phosphorylethanolamine, 10 μg/ml transferrin, 15 nM selenite, 1 ng/ml cholera toxin, 35 μg/ml bovine pituitary extract, 100 units/ml penicillin, 100 μg/ml streptomycin, and 20 μg/ml gentamicin. Cells were maintained in 95% humidified air plus 5% CO_2 and sub-cultured weekly.

Luciferase reporter assays

To measure transcriptional activity of Wnt, NF-κB, iNOS, or NOTCH, we transiently transfected HCT116 cells or HEK293T cells with TOP-FLASH, FOP-FLASH, NF-κB, iNOS, or NOTCH luciferase reporter, along with an internal *Renilla* luciferase reporter plasmid as a control (hRL-null). Transfection was performed with lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA) as according to the manufacturer's manual. The results were normalized to the control *Renilla* activity. The reported data represent the average of three independent experiments.

Cell viability assay

Cell viability was assayed by Sulforhodamine B based *In Vitro* Toxicology Assay Kit (Sigma-Aldrich). Cells were plated in 6-well plates, and after reaching 60-70% confluency, the cells were treated with chemicals at concentrations and durations as indicated in the figures and figure legends. Staining and quantitative analysis were performed according to the manufacturer's manual. All experiments were performed as duplicates.

Immunoblotting

Cells were washed twice with phosphate Buffered Saline (PBS) and lysed with 50 mM Tris, 150 mM NaCl, and 0.5% Nonidet P (NP)-40 buffer with protease inhibitor cocktail (Roche). Equal amounts of total cellular proteins per sample were subjected to SDS-PAGE and transferred to a nitrocellulose membrane (Bio-Rad Laboratories). For western blotting, the following primary antibodies were used: Active β-catenin (Millipore), β-catenin (BD Transduction Laboratories™) (Cell Signaling), Axin 2 (Cell Signaling), cyclin D1 (Santa Cruz), c-Myc (Santa Cruz), β-actin (Sigma) and Lamin B (Santa Cruz). Bands were detected with Western Lightning Plus ECL (PerkinElmer).

Animal Experiments

For xenograft tumor models, cancer cell line HCT116, HT115, H23, or H460 ($2x\ 10^6$) was injected subcutaneously (s.c.) into the flanks of athymic nude mice (NCr nude, 5-6 week old). We also used MMTV-Wnt1 transgenic mice. About 15% of these mice develop mammary tumors between 6 weeks and 3 months of age in MMTV-Wnt1 transgenic mice model. Tumors were allowed to grow to 40 mm^3 prior to intraperitoneal injection. One week after cellular inoculation, mice were treated by intraperitoneal injection with vehicle or MSAB (10 or 20 mg/kg) every day for two weeks. Two weeks after intraperitoneal injection, the mice were euthanized and tumor weight was measured. Tumor dimensions were measured, and volume was calculated by length (L) and width (W) using the formula (volume = $\pi/6 \times L \times W^2$). All animal experiments were reviewed and approved by the Massachusetts General Hospital Subcommittee on Research Animal Care.

Total RNA extraction and Quantitative RT-PCR analysis
Total RNA was extracted from cells using Qiagen RNA extraction kit, and reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer's protocol. Synthesized cDNA was analyzed by q-PCR using gene-specific primers. Primer sequences used were as follows: Axin 2 (FW; ACTGCCCACACGATAAGGAG, RV; CTGGCTATGTCTTTGGACCA), c-Myc (FW; GCTGCTTAGACGCTGGATTT, RV; CACCGAGTCGTAGTCGAGGT), cyclin D1 (FW; CCATCCAGTGGAGGTTTGTC, RV; AGCGTATCGTAGGAGTGGGA), BMP4 (FW; ATCAAACTAGCATGGCTCGC, RV; TGGCTGTCAAGAATCATGGA), 36B4 (FW; GCAATGTTGCCAGTGTCTGT, RV; GCCTTGACCTTTTCAGCAAG). q-PCR was performed using an iCycler iQ^{TM}5 real time detection system (Bio-Rad Laboratories) with LightCycler 480 SYBR Green I Master according to the manufacturer's instructions. The quantitative value was normalized by 36B4 expression level. All experiments were performed in triplicates. Gene expression analysis was performed using the comparative CT method with the housekeeping gene, 36B4, for normalization.

Chromatin Immunoprecipitation (ChIP)
Cells were seeded in 10 cm dishes and compound was treated beginning at 70 % confluency for 20 h. Ten million cells were treated with 1% formaldehyde for 15 min, then harvested, and ChIP was carried out according to the manufacturer's instructions using Chromatin Immunoprecipitation Assay Kit (EMD Millipore). Immunoprecipitation was performed at 4°C overnight with anti-β-catenin antibody (BD Transduction Laboratories^{TM}). Final purified DNA was used for PCR using the following primers, Axin2 (FW; CTGGAGCCGGCTGCGCTTTGAT, RV; CGGCCCCGAAATCCATCGCTCT), c-Myc (FW; GCTCTCCACTTGCCCCTTTTA, RV; GTTCCCAATTTCTCAGCC), cyclin D1 (FW; AGGCGCGGCGGCTCAGGGAT, RV; ACTCTGCTGCTCGCTGCTACT). The value obtained from co-precipitating DNA was normalized over that of input in order to calculate fold-enrichment level. All experiments were performed in triplicates.

Compound affinity purification and mass spectrometry analysis
The general procedure for preparing cell lysates for the detection of binding proteins was carried out as previously described (Ki et al., 2000; Wan et al., 2004) with modifications. The lysates were incubated with Biotin-MSAB or Biotin-nc-MSAB. After incubation for 1 h at 4°C, proteins associated with Biotin-MSAB or Biotin-nc-MSAB were pulled down with streptavidin-agarose (Thermo Pierce). The bound proteins were eluted with SDS/PAGE loading buffer, separated by a 4-20% gradient polyacrylamide gel, and visualized by silver staining. In-gel trypsin digestion was carried out and the eluted tryptic peptides were subjected to tandem mass spectrometry analysis for identification. The level of protein co-precipitating with Biotin-MSAB or Biotin-nc-MSAB was also examined through western blot analysis.

Recombinant protein expression and purification
Plasmid carrying full-length (amino acid residues: 1-781 ; plasmid # 17198), N-terminal (1-137 ; plasmid # 17203), or C-terminal (666-781 ; plasmid # 17203) human β-catenin was purchased from Addgene. Armadillo repeat (136-686) human β-catenin construct was kindly provided by Wenqing Xu in University of Washington. Armadillo repeat (142-305), or (301-670) human β-catenin construct was subcloned into pET28a. *E. coli* BL21(DE3) cells transformed with this plasmid were cultured in LB media supplemented with 50 μg/ml kanamycin until OD_{600} was approximately 0.4, and then protein expression was induced with 0.2 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30°C for 6 h. Cells were lysed by sonication and the proteins were purified by Ni-NTA agarose affinity chromatography (TALON Metal Affinity Resin, Clontech).

Surface Plasmon Resonance (SPR) spectroscopy
Protein-compound interactions were measured with Biacore 3000 surface plasmon resonance instrument (GE Healthcare). Proteins were covalently coupled to the sensor chip CM5 as follows: 100 mM N-hydroxysuccinimide (NHS) and 400 mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were mixed 1:1 and injected at 5 μl/min for 10 min over the flow cells. The protein to be coupled was diluted in 10 mM sodium acetate buffer (pH 5.5) to a concentration of 100 μg/ml and subsequently injected at 5 μl/min with a manual mode until an intended level of protein was immobilized. One flow cell per chip did not receive any protein at this step and served as a reference channel. To quench any remaining activated

chip surface, 1M ethanolamine was injected at 5 μl/min for 7 min into all flow cells. The channels were subsequently washed with PBS until a stable baseline was observed. Subtracting this baseline from the resonance unit (RU) obtained prior to adding protein gave a measure of protein immobilized in each flow cell. A four flow cell revealing capture of 2,000 response unit (RU) of β-catenin was used to measure the interaction with compounds. Various concentrations (0-125 μM) of compounds in running buffer (BSA (100 μg/ml), 0.01 % Triton X-100, 2% DMSO in PBS, pH 7.4) were injected for 90 sec at a flow rate of 40 μl/min, and each series of experiments was tested in duplicates. The sensor chips were regenerated by a 90 sec injection of 50 mM NaOH and 1M NaCl. All Biacore experiments were performed at 25°C. Analyte samples were injected in a randomized order in duplicates. To compensate nonspecific binding of compound to chip surface, binding signal from the reference channel containing no protein was subtracted from binding signal from the flow cell containing immobilized β-catenin. Sensorgram analyses were carried out through BIAevaluation software (GE Healthcare).

Statistical analysis

Statistics were calculated using Prism 6.0 software (GraphPad Software). Significant differences between mean values were evaluated using the Student's t-test for unpaired results or two-way ANOVA followed by Bonferroni's post-tests with more than two experimental groups.

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