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Supplementary Materials for

Pharmacological inhibition of β-catenin/BCL9 interaction overcomes resistance to immune checkpoint blockades by modulating T_{reg} cells

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Supplementary Materials and Methods

Protein binding assay

HTRF was performed according to protocol (Cisbio) and previous publication reports, with modification of settings to optimize assay conditions (Zhang *et al.*, J Biomol Screen, 1999,4, 67-73). Reaction buffer was prepared with 50 mM MES (pH 6.5), 150 mM NaCl, 0.1% BSA, 1 mM DTT, and 0.1% Tween 20. His-tagged β-cat, Eu-labeled mAb (for His-tag), and Streptavidin-XL655 conjugates were then dissolved in buffer at concentrations of 500 nM, 0.5 µL/200 µL mAb, and 2 µL/200 µL respectively. Optimized assay concentrations were determined following cross-titrations between β-cat protein and BCL9 peptide, as well as testing of three combinations between Eu-labeled mAb and Streptavidin-XL655 (the Max average signal in HTRF assay was 4068.5 with a standard deviation of 98.18, Min average signal was 911.3 with a standard deviation of 25.53, and the Z-factor was 0.8). β-cat protein and biotinylated BCL9 peptide were titrated to determine concentrations yielding optimal fluorescent readout, while procedures without β-cat and BCL9 served as respective negative controls. For both β-cat and BCL9, a concentration of 500 nM was implemented for subsequent HTRF studies. Biotinylated hsBCL9$_{CT}$-24 was added at concentrations of 9000, 4500, and 2250 nM; following optimization, hsBCL9$_{CT}$ derivatives and controls were serially diluted to determine Kd values. After preparation of reagents, 5 µL of His-tagged β-cat was added to each well in Sigma-Aldrich low-volume 384-well plates, followed by 5 µL of biotinylated hsBCL9$_{CT}$-24, 10 µL Eu-labeled mAb, and Streptavidin-XL655 detection mixture to the same wells. Plates were incubated for 60 minutes in the dark before fluorescence readout via a microplate reader (Molecular Devices), followed by determination of Kd values using nonlinear regression analysis with Prism software (GraphPad). All assays were performed in triplicates and repeated at least twice with freshly prepared reagent solutions.

Amplified Luminescence Proximity Homogeneous Assay (ALPHA) screening (Perkin Elmer) was performed according to standard protocol, with modifications to account for optimized readout. The Max average signal in ALPHA assay was 31618.9 with a standard deviation of 2872.1, Min average signal was 526.4 with a standard deviation of 68.6, and the Z-factor was 0.7. Biotinylated SAH-BCL9$_B$ and hsBCL9$_{CT}$-24 were conjugated with streptavidin-coated donor beads via PEG linker, customized to allow greater binding area between BCL9 peptides and β-cat, while β-cat was conjugated with protein A-coated acceptor beads through β-cat antibody (abcam ab32572). Following cross-titrations, 100 nM BCL9-HD2$_B$ (biotinylated BCL9-HD2 peptide), which omits the PEG tag in comparison to BCL9-HD2$_A$, and 100 nM β-cat were used in further studies. ALPHA assay readout was performed on EnVision Multimode Plate Reader and Enspire Multimode Plate Reader (Perkin Elmer). All assays were performed in duplicates and repeated at least twice with freshly prepared reagent solutions.

Reporter assay

The following signaling pathways and cell lines were analyzed: cAMP/PKA pathway tested in CRE-bla HEK 293T (Life Technologies) with Forskolin stimulation, cortisol pathway tested in MMTV-bla HeLa (Life Technologies) with Dexamethasone stimulation, JAK/STAT pathway in SIE-bla ME-180 cells (Life Technologies) with IL-6 stimulation, PI3K/AKT/FOXO3 pathway in T-REx FOXO3 DBE-bla HeLa cells (Life Technologies) with insulin stimulation, TGF-β pathway in SBE-bla HEK 293T cells (Life Technologies) with TGF-β1 stimulation, TNF-α/JNK pathway in AP-1-bla ME-180 cells (Life Technologies) with TNF-α stimulation, and Nrf2-induced oxidative stress pathway in ARE-bla HepG2 cells (Life Technologies). All assays were performed in duplicates and repeated at least twice with freshly prepared reagent solutions.

a. cAMP/PKA - CRE-bla Jurkat - Inhibitor Screen, Activated by Forskolin

CRE-bla Jurkat cells were thawed and prepared as described in the manufacturer's protocol. 4 µL of a 10x serial dilution of H-89 (control inhibitor starting concentration: 10,000 nM) or compound was added to appropriate wells of a TC-treated assay plate. Then, 32 µL cell suspension was added to each well, and the plate was pre-incubated at 37ºC/5% CO_2 in a humidified incubator with compound and control inhibitor titrations for 30 minutes. Next, 4 µL of 10x control activator Forskolin at the pre-determined EC_{80} concentration was added to wells containing the control inhibitor or compounds. The plate was incubated for 5 hours at 37ºC/5% CO_2 in a humidified incubator before 8 µL of 1 µM substrate loading solution was added to each well, followed by incubation for 2 hours at room temperature. The plate was read on a fluorescence plate reader.

b. Cortisol - MMTV-bla HeLa - Inhibitor Screen, Activated by Dexamethasone

MMTV-bla HeLa cells were thawed and prepared as described in the manufacturer's protocol. 32 µL of cell suspension was added to each well of a 384-well TC-treated assay plate. Cells in assay media were incubated for 16-24 hours in the plate at 37ºC/5% CO_2 in a humidified incubator. Then, 4µL of a 10x serial dilution of RU-486 (control inhibitor starting concentration: 100 nM) or compound was added to appropriate wells, and the plate was pre-incubated at 37ºC/5% CO_2 in a humidified incubator for 30 minutes. Next, 4 µL of 10x control activator Dexamethasone at the predetermined EC_{80} concentration was added to wells containing the control inhibitor or compounds. The plate was incubated for 5 hours at 37ºC/5% CO_2 in a humidified incubator before 8 µL of 1 µM substrate + solution D loading solution was added to each well, followed by incubation for 2 hours at room temperature. The plate was read on a fluorescence plate reader.

c. JAK/STAT - SIE-bla HEK 293T - Inhibitor Screen, Activated by IL-6

SIE-bla HEK 293T cells were thawed and prepared as described in the manufacturer's protocol. 32 µL of cell suspension was added to each well of a 384-well TC-treated assay plate. Cells in assay media were incubated for 16-24 hours in the plate at 37ºC/5% CO_2 in a humidified incubator. Then, 4µL of a 10x serial dilution of

JAK Inhibitor I (control inhibitor starting concentration: 10,000 nM) or compound was added to appropriate wells, and the plate was pre-incubated at 37ºC/5% CO_2 in a humidified incubator for 30 minutes. Next, 4 µL of 10x control activator IL-6 at the predetermined EC_{80} concentration was added to wells containing the control inhibitor or compounds. The plate was incubated for 5 hours at 37ºC/5% CO_2 in a humidified incubator before 8 µL of 1 µM substrate loading solution was added to each well, followed by incubation for 2 hours at room temperature. The plate was read on a fluorescence plate reader.

d. PI3K/AKT/FOXO3 - TREx FOXO3-DBE-bla HeLa - Inhibitor Screen, Activated by Insulin

TREx FOXO3-DBE-bla HeLa cells were thawed and prepared as described in the manufacturer's protocol. 4 µL of a 10x serial dilution of Triciribine (control inhibitor starting concentration: 1,000 nM) or compound was added to appropriate wells of a TC-treated assay plate. Then, 32 µL cell suspension was added to the wells and pre-incubated at 37ºC/5% CO_2 in a humidified incubator with compound and control inhibitor titrations for 30 minutes. Next, 4 µL of 10x control activator Insulin at the pre-determined EC_{80} concentration was added to wells containing the control inhibitor or compounds. The plate was incubated for 16-24 hours at 37ºC/5% CO_2 in a humidified incubator before 8 µL of 1 µM substrate loading solution was added to each well, followed by incubation for 3 hours at room temperature. The plate was read on a fluorescence plate reader.

e. TGF-β - SBE-bla HEK 293T - Inhibitor Screen, Activated by TGF-β1

SBE-bla HEK 293T cells were thawed and prepared as described in the manufacturer's protocol. 32 µL cell suspension was added to each well of a 384-well Poly-D-Lysine assay plate. Cells in assay media were then incubated for 16-24 hours in the plate at 37ºC/5% CO_2 in a humidified incubator. Next, 4 µL of a 10x serial dilution of TGF-β R1 (control inhibitor starting concentration: 10,000 nM) or compound was added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO_2 in a humidified incubator for 30 minutes. Then, 4 µL of 10x control activator TGF-β1 at the pre-determined EC_{80} concentration was added to each well. The plate was incubated for 5 hours at 37ºC/5% CO_2 in a humidified incubator before 8 µL of 1 µM substrate loading solution was added to each well, followed by incubation for 2 hours at room temperature. The plate was read on a fluorescence plate reader.

f. TNF-α/JNK - AP1-bla ME-180 - Inhibitor Screen, Activated by TNF-α

AP1-bla ME-180 cells were thawed and prepared as described in the manufacturer's protocol. 32 µL cell suspension was added to each well of a 384-well TC-treated assay plate. Cells in assay media were incubated for 16-24 hours in the plate at 37ºC/5% CO_2 in a humidified incubator. Next, 4µL of a 10x serial dilution of JNK Inhibitor II (control inhibitor starting concentration: 100,000 nM) or compound was added to appropriate wells of the plate and pre-incubated at 37ºC/5% CO_2 in a humidified incubator for 30 minutes. Then, 4 µL of 10x control activator TNF-α at the pre-determined EC_{80} concentration was added to each well. The plate was

incubated for 5 hours at 37ºC/5% CO_2 in a humidified incubator before 8 µL of 1 µM substrate loading solution was added to each well, followed by incubation for 2 hours at room temperature. The plate was read on a fluorescence plate reader.

g. Nrf2 - AP1-bla HepG2 - Inhibitor Screen, Activated by tBHQ

ARE-bla HepG2 cells were resuspended in assay media (DMEM, 10% dialyzed FBS, 25 mM HEPES pH 7.3, 0.1 mM NEAA, 100 U/mL/100 µg/mL Pen/Strep) to a concentration of 375,000 cells/mL. 32 µL of cell suspension (12,000 cells) was added to each well of a 384-well Poly-D-Lysine assay plate and incubated for 16-24 hours in the plate at 37ºC/5% CO_2 in a humidified incubator. Next, 4 µL of a 10x serial dilution of tBHQ (control activator starting concentration: 150,000 nM) or compound was added to appropriate wells of the plate. Then, 4 µL assay media was added to all wells to bring the final assay volume to 40 µL. The plate was incubated for 5 hours at 37ºC/5% CO_2 in a humidified incubator. 8 µL of 1 µM substrate + Solution D loading solution was added to each well, followed by incubation for 2 hours at room temperature. The plate was read on a fluorescence plate reader.

Immunofluorescence staining

To measure intracellular uptake of hsBCL9$_{CT}$-FITC peptide, CT26 cells were treated for two hours with vehicle control or hsBCL9$_{CT}$-24-FITC at 5 µM, prepared using a cytocentrifuge (Thermo Shandon) and fixed as described in our previous studies[2]. Images were obtained using confocal microscopy (Carl Zeiss LSM710) and visualized under 20x magnification following staining.

Toxicokinetic studies

In hematoxylin and eosin (H&E) staining for histopathological analysis, a total of 16 tissues from brain, lung, kidney, liver, heart, spleen, small intestine, and colon regions were analyzed in female BALB/c mice treated with vehicle control or hsBCL9$_{CT}$-24 at 10 mg/kg, i.p., QD over 14 days. Tissues were trimmed, fixed with 10% neutral-buffered formalin (NBF), processed in alcohol and xylene, and embedded in paraffin (Leica EG1150H+C Paraffin Embedding Station). Sections of 3 µm were then created before H&E staining (Leica Biosystems) in non-GLP study.

Toxicology study and toleration of hsBCL9$_{CT}$-24 were then examined in female BALB/c nude mice (Shanghai BK Animal Ltd., SCXK2013001812536). Four cohorts (n = 5) were treated with vehicle control (4% ethanol, 8% Tween80 in PBS) or hsBCL9$_{CT}$-24 (10, 15, and 20 mg/kg) via i.p. injection, QD over 14 days. In this first arm, clinical observation was performed twice daily, while body weight and food consumption were recorded once daily, culminating in clinical pathology and gross necropsy on Day 15. In the second arm, three cohorts (n = 3) were administered i.p. injection of hsBCL9$_{CT}$-24 (10, 15, and 20 mg/kg) QD over 14 days, during which

TK sampling occurred at pre-dose, 0.5, 2, 4, and 24-hour timepoints on Day 1 and 14. Food consumption, taken daily from Day 1 to 14, was determined by measuring the difference in food amount present per cage with each subsequent day. All studies were conducted in compliance with animal welfare regulations and IACUC approval.

Solubility, stability, and purity testing

Preliminary studies on solubility, stability, and salt selection were conducted with hsBCL9$_{CT}$-24 (CPC Scientific). In solubility testing, water, DMSO, or PBS was added dropwise into a tube of 0.5 mg peptide. If the solution was not immediately clear, sonication for 2 minutes at 220V, 40 Hz, and 300 W was performed. In stability and purity testing, hsBCL9$_{CT}$-24 peptide was analyzed via high-performance liquid chromatography (HPLC) (HP 1090/QC-HPLC-1). For reconstitution, 1 mg of peptide was dissolved in Water/Acetonitrile (50/50), then brought to the final concentration with purified water to 1 mg/mL. The gradient pump was set at 40-100% Water/ACN over 20 minutes, with column Supelco C18 (25×4.6 cm, 5 µm particle size) heated to 49°C. During mobile phase A and B, samples were tested at 0.1% TFA in water and 0.1% TFA in acetonitrile, respectively, at injection volumes of 10 µL. HPLC conditions for analysis consisted of a flow rate of 1.0 mL/min, column temperature of 50°C, and retention time of 16.3-16.5 minutes.

In addition, plasma stability assay was conducted according to standard protocol (Cyprotex) to assess hsBCL9$_{CT}$-24 and Procaine percent remaining in BALB/c nude mice following a 60 minute time period. Initially, 0.05 M sodium phosphate and 0.07 M NaCl buffer (pH 7.4) were preheated, while 14.505 g/L $Na_2HPO_4 \cdot 12H_2O$, 1.483 g/L $Na_2HPO_4 \cdot 2H_2O$, and 4.095 g/L NaCl was dissolved in deionized water. The basic solution was titrated with phosphoric acid to pH 7.40 ± 0.1, and plasma was centrifuged to remove clots and pool supernatant (only plasma with pH 7.4 to 8.0 was used). Next, 0.5 mM test compound spiking solution A in DMSO and 0.02 mM spiking solution B in 0.05 mM Na_2HPO_4 buffer with 0.5% BSA were prepared. 10 µL spiking solution B was added to 90 µL pre-warmed plasma, followed by addition of 400 µL ACN containing IS, to stop the reaction at 0, 15, 30, and 60-minute timepoints. After quenching, plates were shaken and centrifuged before transfer for LC/MS analysis.

Liquid chromatography–mass spectrometry (LC-MS)

The following settings were used. Mobile phase: A = water (0.01% TFA), B = CAN (0.01% TFA), gradient = 5% B increase to 95% B within 1.2 minutes, 95% B for 1.3 minutes, and back to 5% B within 0.01 minutes. Flow rate = 2.0 mL/min, column = SunFire C18, 4.6×50 mm, 3.5 µm, and column temperature = 40˚C.

Circular Dichroism (CD) analysis

Compounds were dissolved in aqueous 50 mM potassium phosphate solution pH 7 to concentrations of 25-50 μM. CD spectra were obtained on a JascoJ-710 spectropolarimeter at 20°C using the following standard measurement parameters: wavelength = 190-260 nm, step resolution = 0.5 nm, speed = 20 nm/sec, accumulations = 10, response = 1 sec, bandwidth = 1 nm, and path length = 0.1 cm. The α-helical content of each peptide was calculated by dividing the mean residue ellipticity [θ]222 obs by the reported [θ]222 obs for a model helical decapeptide.

Supplementary Figures

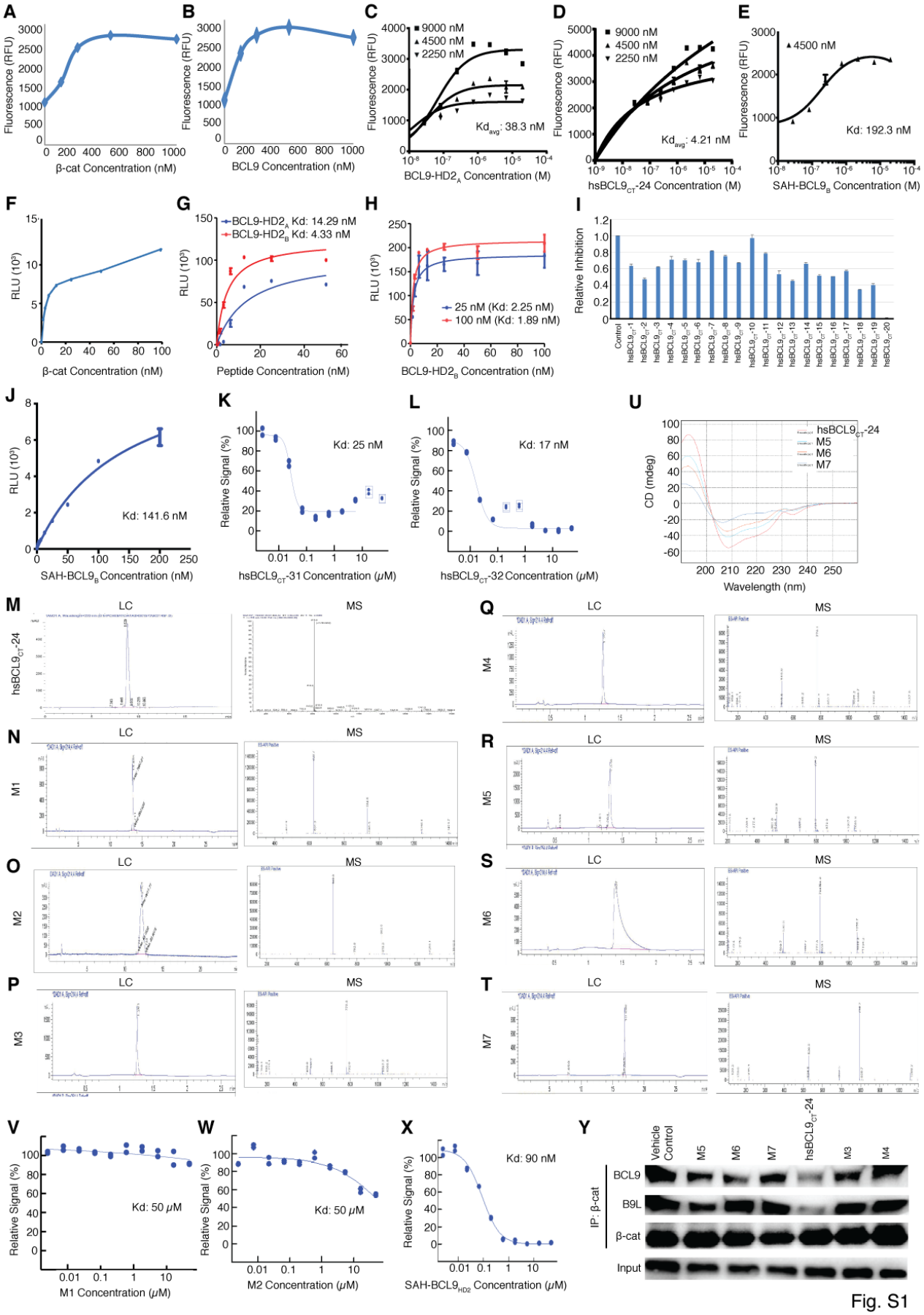

Fig. S1

Fig. S1. Biochemical profile and LC-MS analysis of hsBCL9$_{CT}$ peptides. (**A**) β-cat protein and (**B**) biotinylated BCL9 peptide were titrated to determine concentrations yielding optimal fluorescent readout in HTRF assay. HTRF procedures without β-cat and BCL9 served as respective negative controls. In these two assays, average Z' was 0.8. For both β-cat and BCL9, concentrations of 500 nM produced the best results and were implemented for future HTRF studies. Kd determination using the above HTRF assay conditions for (**C**) biotinylated BCL9-HD2$_A$ (Kd = 38.3 nM), (**D**) hsBCL9$_{CT}$-24 (Kd = 4.21 nM), and (**E**) SAH-BCL9$_B$ (Kd = 192.3 nM). (**F**) β-cat protein was titrated to determine the best concentration for fluorescent readout in ALPHA assay. A 100 nM biotinylated BCL9-HD2$_B$ control was used, with results suggesting selection of 100 nM β-cat for further ALPHA assays. Average Z' was 0.7. (**G**) In ALPHA assay, BCL9-HD2$_A$ and BCL9-HD2$_B$ yielded Kd values of 14.29 nM and 4.33 nM, respectively. (**H**) In ALPHA assay, varying concentrations of BCL9-HD2$_B$ yielded a Kd value of approximately 2 nM. (**I**) In ALPHA assay**,** peptides from hsBCL9$_{CT}$-1 through hsBCL9$_{CT}$-20 were screened. (**J**) In ALPHA assay, biotinylated SAH-BCL9$_B$ peptide yielded a Kd of 141.6 nM. In ALPHA assay, (**K**) hsBCL9$_{CT}$-31 and (**L**) hsBCL9$_{CT}$-32 showed Kd values of 25 nM and 17 nM, respectively. Liquid chromatography (LC) (left) and mass spectrometry (MS) (right) of (**M**) hsBCL9$_{CT}$-24 and mutants (**N**) hsBCL9$_{M1}$ (M1), (**O**) hsBCL9$_{M2}$ (M2), (**P**) hsBCL9$_{M3}$ (M3), (**Q**) hsBCL9$_{M4}$ (M4), (**R**) hsBCL9$_{M5}$ (M5), (**S**) hsBCL9$_{M6}$ (M6), and (**T**) hsBCL9$_{M7}$ (M7). (**U**) Circular dichroism (CD) analysis of α-helical stabilization of hsBCL9$_{CT}$-24, M5, M6, and M7. [θ] ellipticity (deg×cm^2/dmol). Mutation of key hydrophobic amino acids (Leu/Ile to Asp) in the hsBCL9$_{CT}$ series abolished biochemical activity in ALPHA assays. M1 (**V**) and M2 (**W**) showed minimal inhibition of β-cat/BCL9 binding activity (Kd = 50 μM), while (**X**) SAH-BCL9$_{HD2}$ showed a Kd of 90 nM in ALPHA assay. (**Y**) Effect of hsBCL9$_{CT}$-24, M3, M4, M5, M6, and M7 peptides at 1.67 μM on the native association of β-cat with BCL9 or B9L by immunoprecipitation (IP) in Colo320DM cells. Results were denoted as mean ± SEM for assays performed in duplicate. Each experiment was repeated three times and statistical significance of differences between groups was determined by unpaired Student's t-test.

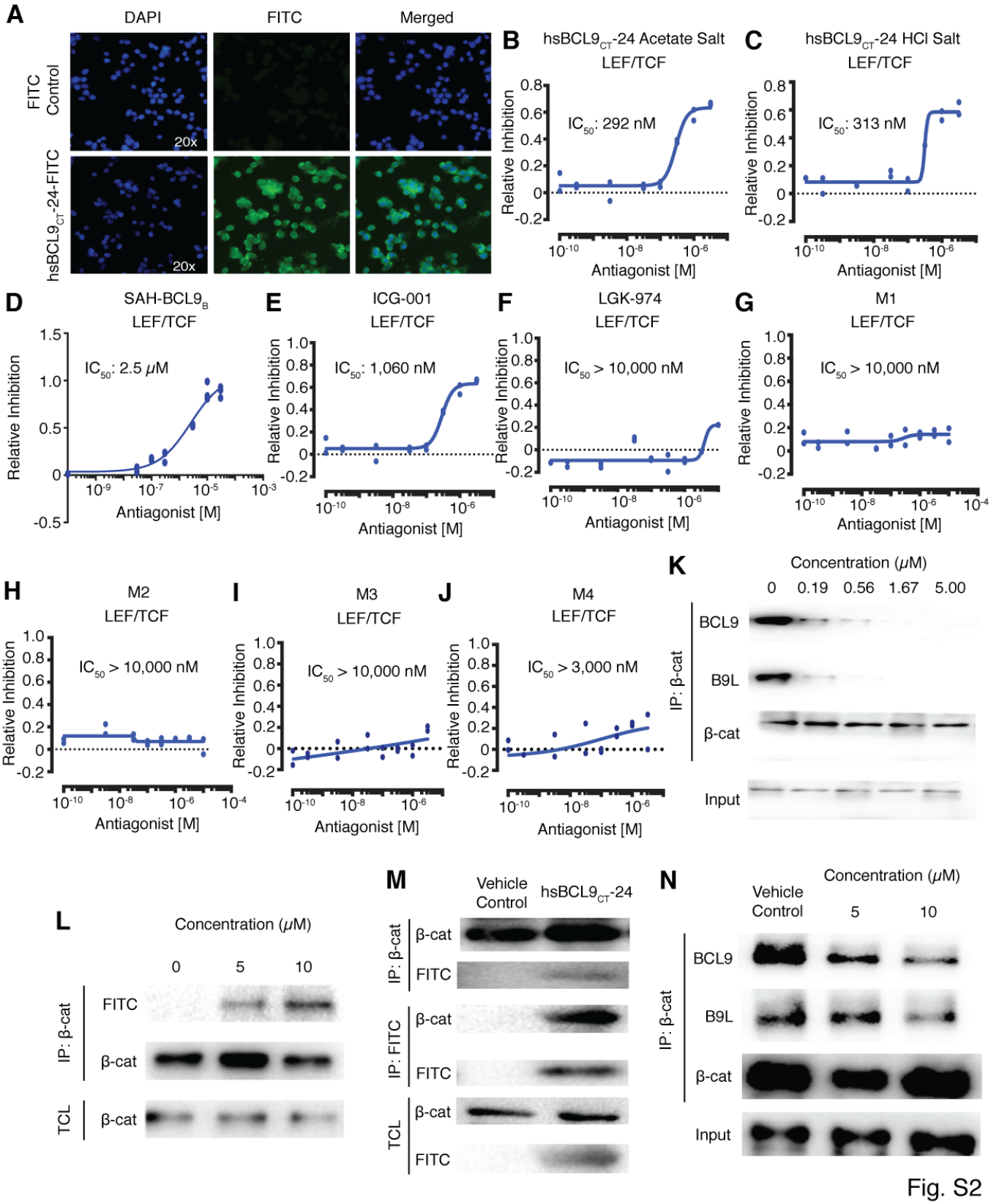
A
DAPI
FITC
Merged
FITC Control
hsBCL9CT-24-FITC
20x
20x
B
hsBCL9CT-24 Acetate Salt
LEF/TCF
IC50: 292 nM
C
hsBCL9CT-24 HCl Salt
LEF/TCF
IC50: 313 nM
Relative Inhibition
Antiagonist [M]
D
SAH-BCL9B
LEF/TCF
IC50: 2.5 μM
E
ICG-001
LEF/TCF
IC50: 1,060 nM
F
LGK-974
LEF/TCF
IC50 > 10,000 nM
G
M1
LEF/TCF
IC50 > 10,000 nM
H
M2
LEF/TCF
IC50 > 10,000 nM
I
M3
LEF/TCF
IC50 > 10,000 nM
J
M4
LEF/TCF
IC50 > 3,000 nM
K
Concentration (μM)
0 0.19 0.56 1.67 5.00
BCL9
B9L
β-cat
Input
IP: β-cat
L
Concentration (μM)
0 5 10
IP: β-cat
FITC
β-cat
TCL
β-cat
M
Vehicle Control
hsBCL9CT-24
IP: β-cat
β-cat
FITC
IP: FITC
β-cat
FITC
TCL
β-cat
FITC
N
Vehicle Control
Concentration (μM)
5
10
IP: β-cat
BCL9
B9L
β-cat
Input

Fig. S2

Fig. S2. Cellular uptake, Wnt reporter, and coimmunoprecipitation analysis of hsBCL9$_{CT}$ peptides. (**A**) Live cell confocal microscopy showing immunofluorescence (IF) staining for the cellular uptake of hsBCL9$_{CT}$-24-FITC (5 μM) in CT26 cells after two-hour treatment. (**B**) LEF/TCF HCT116 reporter assays showed the IC_{50} values of hsBCL9$_{CT}$-24 were 292 nM in the form of acetate salt and (**C**) 313 nM in the form of HCl salt. (**D**) β-cat reporter assay conducted in LEF-TCF-bla HCT116 cells (Ser45 deletion in one allele of the *CTNNB1* gene) treated with SAH-BCL9$_B$ (IC_{50} = 2.5 μM). (**E**) LEF/TCF HCT116 reporter assays showed the IC_{50} values of ICG-001 and (**F**) LGK-974 were 1060 nM and > 10,000 nM, respectively. HCT116 LEF/TCF reporter assays showed that mutated hsBCL9$_{CT}$ peptides (**G**) M1, (**H**) M2, (**I**) M3, and (**J**) M4 did not inhibit Wnt pathway activity. (**K**) Effect of hsBCL9$_{CT}$-24 peptide at various concentrations on the native association of β-cat with BCL9 or B9L in Colo320DM cells. (**L**) Immunoprecipitation of β-cat from lysates of Colo320DM cells treated with hsBCL9$_{CT}$-24-FITC peptide (0, 5, and 10 μM) using anti-FITC and anti-β-cat antibodies. TCL (total cellular lysate). (**M**) Immunoprecipitations of β-cat and FITC from lysates of Colo320DM cells treated with 0 and 5 μM FITC labeled hsBCL9$_{CT}$-24 peptide using anti-FITC and anti-β-cat antibodies. (**N**) Effect of the parental peptide SAH-BCL9$_B$ at various concentrations on interaction between β-cat and BCL9 or B9L by immunoprecipitation in Colo320DM cells. Results were denoted as mean ± SEM for assays performed in duplicate. Each experiment was repeated three times and statistical significance of differences between groups was determined by unpaired Student's t-test.

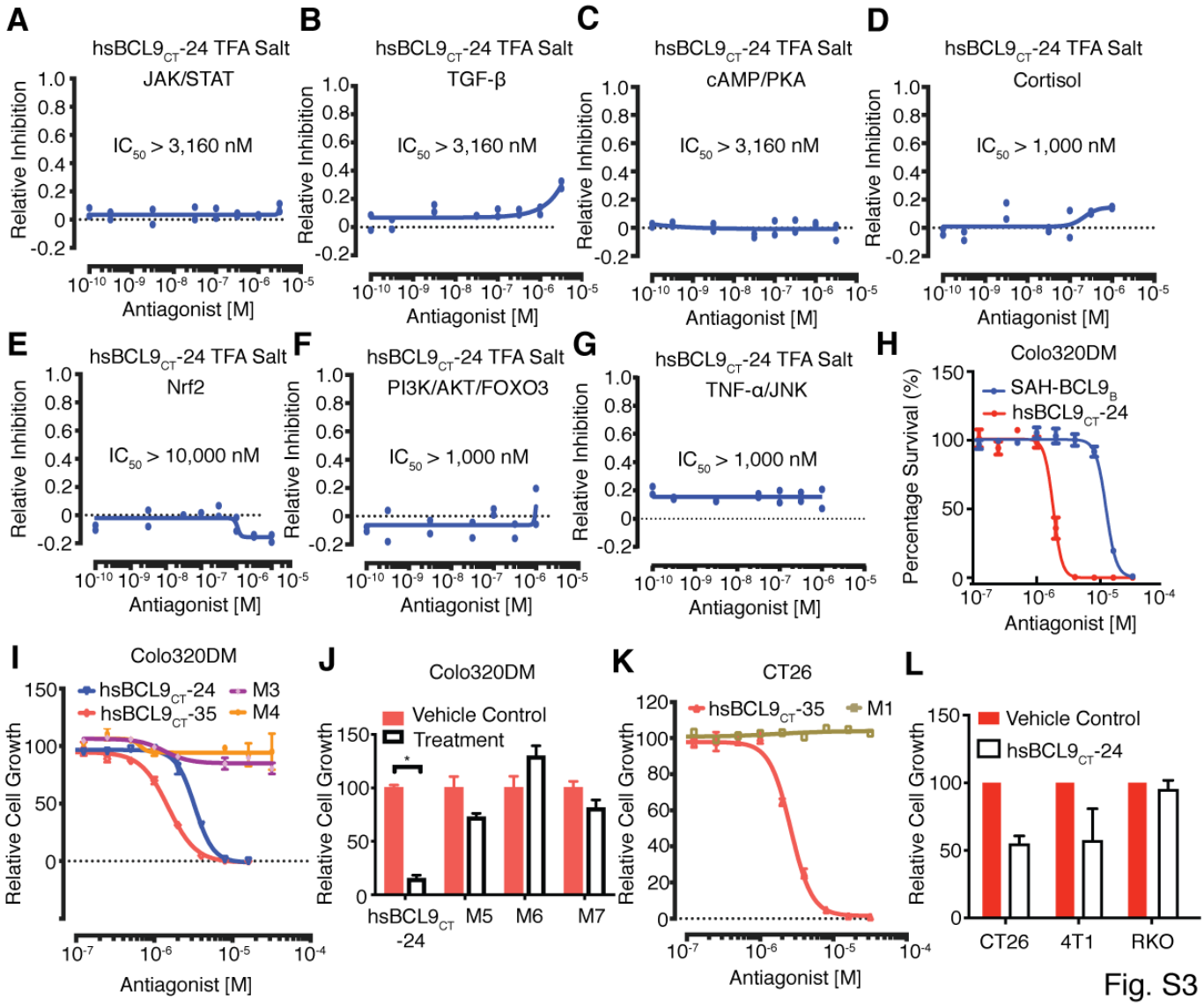

Fig. S3. Selectivity in multiple signaling pathways and antiproliferation assay of hsBCL9$_{CT}$ peptides. Dose response curves of hsBCL9$_{CT}$-24 trifluoroacetic acid (TFA) salt in inhibiting the (**A**) JAK/STAT, (**B**) TGF-β, (**C**) cAMP/PKA, (**D**) Cortisol, (**E**) Nrf2, (**F**) PI3K/AKT/FOXO3, and (**G**) TNF-α/JNK signaling pathways. The IC_{50} value was determined for each pathway shown and indicate hsBCL9$_{CT}$-24 has minimal off-target effects. (**H**) Dose response curves comparing SAH-BCL9$_B$ with hsBCL9$_{CT}$-24 treatment in Colo320DM cell viability assay. (**I**) Dose response curves comparing hsBCL9$_{CT}$-24, hsBCL9$_{CT}$-35, M3, and M4 treatment in Colo320DM cell viability assay. (**J**) BrdU cell proliferation assay of Colo320DM cells treated with vehicle control or 10 μM hsBCL9$_{CT}$-24, M5, M6, or M7 over 24 hours. Relative cell growth was measured based on ^{3}H-thymidine incorporation. (**K**) Dose response curves of hsBCL9$_{CT}$-35 and M1 in CRC cell line CT26 demonstrate that key hydrophobic mutations abrogate Wnt/β-cat dependent cell growth. (**L**) BrdU cell proliferation assay of 4T1, CT26, and RKO cell lines treated with 8 μM hsBCL9$_{CT}$-24 or vehicle control over 24 hours. Results were denoted as mean ± SEM for assays performed in duplicate. Each experiment was repeated three times and statistical significance of differences between groups was determined by unpaired Student's t-test or two way ANOVA.

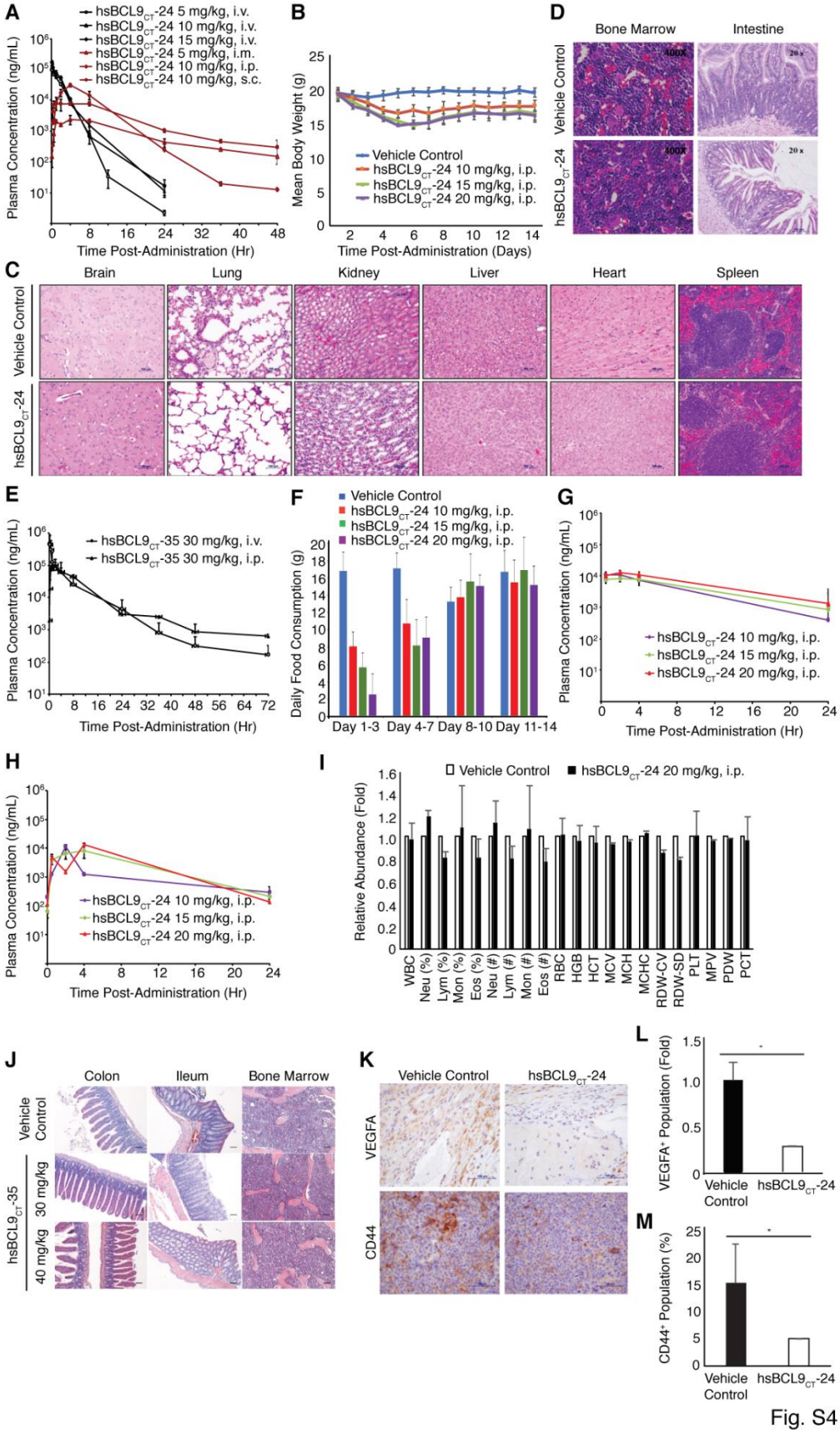
A
hsBCL9CT-24 5 mg/kg, i.v.
hsBCL9CT-24 10 mg/kg, i.v.
hsBCL9CT-24 15 mg/kg, i.v.
hsBCL9CT-24 5 mg/kg, i.m.
hsBCL9CT-24 10 mg/kg, i.p.
hsBCL9CT-24 10 mg/kg, s.c.
Plasma Concentration (ng/mL)
Time Post-Administration (Hr)
B
Mean Body Weight (g)
Vehicle Control
hsBCL9CT-24 10 mg/kg, i.p.
hsBCL9CT-24 15 mg/kg, i.p.
hsBCL9CT-24 20 mg/kg, i.p.
Time Post-Administration (Days)
D
Bone Marrow
Intestine
Vehicle Control
hsBCL9CT-24
C
Brain
Lung
Kidney
Liver
Heart
Spleen
Vehicle Control
hsBCL9CT-24
E
hsBCL9CT-35 30 mg/kg, i.v.
hsBCL9CT-35 30 mg/kg, i.p.
Plasma Concentration (ng/mL)
Time Post-Administration (Hr)
F
Vehicle Control
hsBCL9CT-24 10 mg/kg, i.p.
hsBCL9CT-24 15 mg/kg, i.p.
hsBCL9CT-24 20 mg/kg, i.p.
Daily Food Consumption (g)
Day 1-3
Day 4-7
Day 8-10
Day 11-14
G
Plasma Concentration (ng/mL)
hsBCL9CT-24 10 mg/kg, i.p.
hsBCL9CT-24 15 mg/kg, i.p.
hsBCL9CT-24 20 mg/kg, i.p.
Time Post-Administration (Hr)
H
Plasma Concentration (ng/mL)
hsBCL9CT-24 10 mg/kg, i.p.
hsBCL9CT-24 15 mg/kg, i.p.
hsBCL9CT-24 20 mg/kg, i.p.
Time Post-Administration (Hr)
I
Vehicle Control
hsBCL9CT-24 20 mg/kg, i.p.
Relative Abundance (Fold)
WBC
Neu (%)
Lym (%)
Mon (%)
Eos (%)
Neu (#)
Lym (#)
Mon (#)
Eos (#)
RBC
HGB
HCT
MCV
MCH
MCHC
RDW-CV
RDW-SD
PLT
MPV
PDW
PCT
J
Colon
Ileum
Bone Marrow
Vehicle Control
hsBCL9CT-35
30 mg/kg
40 mg/kg
K
Vehicle Control
hsBCL9CT-24
VEGFA
CD44
L
VEGFA+ Population (Fold)
Vehicle Control
hsBCL9CT-24
M
CD44+ Population (%)
Vehicle Control
hsBCL9CT-24

Fig. S4

Fig. S4. PK, toxicology, and histology analysis of hsBCL9$_{CT}$-24 and hsBCL9$_{CT}$-35. (**A**) Plasma concentration of hsBCL9$_{CT}$-24 over a 48-hour period after peptide administration via i.v., i.p., s.c., or i.m. injection in BALB/c mice (*n* = 2 per cohort). (**B**) Mean body weight changes recorded in BALB/c mice administered vehicle control or hsBCL9$_{CT}$-24 at 10, 15, or 20 mg/kg via i.p. injection, QD over 14 days (*n* = 5 per cohort). (**C**) Histology study in female BALB/c mice (*n* = 16) treated with vehicle control or hsBCL9$_{CT}$-24 at 10 mg/kg, QD over 14 days. Representative images are shown. (**D**) Histological study in bone marrow and intestinal tissue in C57BL/6 mice treated with hsBCL9$_{CT}$-24 at 30 mg/kg by s.c. injection over 7 days (scale bar denotes 5 μm in bone marrow and 100 μm in intestinal tissue). (**E**) Profiling mean plasma concentration of hsBCL9$_{CT}$-35 over a 72-hour period after peptide administration via i.v. or i.p. injection in BALB/c mice (*n* = 2 per cohort). (**F**) Food intake of BALB/c mice (*n* = 5 per cohort) administered vehicle control or hsBCL9$_{CT}$-24 at 10, 15, or 20 mg/kg via i.p. injection, QD over 14 days. Bars during each treatment period represent the mean values (g) per cohort. TK study of plasma concentration in hsBCL9$_{CT}$-24 BALB/c mice administered 10, 15, or 20 mg/kg via i.p. injection, QD over 14 days (*n* = 5 per cohort). Periodic measurement on (**G**) Day 1 (118,033 to 162,333 hr×ng/mL) and (**H**) Day 14 showed comparable AUC values (107,420 to 159,976 hr×ng/mL). (**I**) Blood cell count of BALB/c mice treated with vehicle control or hsBCL9$_{CT}$-24 at 20 mg/kg, QD over 14 days (*n* = 5 per cohort). Cell types analyzed include WBC (white blood cells), Neu (neutrophils), Lym (lymphocytes), Mon (monocytes), Eos (eosinophils), RBC (red blood cells), HGB (hemoglobin), HCT (hematocrit), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular HGB concentration), RDW (red cell distribution width), PLT (platelet), MPV (mean platelet volume), PDW (platelet distribution width), and PCT (platelet hematocrit). (**J**) Histology study on colon, ileum, and bone marrow tissue of BALB/c mice administered vehicle control or hsBCL9$_{CT}$-35 at 30 and 40 mg/kg (scale bar denotes 100 μm). (**K**) IHC staining of VEGFA and CD44 in tumor samples from Colo320DM xenograft mouse models, administered vehicle control or hsBCL9$_{CT}$-24 at 15 mg/kg. Representative images from the treatment group displayed significantly reduced VEGFA and CD44 expression following hsBCL9$_{CT}$-24 administration. (**L**) Quantification of VEGFA expression (*$p < 0.05$) in (K). (**M**) Quantification of CD44 expression (*$p < 0.05$) in (K). Results were denoted as mean ± SEM for experiments performed in triplicates. Each experiment was repeated three times and statistical significance of differences between groups was determined by unpaired Student's t-test.

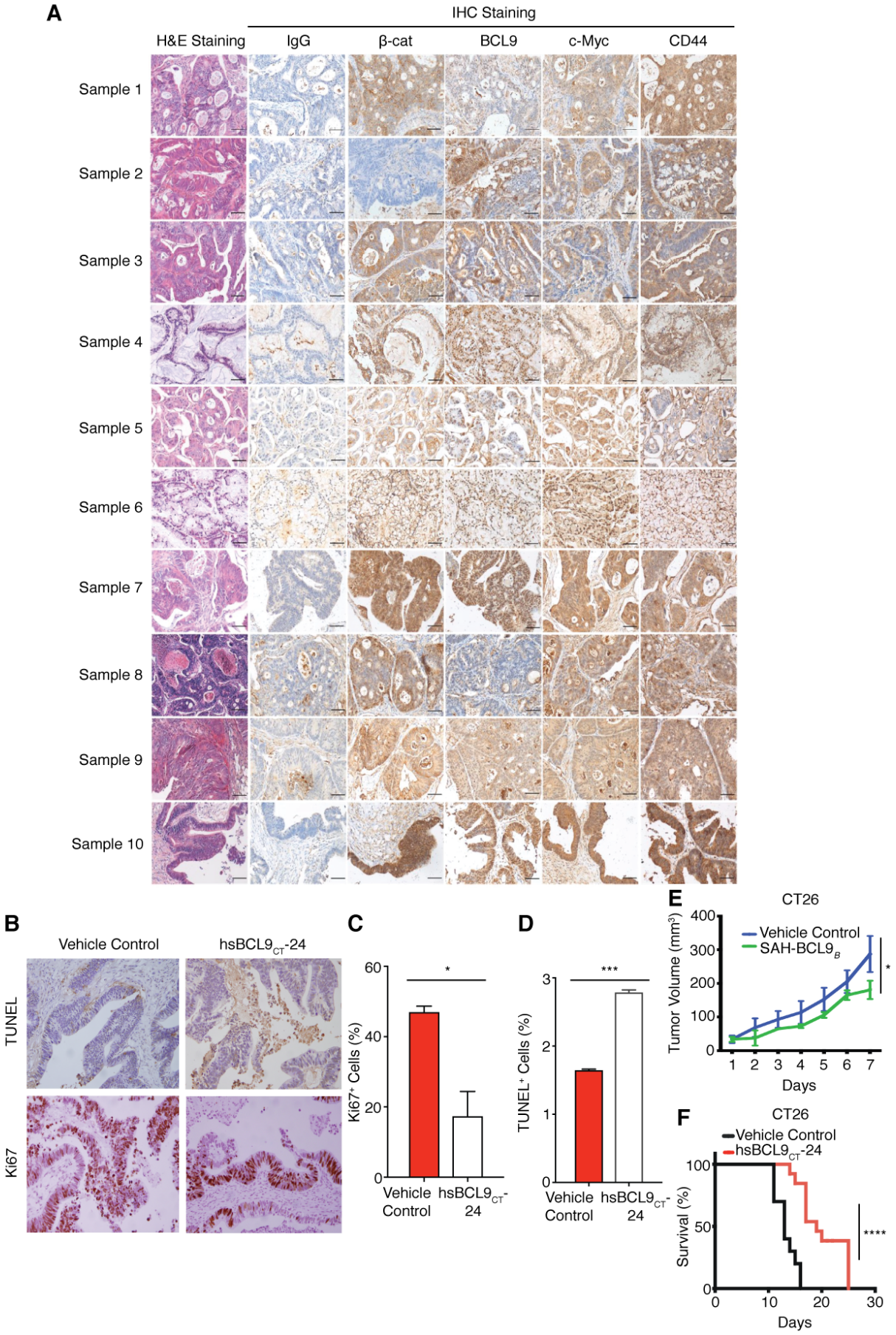
A
IHC Staining
H&E Staining
IgG
β-cat
BCL9
c-Myc
CD44
Sample 1
Sample 2
Sample 3
Sample 4
Sample 5
Sample 6
Sample 7
Sample 8
Sample 9
Sample 10
B
Vehicle Control
hsBCL9CT-24
TUNEL
Ki67
C
Ki67+ Cells (%)
Vehicle Control
hsBCL9CT-24
D
TUNEL+ Cells (%)
Vehicle Control
hsBCL9CT-24
E
CT26
Tumor Volume (mm3)
Vehicle Control
SAH-BCL9B
Days
F
CT26
Vehicle Control
hsBCL9CT-24
Survival (%)
Days

Fig. S5

Fig. S5. IHC staining in patient tumor tissues and PDX tumor models. (**A**) IHC staining for the expression of β-cat, BCL9, c-Myc, and CD44 in ten patient-derived CRC tumor samples (scale bar denotes 100 μm). (**B**) TUNEL and Ki67 staining in vehicle control- and hsBCL9$_{CT}$-24-treated tumor tissues from the PDX model. (**C**) Quantification of Ki67 positive cells (*$p < 0.05$) from (B). (**D**) Quantification of TUNEL positive cells (*$p < 0.05$) from (B). (**E**) CT26 cells were inoculated in BALB/c mice before treatment with vehicle control or SAH-BCL9$_B$ at 25 mg/kg via i.p. injection ($n = 4$ per cohort), QD for 7 days (*$p < 0.05$). (**F**) Kaplan-Meier survival curves of CT26 syngeneic mice ($n = 8$ per cohort) administered vehicle control or hsBCL9$_{CT}$-24 at 25 mg/kg, i.p. over 22 days. A cutoff value of 1000 mm^3 in tumor volume was used (****$p < 0.0001$). Results were denoted as mean ± SEM for experiments performed in triplicates. Each experiment was repeated three times and statistical significance of differences between groups was determined by unpaired Student's t-test.

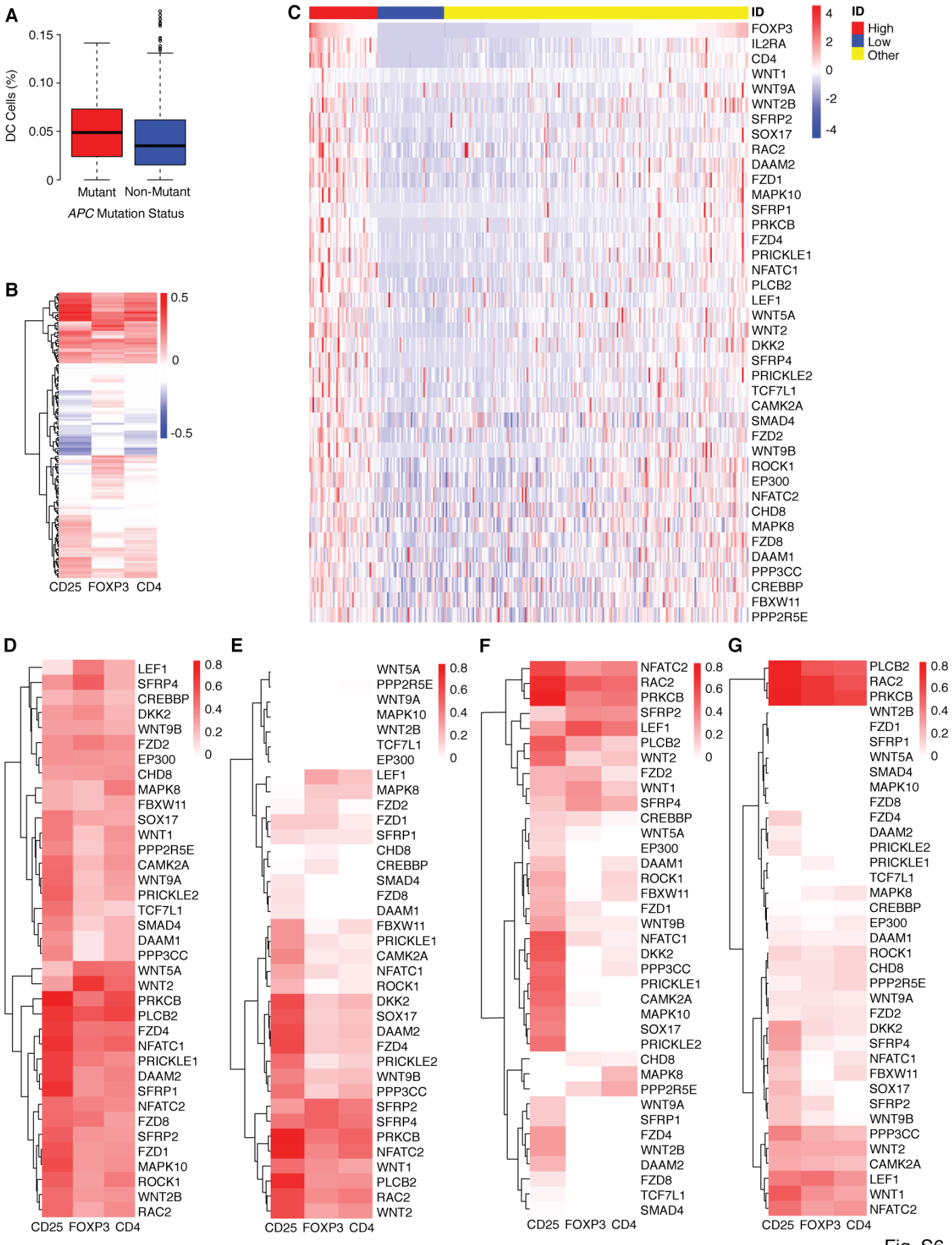
A
DC Cells (%)
0.15
0.10
0.05
0
Mutant
Non-Mutant
APC Mutation Status
B
0.5
0
-0.5
CD25 FOXP3 CD4
C
ID
FOXP3
IL2RA
CD4
WNT1
WNT9A
WNT2B
SFRP2
SOX17
RAC2
DAAM2
FZD1
MAPK10
SFRP1
PRKCB
FZD4
PRICKLE1
NFATC1
PLCB2
LEF1
WNT5A
WNT2
DKK2
SFRP4
PRICKLE2
TCF7L1
CAMK2A
SMAD4
FZD2
WNT9B
ROCK1
EP300
NFATC2
CHD8
MAPK8
FZD8
DAAM1
PPP3CC
CREBBP
FBXW11
PPP2R5E
4
2
0
-2
-4
ID
High
Low
Other
D
LEF1
SFRP4
CREBBP
DKK2
WNT9B
FZD2
EP300
CHD8
MAPK8
FBXW11
SOX17
WNT1
PPP2R5E
CAMK2A
WNT9A
PRICKLE2
TCF7L1
SMAD4
DAAM1
PPP3CC
WNT5A
WNT2
PRKCB
PLCB2
FZD4
NFATC1
PRICKLE1
DAAM2
SFRP1
NFATC2
FZD8
SFRP2
FZD1
MAPK10
ROCK1
WNT2B
RAC2
0.8
0.6
0.4
0.2
0
CD25 FOXP3 CD4
E
WNT5A
PPP2R5E
WNT9A
MAPK10
WNT2B
TCF7L1
EP300
LEF1
MAPK8
FZD2
FZD1
SFRP1
CHD8
CREBBP
SMAD4
FZD8
DAAM1
FBXW11
PRICKLE1
CAMK2A
NFATC1
ROCK1
DKK2
SOX17
DAAM2
FZD4
PRICKLE2
WNT9B
PPP3CC
SFRP2
SFRP4
PRKCB
NFATC2
WNT1
PLCB2
RAC2
WNT2
0.8
0.6
0.4
0.2
0
CD25 FOXP3 CD4
F
NFATC2
RAC2
PRKCB
SFRP2
LEF1
PLCB2
WNT2
FZD2
WNT1
SFRP4
CREBBP
WNT5A
EP300
DAAM1
ROCK1
FBXW11
FZD1
WNT9B
NFATC1
DKK2
PPP3CC
PRICKLE1
CAMK2A
MAPK10
SOX17
PRICKLE2
CHD8
MAPK8
PPP2R5E
WNT9A
SFRP1
FZD4
WNT2B
DAAM2
FZD8
TCF7L1
SMAD4
0.8
0.6
0.4
0.2
0
CD25 FOXP3 CD4
G
PLCB2
RAC2
PRKCB
WNT2B
FZD1
SFRP1
WNT5A
SMAD4
MAPK10
FZD8
FZD4
DAAM2
PRICKLE2
PRICKLE1
TCF7L1
MAPK8
CREBBP
EP300
DAAM1
ROCK1
CHD8
PPP2R5E
WNT9A
FZD2
DKK2
SFRP4
NFATC1
FBXW11
SOX17
SFRP2
WNT9B
PPP3CC
WNT2
CAMK2A
LEF1
WNT1
NFATC2
0.8
0.6
0.4
0.2
0
CD25 FOXP3 CD4

Fig. S6

Fig. S6. Wnt pathway gene expression is correlated with $CD4^+CD25^+FOXP3^+$ T_{reg} cell infiltration in cancers. (**A**) Correlation study of DC cell infiltration (all DC cell types) and *APC* mutation status using CIBERSORT. (**B**) Wnt signaling genes were divided into three clusters based on hierarchical clustering of expression correlation. (**C**) Relative heat map of Wnt pathway gene expression in colon cancer tumors, stratified by T_{reg} infiltration levels: T_{reg}-hi (CD4-hi CD25-hi FOXP3-hi) versus T_{reg}-lo (CD4-lo CD25-lo FOXP3-lo). All quartiles of the 287 tumors are shown. Each column represents one sample, with samples ranked horizontally by FOXP3 expression. (**D**) Relative heat map of expression correlation between $CD4^+$, $CD25^+$, and $FOXP3^+$ T_{reg} signature genes with Wnt signaling pathway genes in (D) 95 rectum adenocarcinoma (READ) samples, (**E**) 501 lung squamous cell carcinoma (LUSC) samples, (**F**) 517 lung adenocarcinoma (LUAD) samples, and (**G**) 97 triple-negative breast cancer (TNBC) samples.

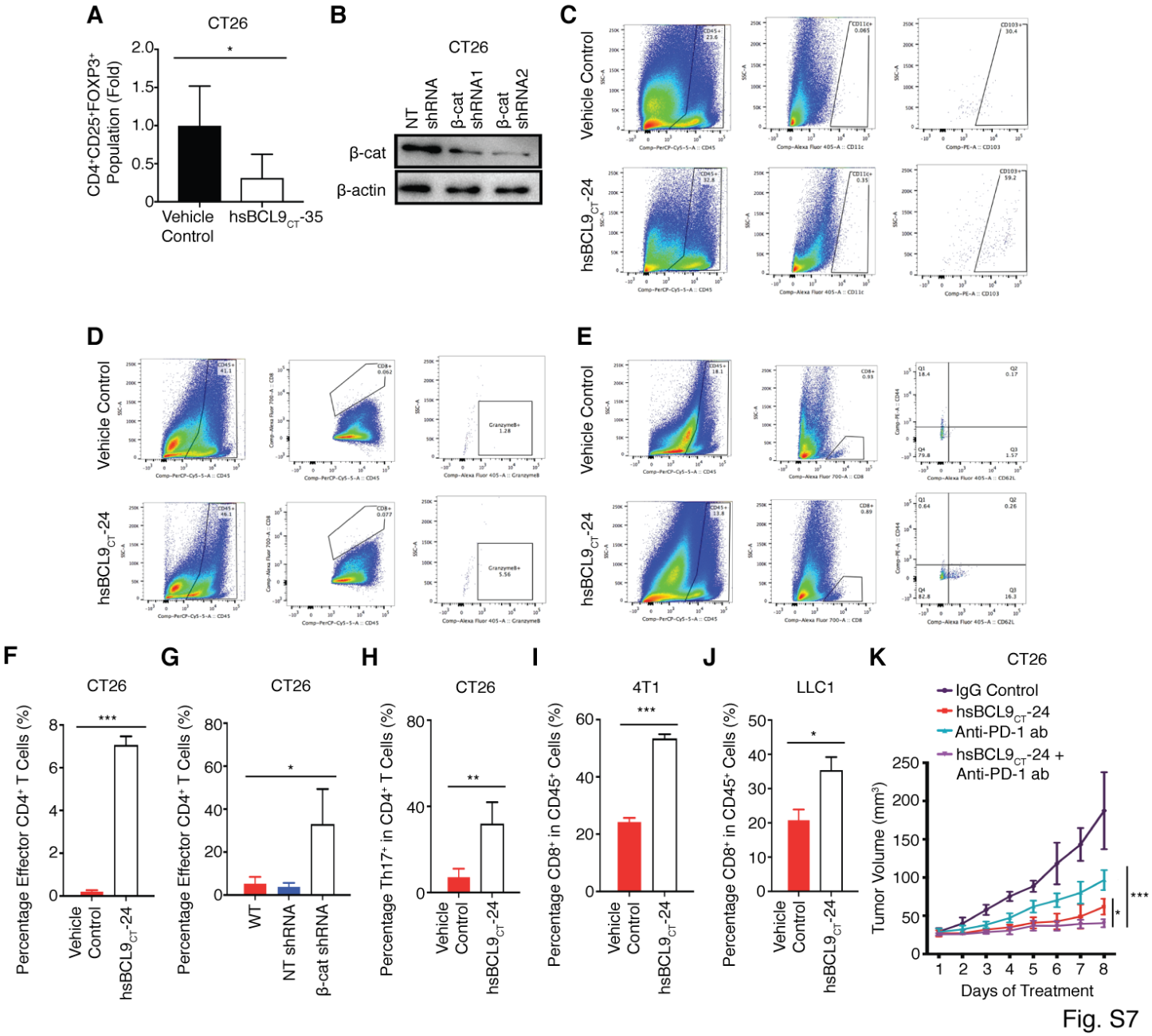

Fig. S7

Fig. S7. Effects of hsBCL9$_{CT}$-24 treatment on anticancer immune cells. (**A**) BALB/c mice inoculated with CT26 tumors were treated with vehicle control or hsBCL9$_{CT}$-35 at 40 mg/kg via i.p. injection, QD over 14 days. The percentage of CD4$^+$CD25$^+$FOXP3$^+$ cells in tumors is shown (*$p < 0.05$). (**B**) Western blot shows β-cat expression in CT26 cells transduced with or without shRNAs targeting β-cat. (**C**) Representative FACS plots showing the gating strategy used to identify CD11c$^+$CD103$^+$ DC cells. (**D**) Representative FACS plots showing the gating strategy used to identify GranzymeB$^+$CD8$^+$ T cells. (**E**) Representative FACS plots showing the gating strategy used to identify CD44$^+$CD62L$^-$ effector CD8$^+$ T cells. (**F**) Percentage of CD44$^+$CD62L$^-$ effector T cells among the overall CD4$^+$ T cell population in CT26 tumors treated with vehicle control or hsBCL9$_{CT}$-24 (***$p < 0.001$). **(G)** Percentage of CD44$^+$CD62L$^-$ effector T cells among the overall CD4$^+$ T cell population in CT26 tumors transduced with or without shRNAs targeting β-cat (*$p < 0.05$). (**H**) Percentage of

$CD196^+CD194^+$ Th17 T-helper cells among the overall $CD4^+$ T cell population in CT26 tumors treated with vehicle control or $hsBCL9_{CT}$-24 (**p < 0.01). (**I**) Percentage of $CD8^+$ T cells among the $CD45^+$ cell population in 4T1 tumors treated with vehicle control or $hsBCL9_{CT}$-24 (***p < 0.001). (**J**) Percentage of $CD8^+$ T cells among the $CD45^+$ cell population in LLC1 tumors treated with vehicle control or $hsBCL9_{CT}$-24 (*p < 0.05). (**K**) Combination treatment of $hsBCL9_{CT}$-24 with anti-PD1 ab resulted in significant tumor reduction in the CT26 model. BALB/c mice were inoculated with CT26 cells and administered IgG, $hsBCL9_{CT}$-24 at 25mg/kg QD, anti-PD-1 ab twice weekly, or $hsBCL9_{CT}$-24 + anti-PD-1 ab as indicated after tumor volume reached 20 mm^3 (*n* = 4 per cohort) (**p < 0.01). Results were denoted as mean ± SEM for experiments performed in triplicate. Each experiment was repeated three times and statistical significance of differences between groups was determined by unpaired Student's t-test. Statistical significance of differences in tumor growth assays was determined by two-way ANOVA.

Supplementary Tables

Table S1. Sequences of hsBCL9$_{CT}$ peptides and related derivatives. Sequences and modifications of all BCL9 derivatives, including hsBCL9$_{CT}$ peptides, mutated peptide derivatives used in binding and inhibition assays (hsBCL9$_{M}$ peptides), and peptide controls (BCL9-HD2$_{A}$, BCL9-HD2$_{B}$, BCL9-HD2$_{C}$) used in HTRF and ALPHA assays. X indicates (S)-2-(4'-pentenyl) Ala, B denotes norleucine (substituted for methionine to optimize activity of the ruthenium catalyst), 2-Nal represents 2-naphthylalanine, and β-Ala indicates β-alanine.

Name	Amino Acid Sequence	Corresponding BCL9 Position	N' Modification	C' Modification
BCL9-HD2	PDGLSQEQLEHRERSLQTLRDIQRMLFPDE	348-377	-	-
BCL9-HD2$_{A}$	LSQEQLEHRERSLQTLRDIQRMLF	351-374	Biotin	-
BCL9-HD2$_{B}$	LSQEQLEHRERSLQTLRDIQRMLF	351-374	Biotin-PEG3	-
BCL9-HD2$_{C}$	LSQEQLEHRERSLQTLRDIQRML	351-373	Ac	2-Nal-β-Ala-β-Ala-NH_2
SAH-BCL9$_{B}$	LSQEQLEHRERSLXTLRXIQRBLF	351-374	Ac	NH_2
hsBCL9$_{CT}$-1	LXQEQXE	351-357	Ac	NH_2
hsBCL9$_{CT}$-2	SXEQLXH	352-358	Ac	NH_2
hsBCL9$_{CT}$-3	QXQLEXR	353-359	Ac	NH_2
hsBCL9$_{CT}$-4	EXLEHXE	354-360	Ac	NH_2
hsBCL9$_{CT}$-5	QXEHRXR	355-361	Ac	NH_2
hsBCL9$_{CT}$-6	LXHREXS	356-362	Ac	NH_2
hsBCL9$_{CT}$-7	EXRERXL	357-363	Ac	NH_2
hsBCL9$_{CT}$-8	HXERSXQ	358-364	Ac	NH_2
hsBCL9$_{CT}$-9	RXRSLXT	359-365	Ac	NH_2
hsBCL9$_{CT}$-10	EXSLQXL	360-366	Ac	NH_2
hsBCL9$_{CT}$-11	RXLQTXR	361-367	Ac	NH_2
hsBCL9$_{CT}$-12	SXQTLXD	362-368	Ac	NH_2
hsBCL9$_{CT}$-13	LXTLRXI	363-369	Ac	NH_2
hsBCL9$_{CT}$-14	QXLRDXQ	364-370	Ac	NH_2
hsBCL9$_{CT}$-15	TXRDIXR	365-371	Ac	NH_2
hsBCL9$_{CT}$-16	LXDIQXB	366-372	Ac	NH_2
hsBCL9$_{CT}$-17	RXIQRXL	367-373	Ac	NH_2
hsBCL9$_{CT}$-18	DXQRBXF	368-374	Ac	NH_2
hsBCL9$_{CT}$-19	RDXQRBXF	367-374	Ac	NH_2
hsBCL9$_{CT}$-20	LRXIQRXL	366-373	Ac	NH_2

hsBCL9$_{CT}$-21	LRXIQRXL	366-374	Ac	2-Nal-NH2
hsBCL9$_{CT}$-22	LRXIQRXL	366-375	Ac	β-Ala-β-Ala-NH2
hsBCL9$_{CT}$-23	LRXIQRXL	366-376	Ac	2-Nal-β-Ala-β-Ala-NH2
hsBCL9$_{CT}$-24	LQTLRXIQRXL	363-373	Ac	2-Nal-NH$_2$
hsBCL9$_{CT}$-31	LQTLRXIQRX	363-372	Ac-GRKKRRQRRRPQ	2-Nal-β-Ala-β-Ala-NH$_2$
hsBCL9$_{CT}$-32	LQTLRXIQRXL	363-373	Ac	2-Nal-β-Ala-β-Ala-GRKKRRXRRRXQ-NH2
hsBCL9$_{CT}$-35	XLQXLRXIQRXL	362-373	Ac	2-Nal-β-Ala-β-Ala-NH$_2$
hsBCL9$_{M1}$ (M1)	XDQXDRXDQRXDH	362-374	Ac	β-Ala-β-Ala-NH$_2$
hsBCL9$_{M2}$ (M2)	XLEXLRXIERXL	362-373	Ac	2-Nal-β-Ala-β-Ala-NH$_2$
hsBCL9$_{M3}$ (M3)	LQTARXAQRXL	363-373	Ac	2-Nal-NH$_2$
hsBCL9$_{M4}$ (M4)	LQTLRXAQRXA	363-373	Ac	2-Nal-NH$_2$
hsBCL9$_{M5}$ (M5)	LQTARXIQRXL	363-373	Ac	2-Nal-NH$_2$
hsBCL9$_{M6}$ (M6)	LQTLRXAQRXL	363-373	Ac	2-Nal-NH$_2$
hsBCL9$_{M7}$ (M7)	LQTLRXIQRXA	363-373	Ac	2-Nal-NH$_2$

Table S2. PK and TK profiles of hsBCL9$_{CT}$-24 and hsBCL9$_{CT}$-35 in mice. (**A**) PK parameters were evaluated and summarized for each hsBCL9$_{CT}$-24 and hsBCL9$_{CT}$-35 treatment group of BALB/c mice (n = 2), with peptide administered i.v., s.c, i.p., and i.m. as indicated. (**B**) PK parameters were evaluated and summarized for hsBCL9$_{CT}$-24 treated BALB/c mice (n = 2) to determine dose-escalation profile, with peptide administered via i.v. at 5, 10, and 15 mg/kg. Evaluated TK parameters were summarized for (**C**) Day 1 and (**D**) Day 14 following hsBCL9$_{CT}$-24 20 mg/kg administration in BALB/c mice (n = 3) via i.p. injection.

(A)

	hsBCL9$_{CT}$-24				**hsBCL9$_{CT}$-35**	
PK Parameters	**i.v.**	**s.c.**	**i.p.**	**i.m.**	**i.v.**	**i.p.**
Dose (mg/kg)	5	10	5	5	30	30
Terminal $t_{1/2}$ (hr)	1.40	13.0	3.09	10.6	7.03	9.81
AUC_{last} (hr×ng/mL)	123,986	140,000	101,978	44,900	996,029	987,148
AUC_{inf} (hr×ng/mL)	125,877	146,000	102,032	45,300	997,708	996,181
F (%)	-	56	81.1	20.6	-	100

(B)

PK Parameters	**i.v. 5 mg/kg**	**i.v. 10 mg/kg**	**i.v. 15 mg/kg**
CL (L/hr/kg)	0.0397	0.0428	0.0576
V_{ss} (L/kg)	0.0692	0.0788	0.0946
Terminal $t_{1/2}$ (hr)	1.40	2.17	1.80
AUC_{last} (hr×ng/mL)	123,986	233,000	260,000
AUC_{inf} (hr×ng/mL)	125,877	233,000	260,000
C_{max} (μM)	41	86	102
AUC (hr×nmol/mL)	76	143	160

(C)

TK Parameters	**i.p. 10 mg/kg**	**i.p. 15 mg/kg**	**i.p. 20 mg/kg**
T_{max} (hr)	1.50	2.17	2.67
C_{max} (ng/mL)	11,390	9,767	12,733
Terminal $t_{1/2}$ (hr)	4.52	7.59	6.47
AUC_{last} (hr×ng/mL)	114,333	118,033	162,333

AUC_{inf} (hr×ng/mL)	117,000	129,100	175,333

(D)

TK Parameters	**i.p. 10 mg/kg**	**i.p. 15 mg/kg**	**i.p. 20 mg/kg**
T_{max} (hr)	2.00	3.33	4.00
C_{max} (ng/mL)	11,360	8,748	13,725
Terminal $t_{1/2}$ (hr)	6.97	4.25	4.55
AUC_{last} (hr×ng/mL)	37,836	107,420	159,976
AUC_{inf} (hr×ng/mL)	41,091	108,735	160,881

Table S3. Additional PK, solubility, and stability investigations with hsBCL9$_{CT}$-24. (**A**) PK parameters of hsBCL9$_{CT}$-24 5 mg/kg treatment in BALB/c syngeneic mice (n = 2), administered via i.v. or i.p. over 36 hours. Results from three different batches (lots) are reported. (**B**) hsBCL9$_{CT}$-24 solubility studies in water, DMSO, and PBS from two different lots. (**C**) hsBCL9$_{CT}$-24 HPLC purity studies, reported under varying temperatures and weekly intervals over a one-month period. (**D**) Plasma stability assay in BALB/c nude mice, conducted over 60 minutes to assess percent remaining of hsBCL9$_{CT}$-24 in plasma. Procaine was used as the control.

(A)

	hsBCL9$_{CT}$-24 i.v. 5 mg/kg			**hsBCL9$_{CT}$-24 i.p. 5mg/kg**		
PK Parameters	**Lot A**	**Lot B**	**Lot C**	**Lot A**	**Lot B**	**Lot C**
CL (L/hr/kg)	0.047	0.0459	0.0368	0.06	-	-
V_{ss} (L/kg)	-	0.0686	0.0693	-	-	-
Vz (L)	0.0050	-	-	0.010	-	-
Terminal $t_{1/2}$ (hr)	2.44	1.11	1.99	2.59	3.19	3.09
AUC_{last} (hr×ng/mL)	105,637	108,000	136,000	77,618	89,300	89,800
AUC_{inf} (hr×ng/mL)	105,709	109,000	136,000	77,849	89,400	89,900
MRT_{inf} (hr)	-	1.49	1.88	-	-	-
T_{max} (hr)	-	-	-	-	2.00	2.00
C_{max} (ng/mL)	47,354.5	-	-	10,452	10,500	19,600
F (%)	100	-	-	73.6	82.0	66.1

(B)

hsBCL9$_{CT}$-24 Lot	**Quantity (mg)**	**pH**	**Solubility (1 mg/mL)**		
			H_2O	**DMSO**	**PBS**
B	24.0	5.00	Soluble	Soluble	Insoluble
C	19.0	5.78	Soluble	Soluble	Insoluble

(C)

hsBCL9$_{CT}$-24 Lot	**Quantity (mg)**	**Temperature (°C)**	**Purity at 0 months (%)**	**Purity at 1 month (%)**
B	24.0	2-8	96.4	96.3
			98.4	98.3
C	19.0	RT	96.4	96.3
			98.4	97.9

(D)

Test Article	Species		Percent Remaining (%)					$T_{1/2}$ (min)
			0 min	15 min	30 min	45 min	60 min	
Procaine	BALB/c Nude Mice	Mean	100.00	8.48	0.20	0.07	0.02	4.21
		RSD of Area Ratio	0.01	0.68	0.09	0.75	0.05	
$hsBCL9_{CT}$-24	BALB/c Nude Mice	Mean	100.00	103.82	91.67	85.76	90.28	307.51
		RSD of Area Ratio	0.02	0.11	0.07	0.10	0.25	