

Additional File

A chemical probe inhibitor targeting STAT1 restricts cancer stem cell traits and angiogenesis in colorectal cancer

Pei-Hsuan Chou¹, Cong-Kai Luo¹, Niaz Wali^{2,3,4}, Wen-Yen Lin⁵, Shang-Kok Ng¹, Chun-Hao Wang⁶, Mingtao Zhao^{7,8,9}, Sheng-Wei Lin¹⁰, Pei-Ming Yang¹¹, Pin-Jung Liu¹², Jiun-Jie Shie^{2*}, Tzu-Tang Wei^{1,4*}

Inventory of Additional File

Figure S1-S2, related to Figure 1

Figure S3, related to Figure 2

Figure S4, related to Figure 3

Figure S5, related to Figure 4

Figure S6, related to Figure 5

Figure S7, related to Figure 6

Figure S8

Table S1

Table S2

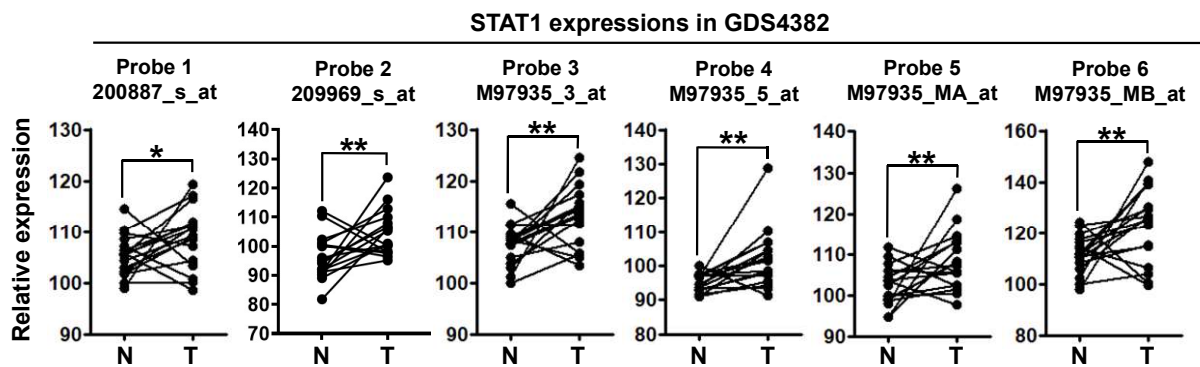
Figure S1

A

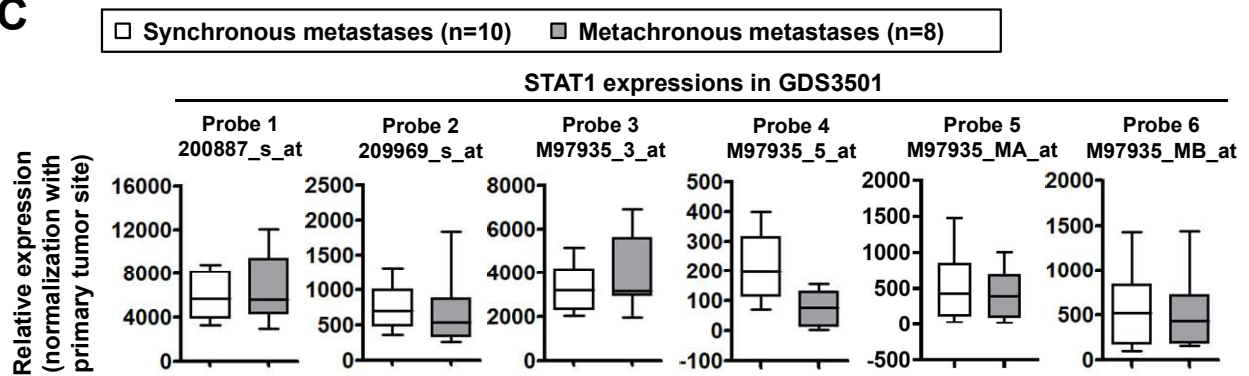
GEO Accession	Dataset	Platform	Description and sample size
GSE32323	GDS4382	GPL570	<ul style="list-style-type: none"> RNA samples were extracted from colonic mucosa of CRC patients. Primary colorectal cancer paired with non-cancerous tissues (17 paired samples).
GSE10961	GDS3501	GPL570	<ul style="list-style-type: none"> RNA samples were extracted from liver metastases of CRC patients. Contained synchronous liver metastasis (10 samples) and metachronous liver metastasis (8 samples).

GPL570, [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array.

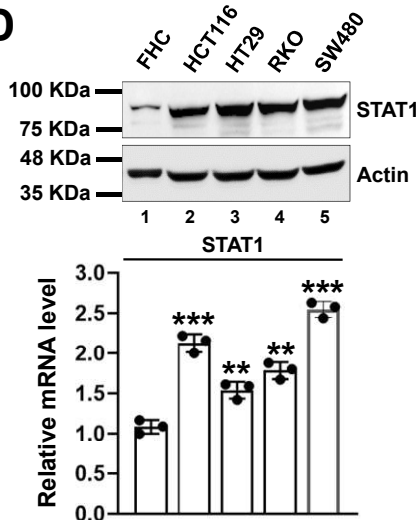
B



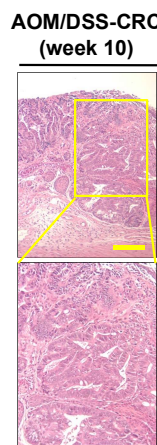
C



D



E



F

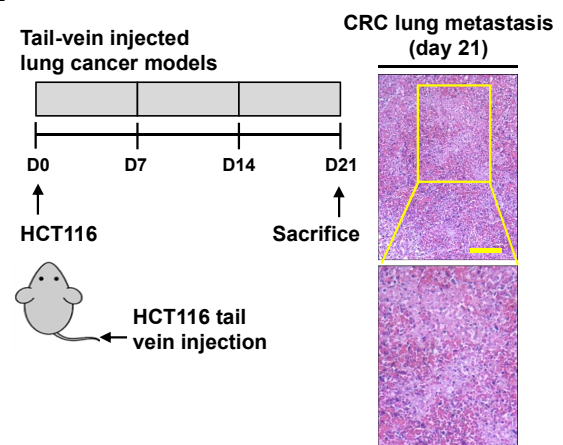


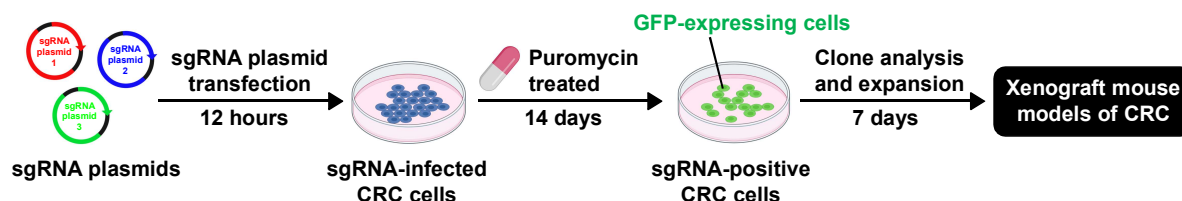
Figure S1. Expressions of STAT1 in primary and metastatic colorectal cancer.

(A) A summary of the each individual microarray datasets from different GEO dataset (<https://www.ncbi.nlm.nih.gov/gds>). (B) The GDS4382 dataset containing microarray data for colorectal tumor tissues and adjacent normal tissues was obtained from the NCBI GEO database. The expression levels of STAT1 are shown. * $p < 0.05$; *** $p < 0.001$. (C) The GDS3501 dataset containing microarray data for patients with metastatic CRC was obtained from the NCBI GEO database. The expression levels of STAT1 are shown. (D) Western blot analysis and Q-PCR analysis for STAT1 expressions in normal human colon epithelium cell line (FHC) and CRC cell lines (HCT116, HT29, RKO, and SW480). Total cell lysates were prepared, and Western blot analysis was performed (upper panel). Q-PCR analyzed the mRNA expressions of STAT1, which were normalized to GAPDH (lower panel). ** $p < 0.01$ versus FHC; *** $p < 0.001$ versus FHC. (E) Tumor sections from the AOM/DSS-induced CRC mouse models were counterstained with H&E, and high-magnification images of the yellow-boxed area are shown. Scale bars: 250 μm . (F) Schematic overview of the experimental design (left panel). Seven-week-old male NOD/SCID mice were intravenously injected with HCT116 cells (1×10^6) through the tail vein. Mice were sacrificed at 3 weeks, and lung segments were fixed by formalin. Lung sections from the tail vein-injected mouse models were counterstained with H&E, and high-magnification images of the yellow-boxed area are shown (right panel). Scale bars: 250 μm .

Figure S2

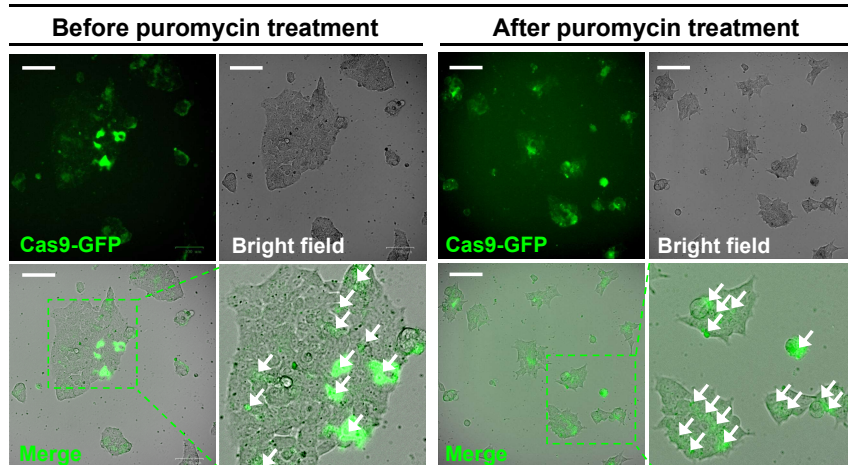
A

Generation of STAT1 knockout CRC cells by CRISPR/Cas9 system:

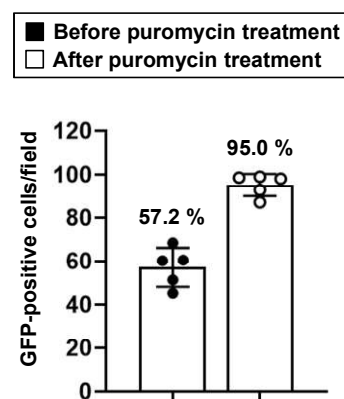


B

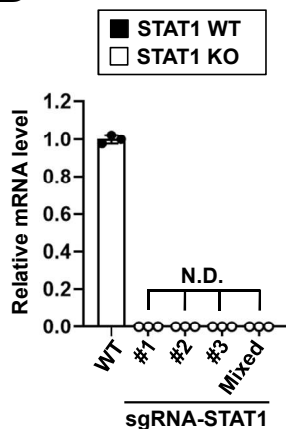
CRISPR/Cas9-mediated STAT1 K.O. HCT116



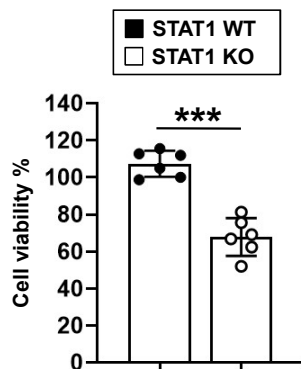
C



D



E



F

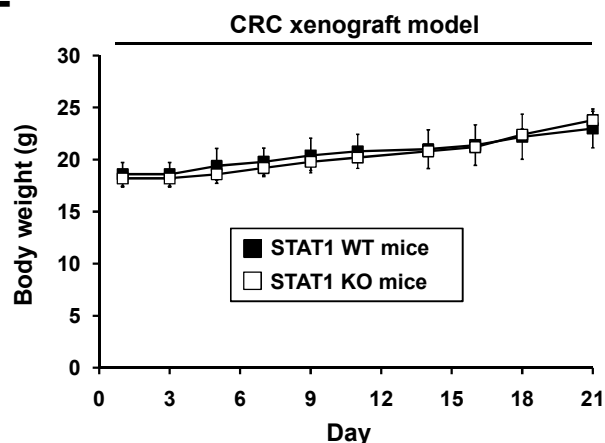


Figure S2. Generation of STAT1 knockout colorectal cancer cells using CRISPR/Cas9

system. (A) Schematic overview of the CRISPR/Cas9 system in CRC cells. CRISPR plasmids expressing Cas9 and STAT1 sgRNAs were transfected into HCT116 cells for 12 hours. Cells were treated with puromycin (0.5 μ g/ml) for 14 days. Single clones were picked according to

GFP expression and expanded to generate monoclonal cell colonies. **(B)** Successful transfection of CRISPR/Cas9 KO plasmids could be visually confirmed by detection of the GFP. The white arrowhead indicates the GFP-positive HCT116 cells. Scale bar: 100 μ m. **(C)** GFP-positive HCT116 cells were calculated. **(D)** The STAT1 mRNA levels in HCT116 cells treated with STAT1 sgRNAs versus control were quantified by qPCR analysis. The mRNA expression was normalized to GAPDH. N.D., not detected for 40 cycles by qPCR. **(E)** Role of STAT1 on cell viability in HCT116 cells. STAT1 WT cells and STAT1 KO cells were incubated, and cell viability was measured at 48 hours by the MTT assay. *** $p < 0.001$. **(F)** Changes of the body weights in HCT116 xenograft mouse models are shown.

Figure S3

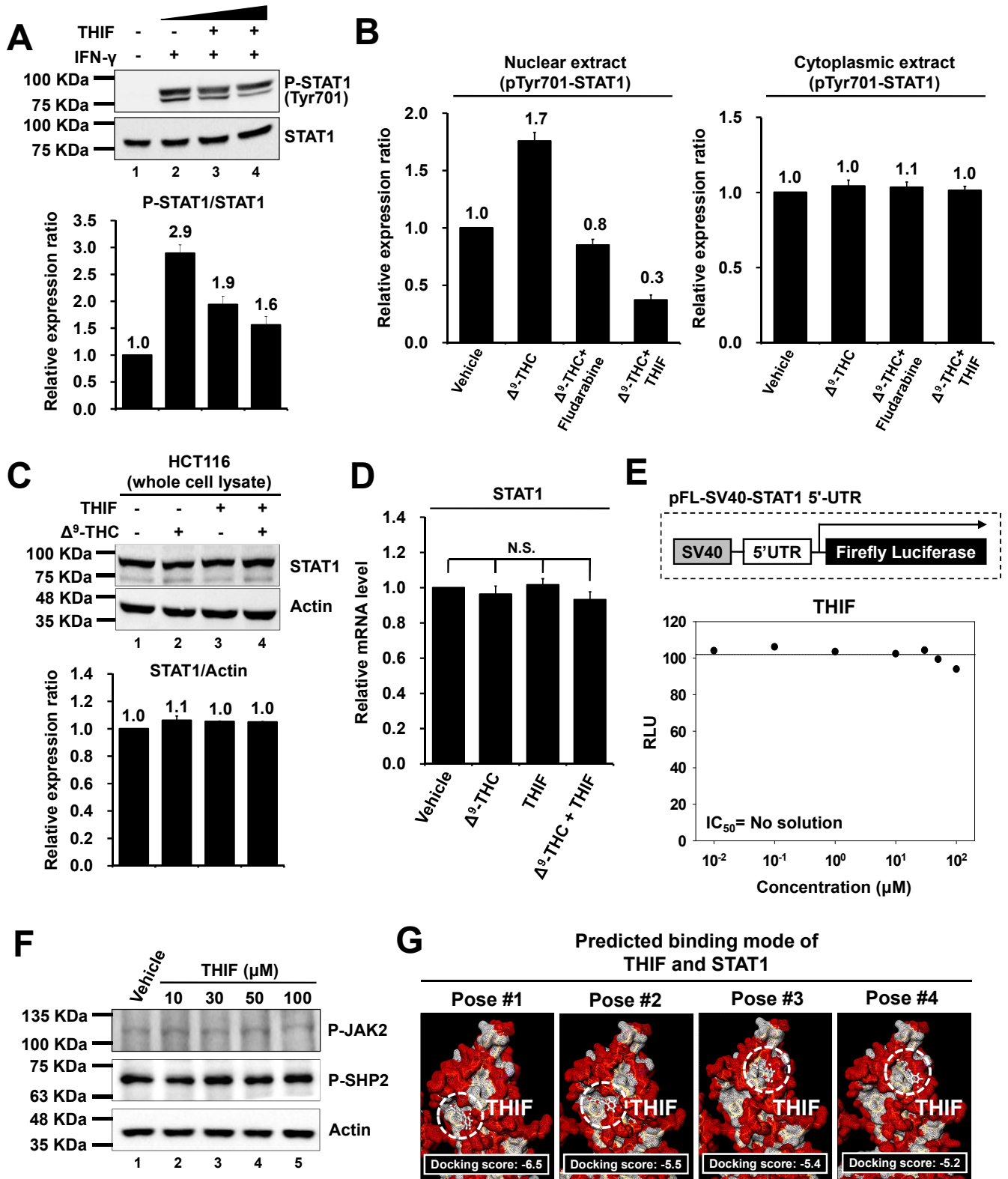
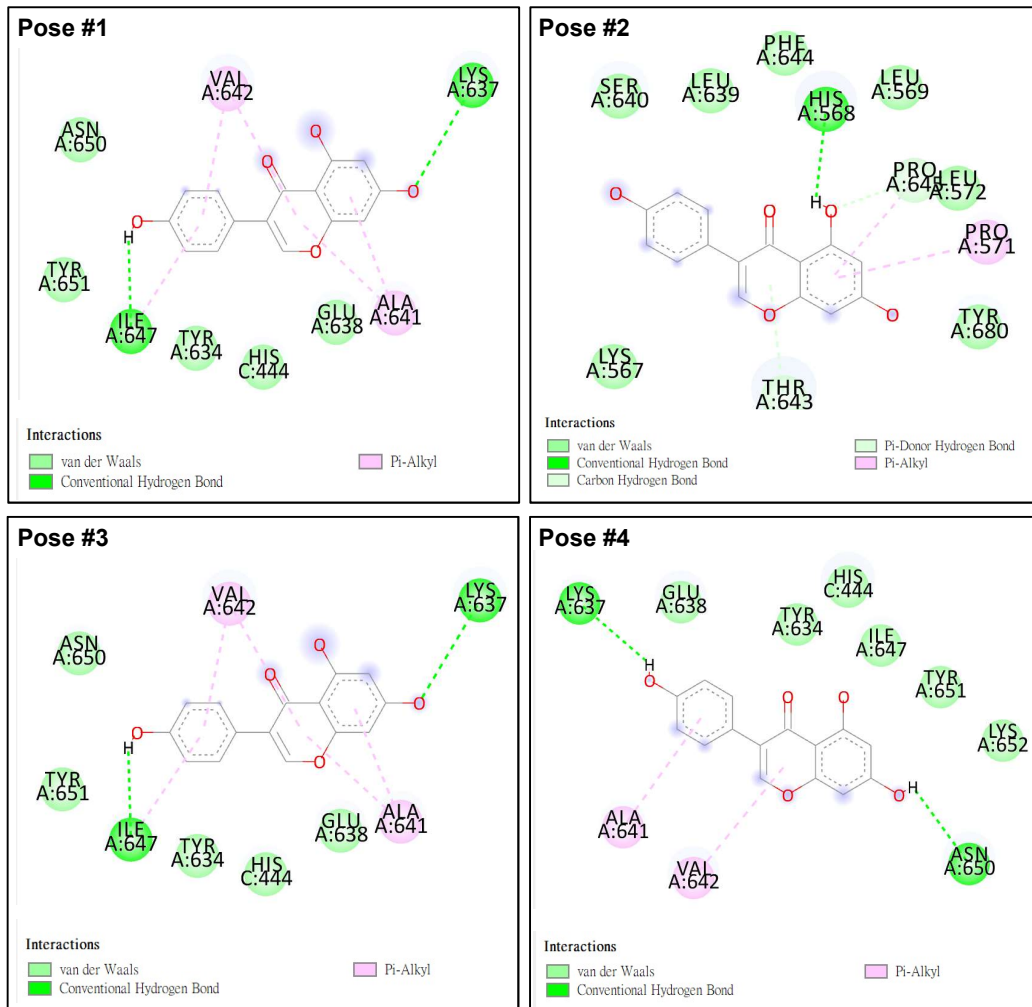


Figure S3 (continue)

H

Receptor-ligand interaction diagrams of THIF and STAT1



I

Interactions between THIF and amino acid residues of STAT1

	Pose #1	Pose #2	Pose #3	Pose #4
HIS444	V		V	V
LYS567		V		
HIS568		V		
LEU569		V		
PRO571		V		
LEU572		V		
TYR634	V		V	V
LYS637	V		V	V
GLU638	V		V	V
LEU639		V		
SER640		V		
ALA641	V		V	V
VAL642	V		V	V
THR643		V		
PHE644		V		
PRO645		V		
ILE647	V		V	V
ASN650	V		V	V
TYR651	V		V	V
LYS652				V
TYR680		V		

STAT1 SH2 Domain

Figure S3. THIF can directly bind to the STAT1 protein. (A) Effect of THIF on STAT1 phosphorylation in CRC cells. HCT116 cells were treated with STAT1 activator (500 ng/ml IFN- γ) or STAT1 activator plus THIF (10 μ M or 50 μ M) for 12 hours. Total protein extract was prepared and Western blot analysis was performed using indicated antibodies (upper panel). Western blot images were quantified by ImageJ software (lower panel). (B) Effect of THIF on STAT1 phosphorylation in CRC cells. HCT116 cells were treated with 5 μ M Δ^9 -THC, Δ^9 -THC plus 10 μ M THIF or Δ^9 -THC plus 10 μ M fludarabine for 12 hours. Protein extracts from the nuclear and cytoplasmic fractions were prepared. Western blot analysis was performed using the indicated antibodies. Western blot images were quantitatively analyzed with ImageJ software. (C and D) HCT116 cells were treated with 5 μ M Δ^9 -THC, 50 μ M THIF or their combination for 48 hours. (C) Protein extract was prepared and Western blot analysis was performed using indicated antibodies (upper panel). Western blot images were quantified by ImageJ software (lower panel). (D) qPCR analysis was performed. The mRNA expression was normalized to GAPDH. N.S., no significance. (E) Dose-response effect of THIF on STAT1 activity. The luciferase activity of STAT1 was measured by the STAT1 reporter assay in 293T cells. Cells were treated with increasing concentrations of THIF for 12 hours. The 50% of inhibition concentration (IC₅₀) of luciferase activity is calculated by SigmaPlot software. (F) HCT116 cells were treated with indicated concentrations of THIF for 48 hours. Protein extract was prepared and Western blot analysis was performed using indicated antibodies. (G-H) Conformations of THIF docked in binding sites on the STAT1 protein. (G) Docking models of THIF in the STAT1 crystal structure (PDB: 1YVL). The predicted binding modes of THIF and STAT1 are shown. The white circle indicates the binding site of THIF. (H) The binding interactions between THIF and amino acid residues in STAT1 protein are shown. (I) The overlapping binding sites of four predicted binding modes of THIF with the STAT1 SH2 domain are highlighted in blue.

Figure S4

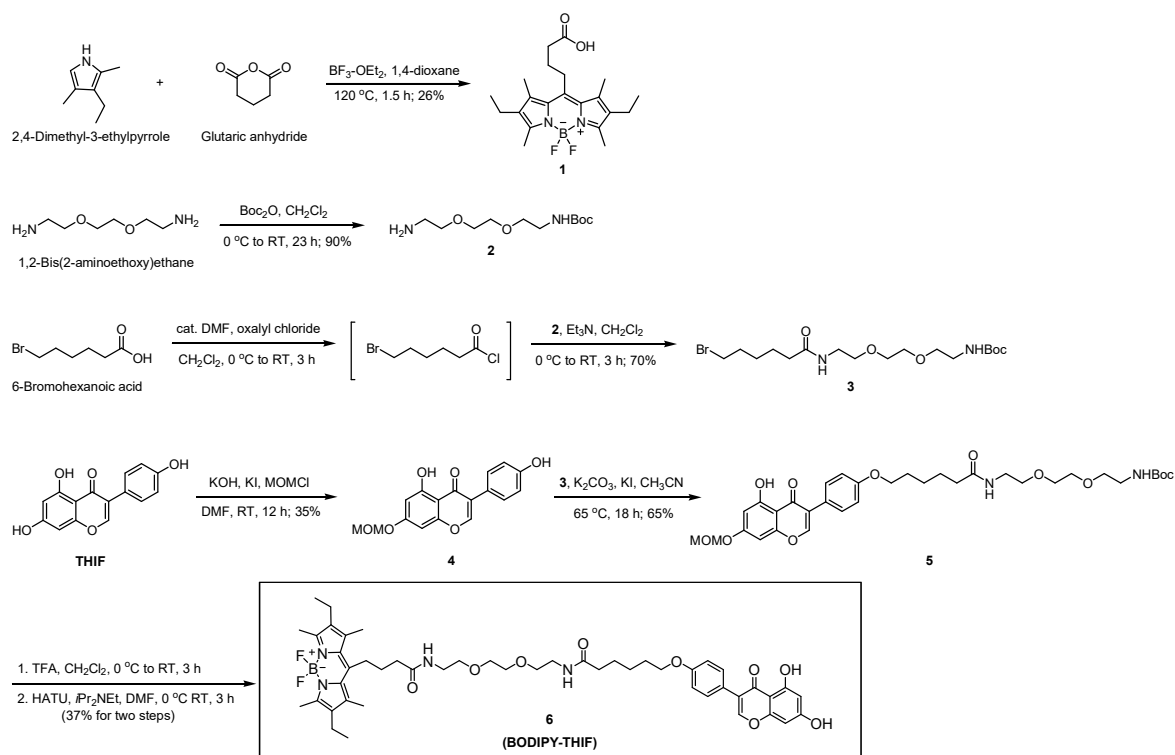
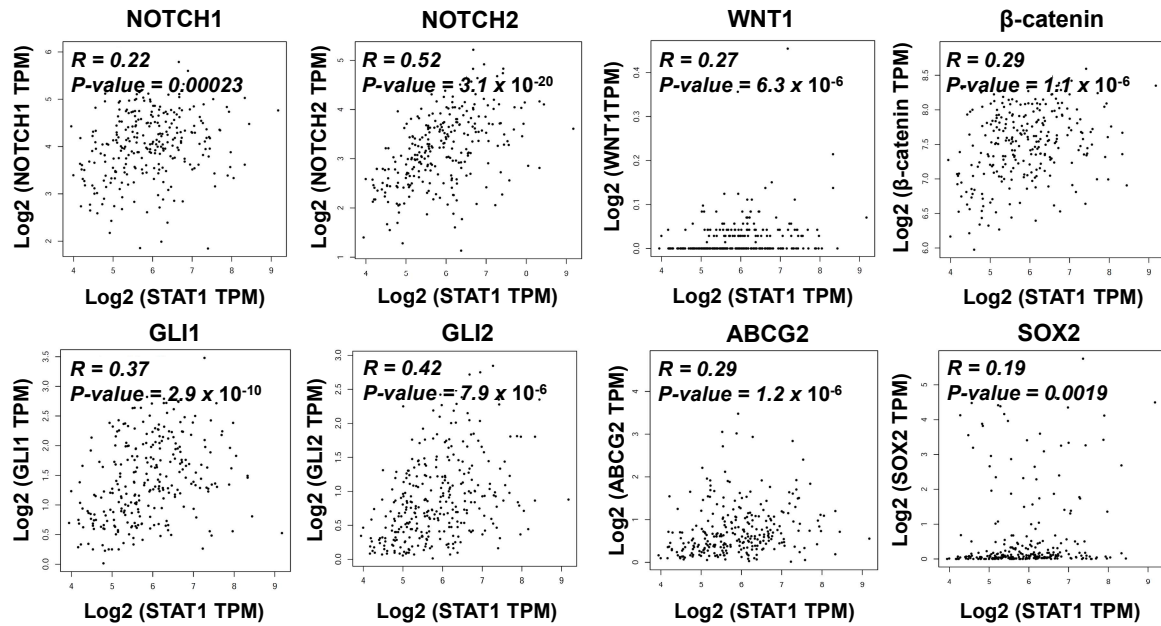


Figure S4. A synthesis plan of fluorescently labeled STAT1 probe inhibitor. Reactions are magnetically stirred and monitored by thin-layer chromatography on silica gel. Flash chromatography is performed on silica gel of 60-200 μm particle size. Yields are reported for spectroscopically pure compounds. Melting points are recorded on a Fargo MP-2D melting point apparatus. ^1H , ^{13}C , and ^{31}P NMR spectra are recorded on Bruker AV 600 (600 MHz), Bruker AV 500 (500 MHz) and Bruker AVIII 17 400 (400 MHz) spectrometers. Chemical shifts are given in δ values relative to tetramethylsilane ($\delta\text{H} = 0$); coupling constants J are given in Hz. Internal standards are CDCl_3 ($\delta\text{H} = 7.24$), CD_3OD ($\delta\text{H} = 3.31$) or DMSO-d_6 ($\delta\text{H} = 2.49$) for ^1H NMR spectra and CDCl_3 ($\delta\text{C} = 77.0$, central line of triplet), CD_3OD ($\delta\text{C} = 49.0$, central line of septet) or DMSO-d_6 ($\delta\text{C} = 39.5$, central line of septet) for ^{13}C NMR spectra. The splitting patterns are reported as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad), and dd (double of doublets). High-resolution electrospray ionization and fast atom bombardment mass spectra were recorded on a JMS-T100LP AccuTOF LC-plus 4G mass spectrometer.

Figure S5

A

Colorectal adenocarcinoma using TCGA database (n=340)



B

Colon tissues using GTEx database (n=308)

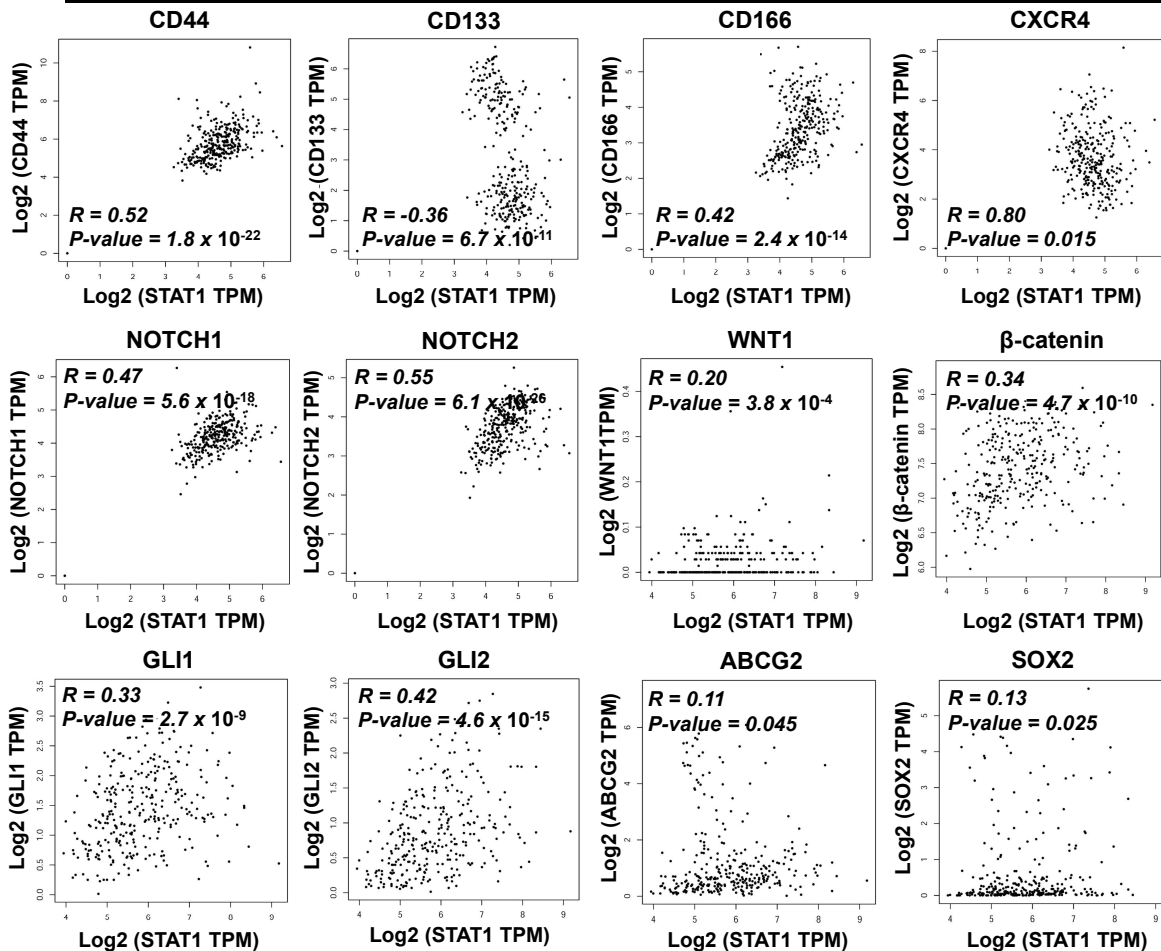


Figure S5 (continue)

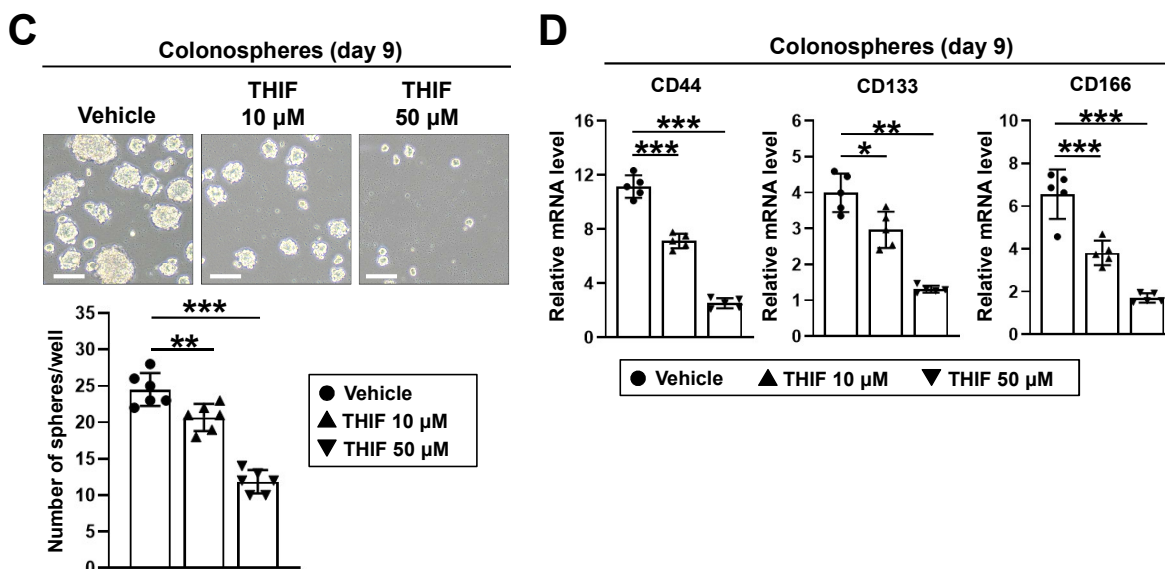


Figure S5. Effect of THIF on cancer stemness. (A) Correlations between STAT1 and common cancer stem cell (CSC) markers are shown in patients with colorectal adenocarcinoma using TCGA database (<https://gdc.cancer.gov/>). (B) Correlations between STAT1 and CSC markers are shown in human colon tissues using the GTEx database (<https://www.gtexportal.org/home/>). (C and D) HCT116 colonospheres were dissociated into single cells and plated at a density of 5×10^4 cells/well in a 6-well Ultra-Low plate. Cells were treated with 10 or 50 μ M THIF for 48 hours. (C) Cell morphology was monitored by microscopy (upper panel). Scale bars: 100 μ m. The number of colonospheres was calculated (lower panel). ** $p < 0.01$; *** $p < 0.001$. (D) The mRNA expression of stemness genes was measured by qPCR. The mRNA expression was normalized to GAPDH. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Figure S6

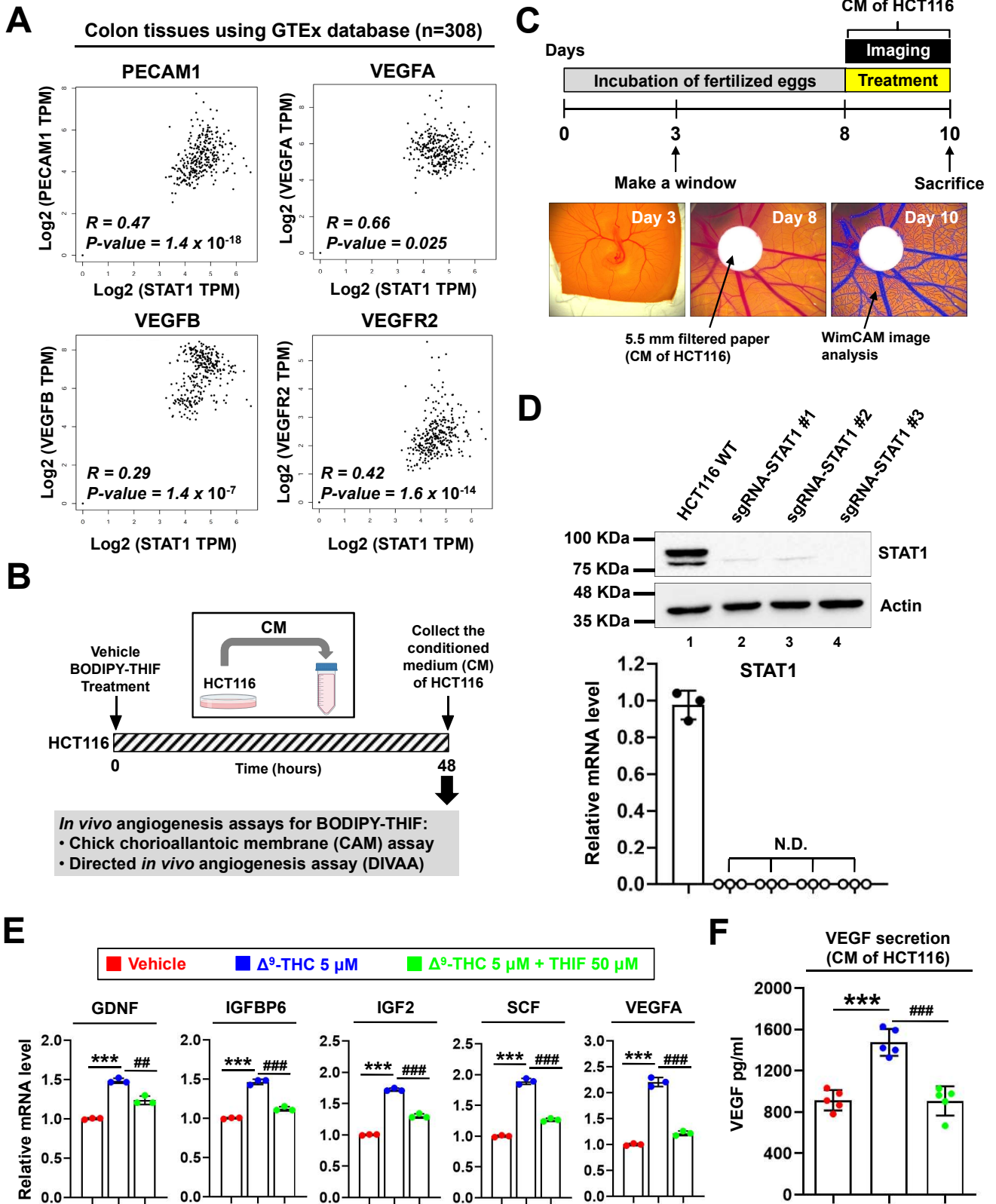


Figure S6. Effect of THIF on angiogenesis in colorectal cancer *in vitro* and *in vivo*.

(A) Correlations between STAT1 and angiogenesis-related markers are shown in human colon tissues using the GTEx database. (B) Schematic overview of the experimental design for *in vivo* angiogenesis assays. (C) HCT116 cells were treated with vehicle (control) or BODIPY-THIF (50 μ M or 100 μ M) for 48 hours, and the CM was collected for the chick chorioallantoic membrane (CAM) assay. Sterilized filter-paper disks were used as a carrier for CM of HCT116 cells. Images were quantified by WimCAM image analysis software. (D) Validation of STAT1 expression by Q-PCR and western blot analysis in STAT1 KO cells. The STAT1 protein levels and mRNA levels in HCT116 cells treated with STAT1 sgRNAs versus control were quantified by Western blot analysis and Q-PCR analysis. Protein extract was prepared and Western blot analysis was performed using indicated antibodies (upper panel). mRNA expression was normalized to GAPDH (lower panel). N.D., not detected for 40 cycles by Q-PCR. Data are representative of three independent experiments and values are expressed in mean \pm SD. (E) Effect of THIF on Δ^9 -THC-induced angiogenesis in CRC cells. HCT116 cells were treated with 5 μ M Δ^9 -THC or 5 μ M Δ^9 -THC plus 50 μ M THIF for 48 hours. The mRNA expressions of the five angiogenesis-related genes were quantified by qPCR analysis and normalized to GAPDH. *** p < 0.001 versus vehicle; ## p < 0.01 versus Δ^9 -THC; ### p < 0.001 versus Δ^9 -THC. (F) THIF reduces the release of VEGF from CRC cells after Δ^9 -THC treatment. HCT116 cells were treated with 5 μ M Δ^9 -THC or 5 μ M Δ^9 -THC plus 50 μ M THIF for 48 hours, and VEGF concentration in conditioned medium (CM) was measured by ELISA. *** p < 0.001 versus vehicle; ### p < 0.001 versus Δ^9 -THC.

Figure S7

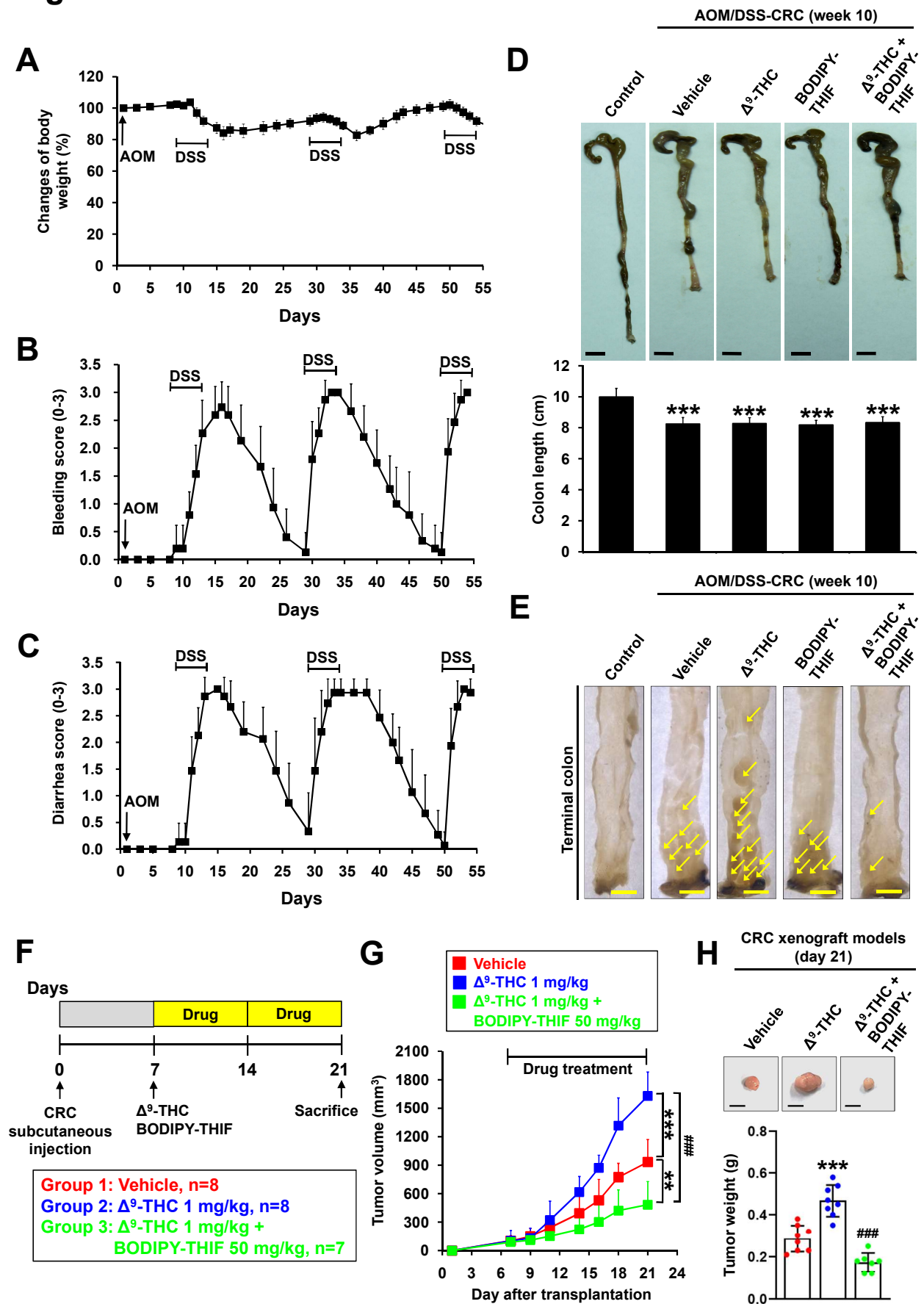


Figure S7. Effects of BODIPY-THIF on tumor growth in AOM/DSS-induced colorectal cancer mouse models. CRC was induced by intraperitoneal injection of AOM (12.5 mg/kg) in conjunction with the DSS stimulus. Mice were maintained with a regular diet and drinking water for 7 days and then subjected to 3 cycles of DSS treatment, with each cycle consisting of the administration of 3.5% DSS for 5 days followed by a 14-day recovery period with regular water. **(A-C)** Colitis symptom in AOM/DSS mouse models during the CRC induction and recovery phases. **(A)** Changes in body weights are shown. **(B)** Clinical bleeding scores are shown. **(C)** Clinical diarrhea scores are shown. **(D)** Representative whole colons (upper panel) and colon length (lower panel) are shown. Scale bar, 2 cm. *** $p < 0.001$ versus healthy control. **(E)** Representative whole colons are depicted, and the yellow arrowhead indicates macroscopic polyps. Scale bars: 5 mm. **(F-H)** Effect of BODIPY-THIF on colorectal tumor growth in xenograft mouse models. HCT116 cells (1×10^7 cells) were subcutaneously implanted into male nude mice. When the tumor volume reached 100 mm^3 , mice were treated with vehicle (control), Δ^9 -THC (1 mg/kg, intraperitoneally), or Δ^9 -THC (1 mg/kg, intraperitoneally) plus BODIPY-THIF (50 mg/kg, orally) every two days for two weeks. The tumor volume was measured every two days after drug treatment. **(F)** Schematic overview of the experimental design in the mouse model. **(G)** The tumor volume was calculated as follows: $V = 0.5 \times (\text{the length of length}) \times (\text{the length of width})^2$. ** $p < 0.01$ versus vehicle; *** $p < 0.001$ versus vehicle; ### $p < 0.001$ versus Δ^9 -THC. **(H)** Gross pictures of xenograft tumors are shown (upper panel). Scale bar: 1 cm. At the end of the experiment, the excised tumors were weighed (lower panel). *** $p < 0.001$ versus vehicle; ### $p < 0.001$ versus Δ^9 -THC.

Figure S8

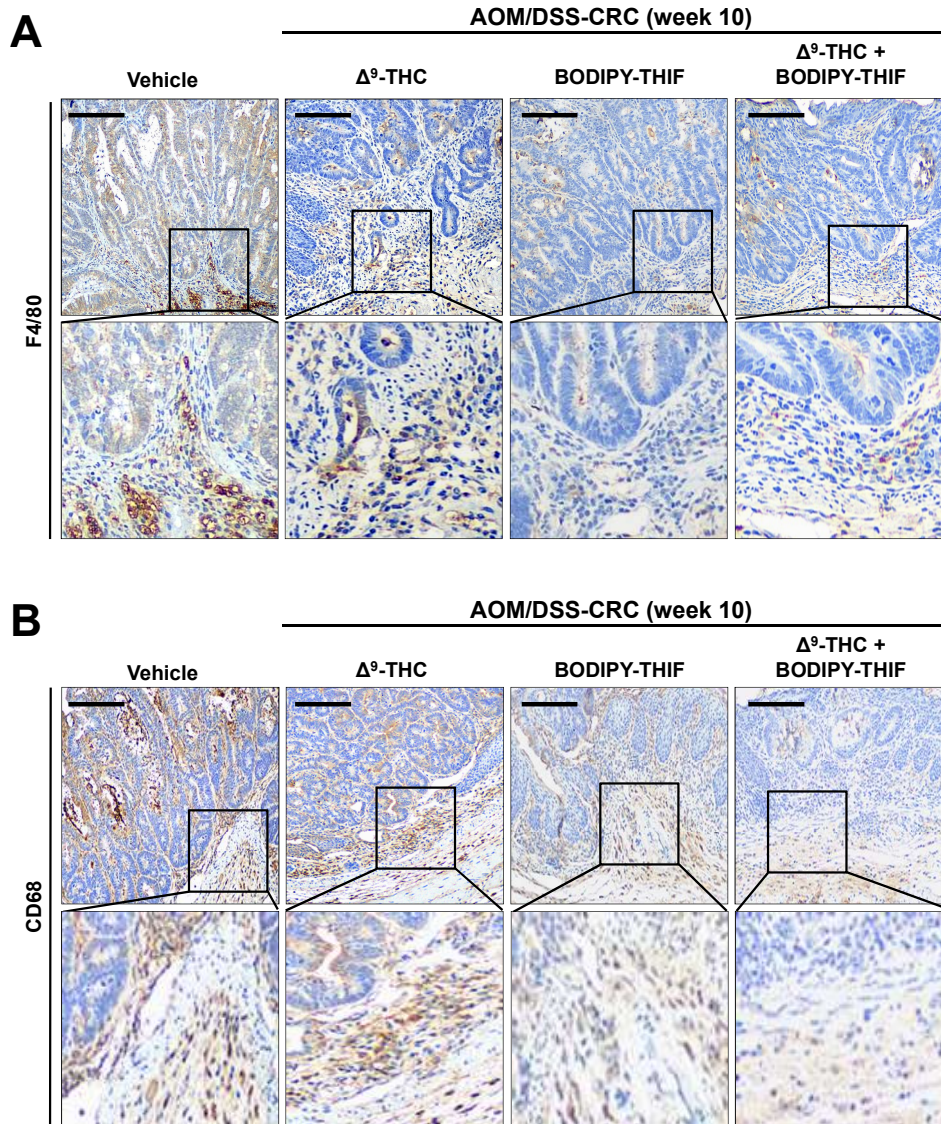


Figure 8. Effects of STAT1 on immune response in colorectal cancer. AOM/DSS-treated mice were randomly divided into four groups. Mice were treated with vehicle (control), Δ^9 -THC (1 mg/kg, intraperitoneally), BODIPY-THIF (50 mg/kg, orally), or Δ^9 -THC (1 mg/kg, intraperitoneally) plus BODIPY-THIF (50 mg/kg, orally) five days a week for two weeks after AOM/DSS treatment. Colon sections were immunostained with anti-F4/80 (**A**) and anti-CD68 (**B**) antibodies, and one representative experiment of three was presented. High-magnification images of the areas in the black boxes are shown. Scale bar, 250 μ m.

Table S1. List of antibodies used in this study.

ANTIBODIES	SUPPLIER NAME	CATALOG NUMBER
Anti-STAT1 antibody	Cell Signaling	#9172
Anti-phospho-STAT1 (Tyr701) antibody	Cell Signaling	#9167
Anti- α -Tubulin antibody	Abcam	ab7291
Anti-lamin B1 antibody	Cell Signaling	#12586
Anti-phospho-SHP-2 (Tyr580) antibody	Abcam	ab75818
Anti-phospho-JAK2 (Tyr1007/1008) antibody	ABclonal	AP0531
Anti-F4/80 antibody	Abcam	ab111101
Anti-CD68 antibody	Bio-Red	MCA1957
Anti- β -actin antibody	GeneTex	GTX109639
Rabbit IgG, polyclonal - Isotype Control	Abcam	ab37415
Rabbit IgG - Normal Control	Santa Cruz	sc-2027

Table S2. Predicted protein targets of THIF from the SwissTargetPrediction Database.

TARGETS ^a	TARGET CLASS
Thromboxane-A synthase	Cytochrome P450
Monoamine oxidase A	Oxidoreductase
Epidermal growth factor receptor erbB1	Kinase
Estrogen receptor alpha	Nuclear receptor
Maltase-glucoamylase	Hydrolase
Serotonin 2a (5-HT2a) receptor	Family A G protein-coupled receptor
Serotonin 2c (5-HT2c) receptor	Family A G protein-coupled receptor
Adenosine A1 receptor	Family A G protein-coupled receptor
Estrogen receptor beta	Nuclear receptor
Adenosine A2a receptor	Family A G protein-coupled receptor

^a Top ten predicted protein targets of THIF from the Swiss Target Prediction Database (<http://www.swisstargetprediction.ch/>).