

Supporting Information

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Transforming Growth Factor Beta Promotes Inflammation and Tumorigenesis in Smad4-Deficient Intestinal Epithelium in a YAP-Dependent Manner

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Supplementary information

TGF- β promotes inflammation and tumorigenesis in Smad4-deficient intestinal epithelium in a Yap-dependent manner

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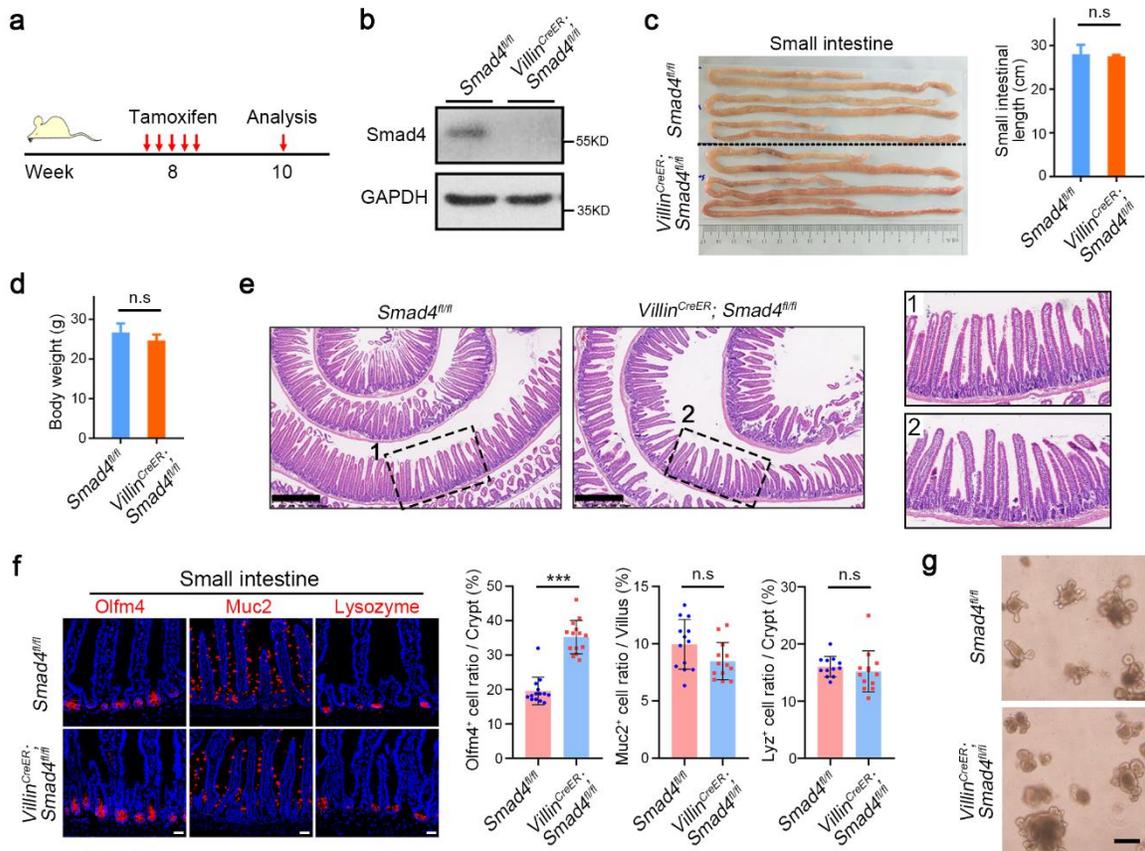


Figure S1. Smad4 deletion in the intestinal epithelium imposes no overt gross phenotypes.

(a) Scheme of tamoxifen-induced gene knockout.

(b) Anti-Smad4 immunoblotting to verify Smad4 expression in crypts derived from *Smad4^{fl/fl}* and *Villin^{CreER};Smad4^{fl/fl}* mice.

(c) Representative images of the small intestine from epithelial *Smad4*-KO and control littermates, with quantification of the colon length (right). n=3 mice for each group.

(d) Quantification of the body weight in epithelial *Smad4*-KO and control littermates. n=3 mice for each group.

(e) Representative images of H&E-stained small intestine sections and enlarged field of the indicated sections (right). n=3 mice for each genotype. Scale bars: 625 μ m.

(f) Immunofluorescence showing the expression of stem cells (Olfm4), goblet cells (Muc2), and Paneth cells (lysozyme) in small intestinal sections, with quantification of the ratios of these cells in the villus-crypt zone. Scale bars: 50 μ m.

(g) Organoids derived from the small intestine of control littermate *Smad4^{fl/fl}* and *Villin^{CreER};Smad4^{fl/fl}* mice. Scale bars: 100 μ m.

Data are presented as means \pm SD with statistical analyses determined by two-tailed Student's *t*-test. *** $p < 0.001$, n.s, no significance.

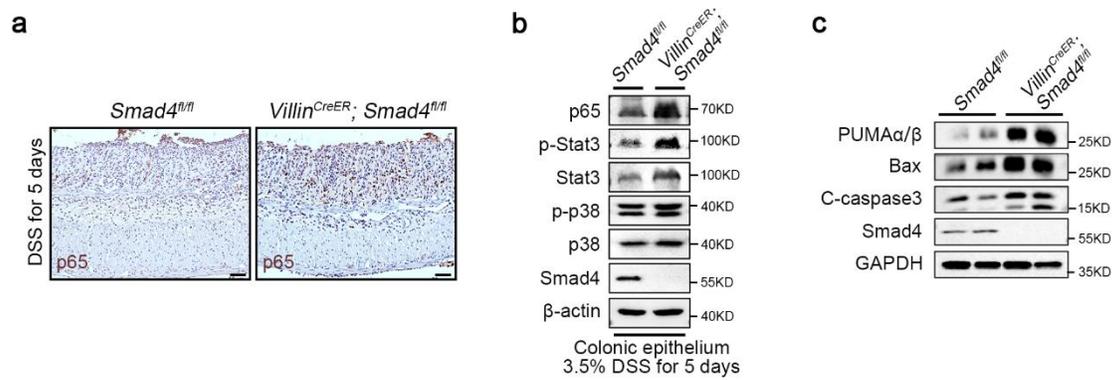


Figure S2. Smad4 loss exacerbates DSS-induced inflammation and disruption of the epithelial barrier.

(a) Anti-p65 immunostaining of colon sections from the indicated mice treated with DSS for five days. Scale bar: 50 μ m

(b) Immunoblotting of colonic epithelium lysates derived from indicated mice after 3.5% DSS treatment for 5 days.

(c) Immunoblotting of proliferation- and apoptosis-related proteins in the colonic epithelium from the indicated mice after DSS treatment. n=2 mice for each genotype.

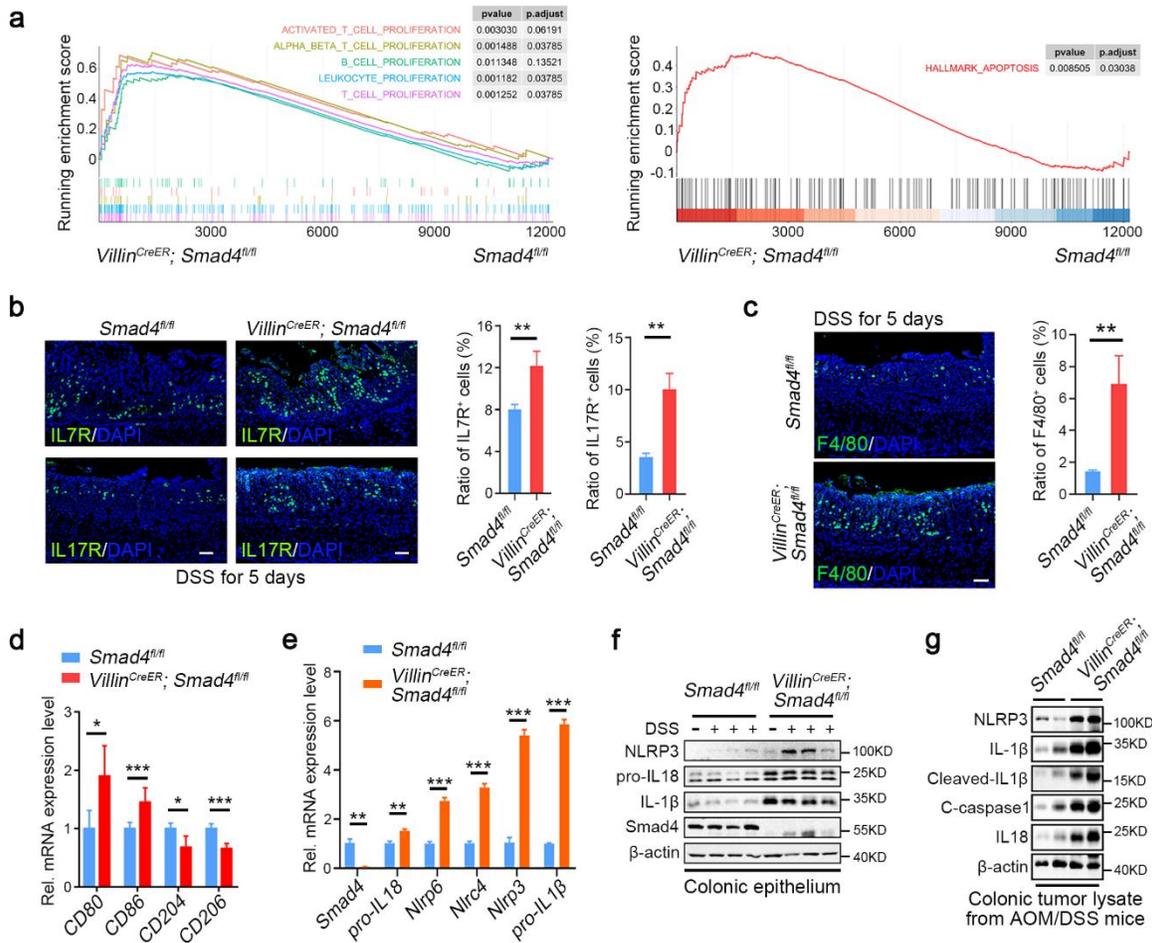


Figure S3. Smad4 deficiency augments immune response.

(a) GSEA analysis of the genes related to immune cell proliferation and epithelial cell apoptosis in littermate control *Smad4^{fl/fl}* and *Villin^{CreER};Smad4^{fl/fl}* mice upon DSS-induced colitis.

(b) Immunofluorescence staining of IL-7R and IL-17R in the distal colon derived from DSS-treated mice and statistical analysis of the cell percentage shown in the right (n=3). Scale bar: 50 μm.

(c) Immunofluorescence and quantification analysis of F4/80 in the colon sections derived from *Smad4^{fl/fl}* and *Villin^{CreER};Smad4^{fl/fl}* mice following DSS treatment for 5 days. n=3. Scale bar: 50 μm.

(d) RT-qPCR analysis of the markers of M1- and M2-macrophages in colonic tissue derived from *Smad4^{fl/fl}* and *Villin^{CreER};Smad4^{fl/fl}* mice following DSS treatment for 5 days. n=3 mice for each genotype.

(e) RT-qPCR analysis of the indicated inflammasome genes in the colonic epithelium from DSS-treated mice. n=3 mice for each genotype.

(f) Immunoblotting of colonic epithelium lysates from the indicated mice with or without DSS treatment. (n=4 mice for each group).

(g) Immunoblotting of colonic tumor lysates from the indicated mice challenged with AOM/DSS. (n=2 mice for each group).

Data are presented as means \pm SD. Statistical significance is determined by unpaired, two-tailed Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

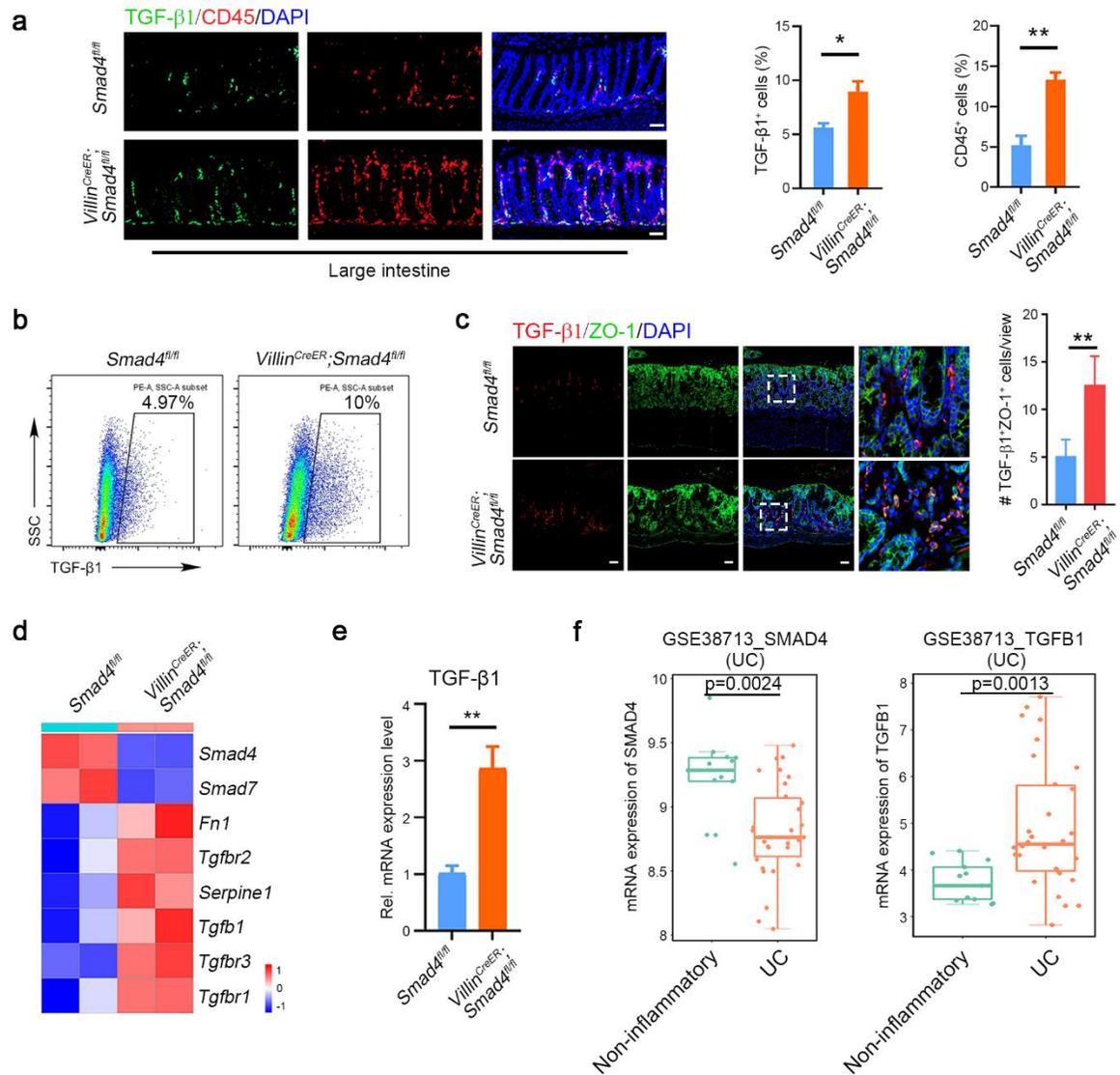


Figure S4. TGF- β is upregulated in *Smad4*^{-/-} colon.

(a) Immunofluorescence staining of colon sections with anti-CD45 and anti-TGF- β 1 antibodies from the indicated adult mice. Right panel shows the quantification of TGF- β 1⁺ and CD45⁺ cells. n=3 mice per group. Scale bar: 50 μ m.

(b) FACS analysis of colon-infiltrated TGF- β 1⁺ cells of DSS-treated littermate control *Smad4*^{fl/fl} and *Villin*^{CreER};*Smad4*^{fl/fl} mice. SSC, side scatter area.

(c) Immunofluorescence co-staining of colon sections with the antibodies against TGF- β 1 or tight junction protein ZO-1, and statistics is shown on the right.

(d) Heatmap of TGF- β target genes in the indicated mice after DSS treatment. n=2 mice per group.

(e) RT-qPCR analysis of TGF- β 1 expression in the indicated mice after DSS treatment. n=3 biological replicates.

(f) Box plots for *SMAD4* and *TGFB1* expression from the datasets of intestinal biopsies from UC and non-inflammatory individuals (GSE38713).

Data are presented as means \pm SD. Statistical significance is determined by unpaired, two-tailed Student's *t*-test. * $p < 0.05$, ** $p < 0.01$,

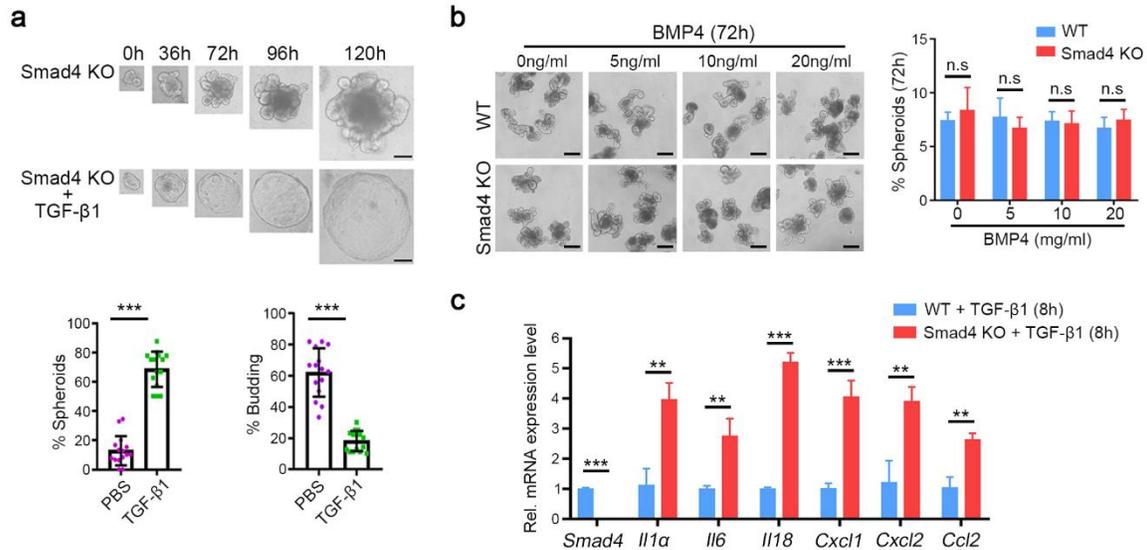


Figure S5. TGF- β induces spheroids and evokes an epithelial-intrinsic immune response in *Smad4*^{-/-} organoids.

(a) Morphology of *Smad4*-deficient organoids with or without 0.5 nM TGF- β 1 treatment. Quantification of spheroids and budding organoids is evaluated (lower panel). Scale bar: 100 μ m.

(b) Morphology of WT and *Smad4*-deficient organoids after stimulating with different concentrations of BMP4. Quantification of the ratio of spheroids is shown on the right. Scale bar: 100 μ m.

(c) RT-qPCR analysis showing gene expression of proinflammatory cytokines and chemokines in the indicated intestinal organoids with TGF- β 1 treatment for 8 hours. Data are presented as means \pm SD. Statistical significance is determined by unpaired, two-tailed Student's *t*-test. ** p < 0.01, *** p < 0.001, n.s., no significance.

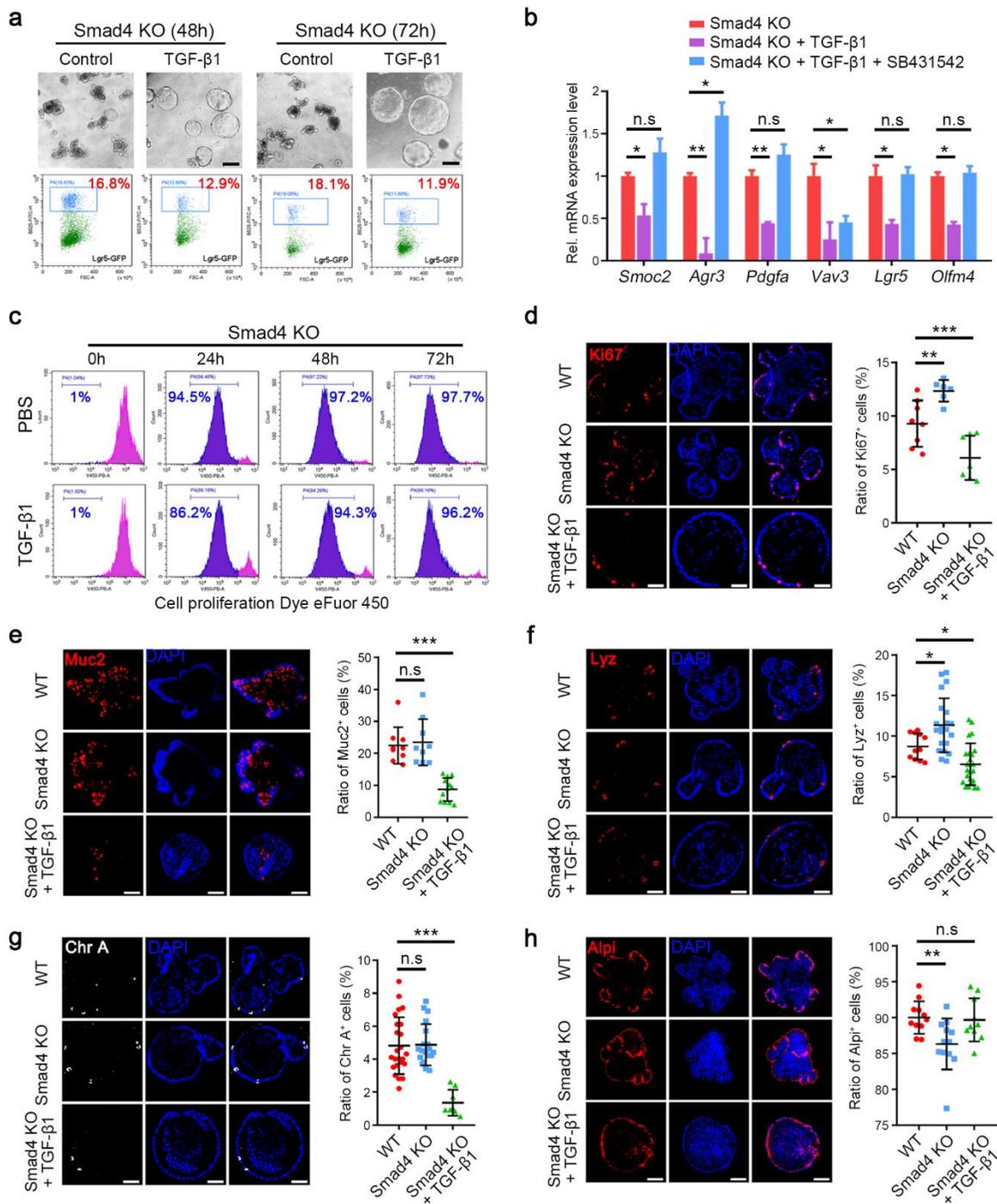


Figure S6. TGF- β 1 impairs ISC proliferation and differentiation in Smad4-deficient organoids.

(a) Morphological change and FACS analysis of Lgr5-GFP⁺ cells in Smad4-deficient organoids with or without 0.5 nM TGF- β 1 stimulation. Scale bar: 100 μ m.

(b) mRNA expression level of intestinal stem cell markers in the organoids with or without TGF- β 1 and TGF β RI inhibitor (SB431542, 10 μ M) treatment. n=3 biological replicates.

(c) Flow cytometric analysis of cell division upon TGF- β 1 stimulation in *Smad4*-deficient organoids using eFlour 450.

(d) Ki67 immunostaining of the indicated organoids and quantification of the Ki67 positive cell. Scale bar: 100 μ m.

(e) Mucin 2 (Muc2) immunostaining and quantification of Muc2⁺ cells. Scale bar: 100 μ m.

(f) Lysozyme (Lyz) immunostaining and quantification of Lyz⁺ cells. Scale bar: 100 μ m.

(g) Immunostaining of Chromogranin A (Chr A) counterstained with DAPI. Right panel shows the quantification of ChrA⁺ cells. Scale bar: 100 μ m.

(h) Alkaline phosphatase (Alpi; Absorptive cells) staining of the indicated organoids with quantification shown in the right. Scale bar: 100 μ m.

Data are presented as mean \pm S.D. Statistical significance is determined by unpaired, two-tailed Student's *t*-test. * p < 0.05, ** p < 0.01, *** p < 0.001, n.s, no significance.

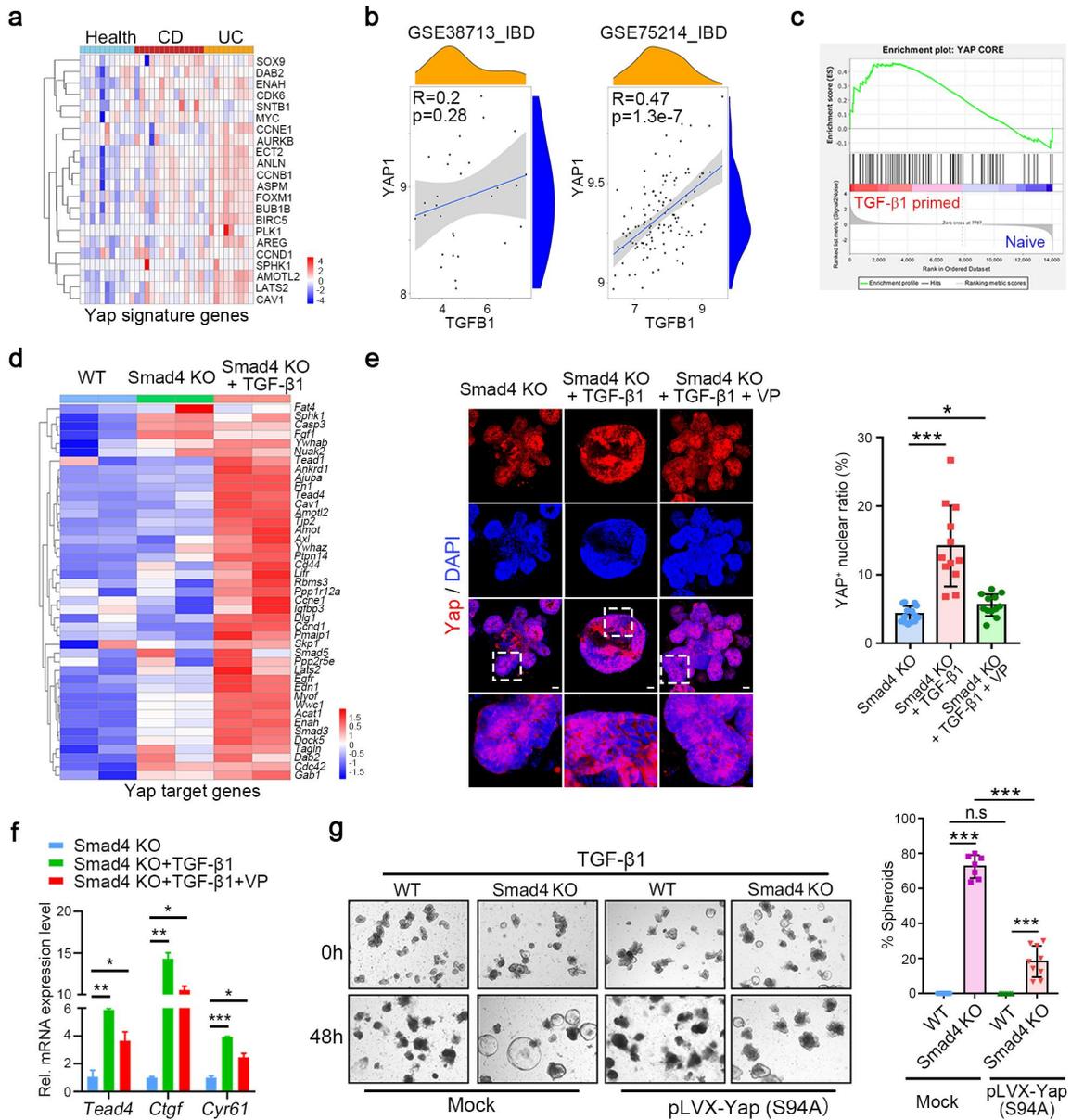


Figure S7. YAP mediates TGF- β effects in Smad4-deficient organoids.

(a) Heatmap of the YAP signature genes in IBD patients based on the dataset GSE10616.

(b) *In silico* analysis of the indicated online database for *YAP1* and its correlation with *TGFB1* expression in human IBD patients.

(c) GSEA of the YAP signature genes from the RNA-seq data derived from *Smad4*-deficient organoids with or without 0.5 nM TGF- β 1 treatment.

(d) Heatmap of the YAP target genes in the indicated organoids with or without TGF- β 1 stimulation. n=2 biological replicates for each group.

(e) Immunostaining of YAP (red) indicating its subcellular distribution in indicated organoids with quantification shown on the right. Scale bars: 50 μ m.

(f) RT-qPCR analysis of the expression of YAP target genes in indicated organoids with or without TGF- β 1 and VP treatment.

(g) Bright field images of WT and *Smad4*-deficient organoids with/without YAP(S94A) overexpression after TGF- β 1 treatment. Quantification is shown on the right. n=3 biological replicates for each timepoint. Scale bar: 50 μ m.

Data are presented as means \pm S.D. Statistical significance is determined by unpaired, two-tailed Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s, no significance.

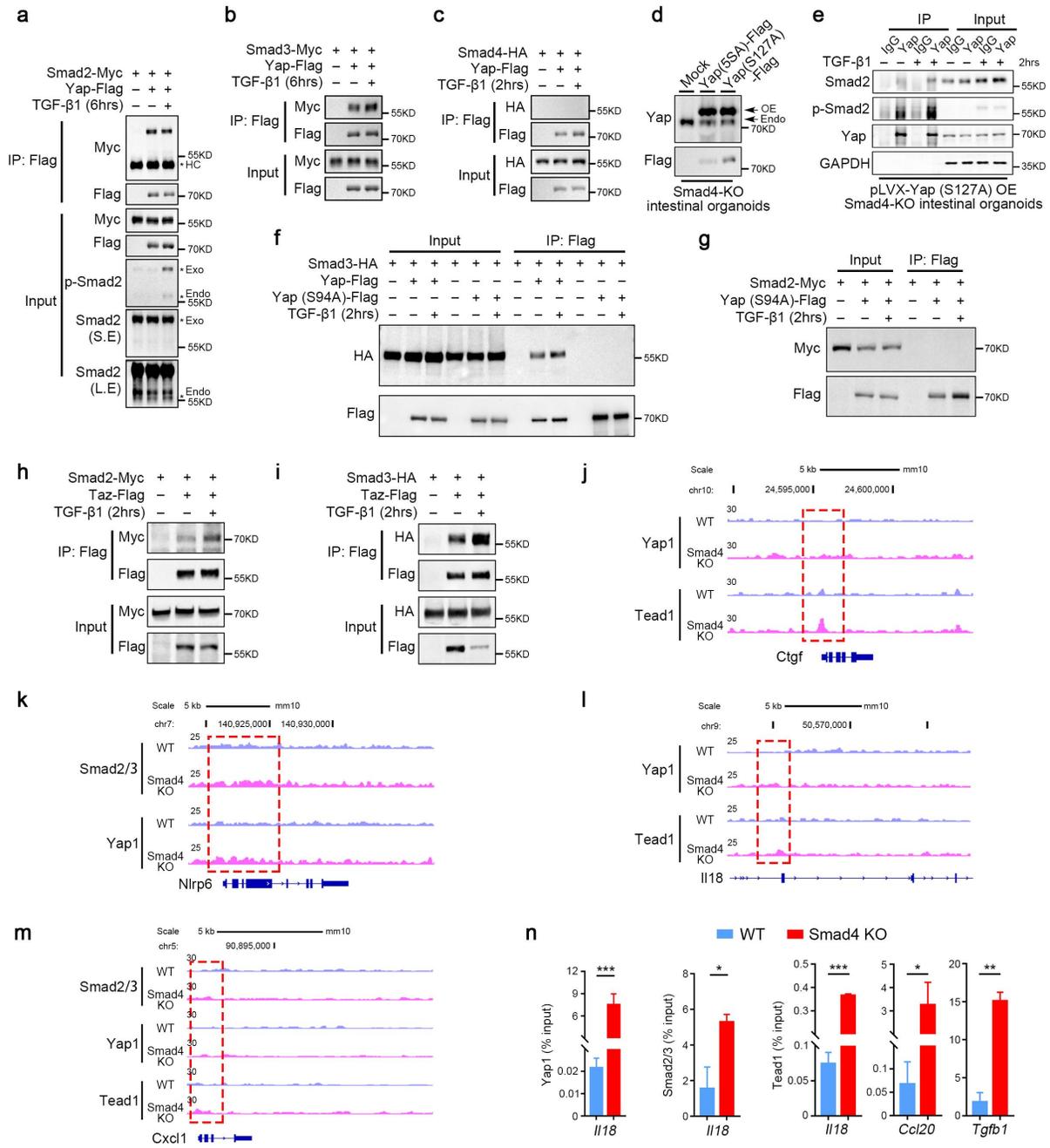


Figure S8. TGF-β enhances the interaction between YAP/TAZ and Smad2/3.

(a, b, c) Co-immunoprecipitation (IP) showing the interaction between overexpressed Yap and Smad2/3/4 in HEK-293T cells with or without 0.5 nM TGF-β1 stimulation.

(d) Immunoblotting showing YAP(5SA) and YAP(S127A) expression in *Smad4*^{-/-} intestinal organoids.

(e) Co-IP analysis of the interaction between overexpressed YAP(S127A) and Smad2 with or without 0.5 nM TGF-β1 treatment in *Smad4* KO organoids.

(f, g) Co-IP showing that Smad2/3 did not interact with YAP(S94A).

(h, i) Exogenous TAZ interacted with Smad2/3, and its interaction was promoted by 0.5 nM TGF- β 1 treatment.

(j, k, l, m) Genomic views of Yap1, Smad2/3 and Tead1 ChIP enrichment in the indicated genes in TGF- β 1-stimulated organoids.

(n) ChIP-qPCR showing Yap1, Smad2/3 and Tead1 occupancy in the indicated genes from colonic epithelium.

Data are presented as means \pm S.D. Statistical significance is determined by unpaired, two-tailed Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

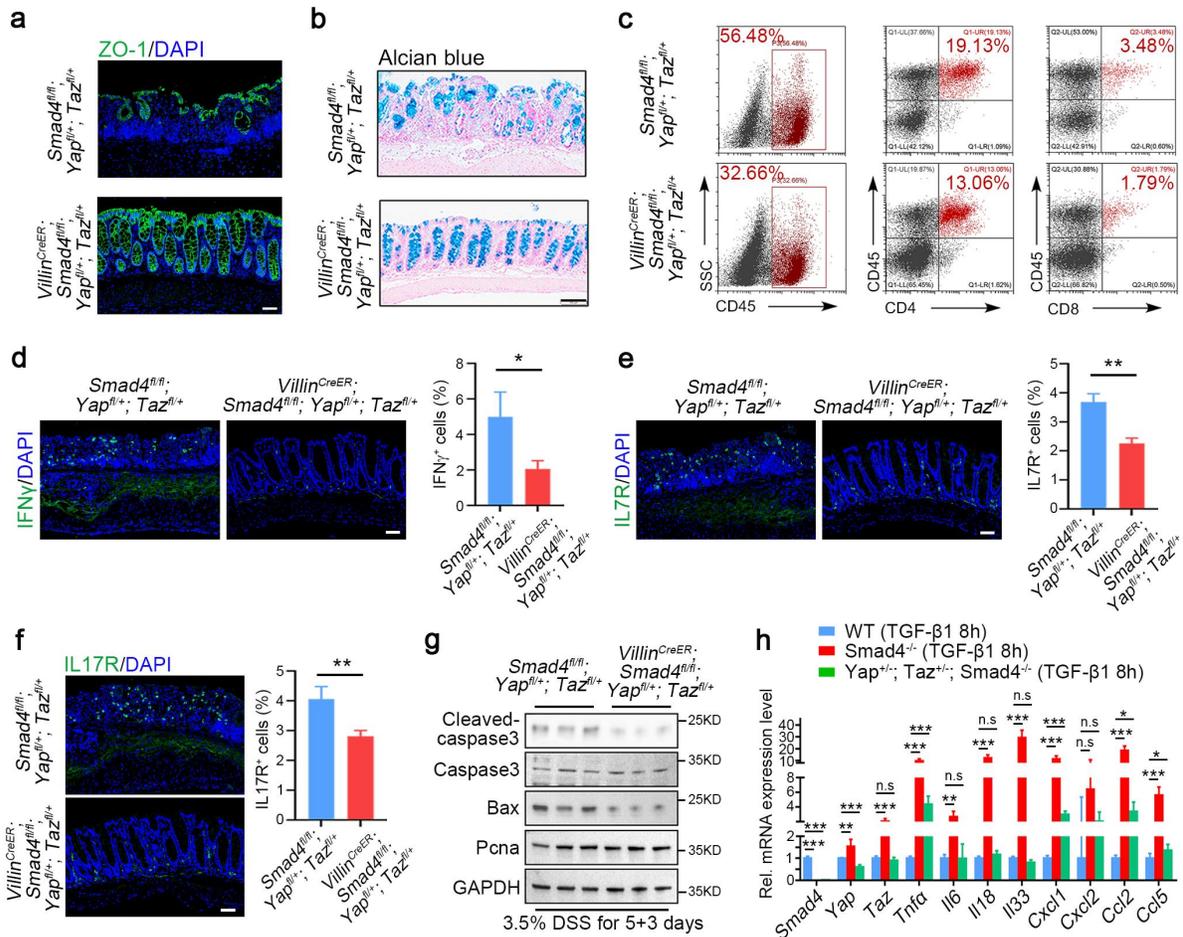


Figure S9. Reducing YAP/TAZ expression attenuates inflammation in *Smad4*^{-/-} intestine.

(a) ZO-1 immunostaining in the colon of the indicated mice after DSS treatment. n=3 mice each genotype. Scale bars: 50 μ m.

(b) Alcian Blue-Periodic acid Schiff (AB-PAS) staining of the colon sections from DSS-treated mice. n=3 mice each genotype. Scale bars: 100 μ m.

(c) Flow cytometric analysis of the colon-infiltrated immune cells from the indicated mice on day 5 after DSS treatment. Data are presented as representative plots.

(d, e, f) Immunostaining and quantification of interferon γ ⁺ (IFN γ ⁺) cells (d), IL7R⁺ cells (e), and IL17R⁺ cells (f) in the colon of the indicated mice following 5 days of DSS treatment. n=3 mice each genotype. Scale bars: 50 μ m.

(g) Immunoblotting of apoptosis- and proliferation-related genes in colonic epithelium derived from control and *Smad4*^{-/-}/*Yap*^{+/-}/*Taz*^{+/-} mice after DSS treatment. n=3 mice each genotype.

(h) RT-qPCR analysis of the expression of proinflammatory cytokines and chemokines in the intestinal organoids with TGF- β 1 treatment for 8 hours.

Data are presented as means \pm S.D. Statistical significance is determined by unpaired, two-tailed Student's *t*-test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, n.s, no significance.

Table S1. Key resources

REAGENT	SOURCE	IDENTIFIER
Antibodies		
IL17R	Santa Cruz	sc-376374
Smad4	Santa Cruz	sc-7966
F4/80	Santa Cruz	sc-377009
ZO-1	Santa Cruz	sc-33725
IFN γ	Santa Cruz	sc-8423
IL7R	Santa Cruz	sc-514445
Pcna	Santa Cruz	sc-56
Bax	Santa Cruz	sc-7480
PUMA α/β	Santa Cruz	sc-374223
Smad2/3	Abcam	Ab202445
Smad2/3	CST	8685
p-Smad2	CST	18338
IL-1 β	CST	31202
Cleaved IL-1 β	CST	63124
NLRP3	CST	15101
Caspase-1	CST	24232
Cleaved caspase-1	CST	89332
AIM2	CST	63660
ASC/TMS1	CST	67824
YAP	CST	14074
YAP1	Novus	NB110-58358SS
YAP/TAZ	CST	93622
Olfm4	CST	39141
Caspase3	CST	9662
Cleaved Caspase3	CST	9664
p65	CST	8242
Stat3	CST	9139
p-Stat3	CST	9145
p38	CST	8690
p-p38	CST	4511
Anti-FLAG	CST	14793
Anti-HA	CST	3724
Anti-Myc	CST	2276
TEAD1	ABclonal	A5218
IL-18	Proteintech	10663-1-AP
β -tubulin	Proteintech	10094-1-AP
β -actin	Abcam	ab8226
CD45	Abcam	ab40763
MUC2	Abcam	ab272692
Lysozyme	Abcam	ab108508
GAPDH	Abcam	ab8245

Ki67	Abcam	ab15580
TGF- β 1	This paper	Chen lab
Chemicals and Reagents		
Dextran Sulfate Sodium, DSS	MP Biomedicals	9011-18-1
Azoxymethane, AOM	Sigma-Aldrich	A5486
Trizol	Thermo Fisher	15596026
HiScript II Q Select RT SuperMix	Vazyme	R232-01
ChamQ SYBR Color qPCR Master Mix	Vazyme	Q431-02
TrypLE	Invitrogen	12604021
4',6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich	28718-90-3
Antifade Mounting Medium	Invitrogen	P36930
in situ cell death detection kit	Roche	12156792910
Protease inhibitor Cocktail	Biotool	B14001
DAB	Sangon Biotech	DB0140
EDTA Antigen Retrieval	Sangon Biotech	E673003
Opti-MEM	Gibco	31985-070
Advanced DMEM/F-12	Gibco	12634028
N-2	Gibco	A1370701
B-27	Gibco	17504044
Matrigel	R&D	BME001-05
GlutaMAX	Gibco	35050061

Table S2. List of Primer Pairs Used for Quantitative Polymerase Chain Reaction Analysis in this Study

Gene	Forward	Reverse
<i>Il6</i>	GCCAGAGTCCTTCAGAGA GA	GGTCTTGGTCCTTAGCC ACT
<i>Tnfa</i>	CCCTCACACTCAGATCATC TTCT	GCTACGACGTGGGCTAC AG
<i>Il-1b</i>	TACCTGTGTCTTTCCCGTG G	TTGTTTTCATCTCGGAGCC TGT
<i>Tgfβ1</i>	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTTGGACAGG ATCTG
<i>Il18</i>	GTCTACCCTCTCCTGTAAG AACA	TGGCAAGCAAGAAAGTG TCC
<i>Nlrp6</i>	CACACCCAGAATGAGACCA G	GTAGCCATAAGCAGCTC CCT
<i>Nlrc4</i>	CTACATTGATGCTGCCTTG G	ATCCGTCACTGCTCACA CAG
<i>Nlrp3</i>	ATTACCCGCCCGAGAAAG G	TCGCAGCAAAGATCCAC ACAG
<i>Smad4</i>	CAGCCATAGTGAAGGACTG TTGC	CCTACTTCCAGTCCAGG TGGTA
<i>Tead4</i>	GCTCTGGATGTTGGAGTTC TCG	TTGGGCTTGACTGGCTG ATGTG
<i>Ctgf</i>	GGGCCTTCTGCGATTTC	ATCCAGGCAAGTGCATT GGTA
<i>Cyr61</i>	CTGCGCTAAACAACACTCAAC GA	GCAGATCCCTTTCAGAG CGG
<i>Agr2</i>	GCAGTTTGTTCCTCAAC CTGG	GTATCGTCCAGTGATGT CTGCC
<i>Muc2</i>	CTACCACCATTACCACCAC TAC	GTCTCTCGATCACCACC ATTT
<i>Tff3</i>	TCCAAGCCAATGTATGGTG CCG	CAGGGCACATTTGGGAT ACTGG
<i>Mmp7</i>	AGGTGTGGAGTGCCAGAT GTTG	CCACTACGATCCGAGGT AAGTC

<i>Lyz1</i>	ACGAGCTACAAACTACAAC CG	GATCTCTCACCACCCTC TTTG
<i>Reg4</i>	CTGGCTATCAGAGAAACCT GCC	CTGGCTTCACTCTTTGTC CTGG
<i>Lct</i>	ACGCTGGATGACACGGAA AGGA	CCACTCGAAGTTGTCCA TCAGC
<i>Alpi</i>	GTCCCACCGCTGGTTACTT T	CTGTGGGCTGAGATGAT GTC
<i>Cbr1</i>	CCTTCCACATTCAAGCAGA GGTG	CTGAGACTCACCATGCT GGACA
<i>Smoc2</i>	GCCAAGTGCAAAGATCCAC AGC	ACACTTGCTGGA ACTCC TTCCG
<i>Agr3</i>	TTCACCACCTGGAGGACTG TCA	GACAAGTTCTTGTCCGT GGTCTC
<i>Pdgfa</i>	CTGGCTCGAAGTCAGATCC ACA	GACTTGTCTCCAAGGCA TCCTC
<i>Vav3</i>	GACCAATGGACTTCGGAGA GCT	CTGCCTGGATATGCAAT GGTGG
<i>Lgr5</i>	CGTAGGCAACCCTTCTCTT ATC	GCACCATTCAAAGTCAG TGTTT
<i>Olfm4</i>	CGAGACTATCGGATTCGCT ATG	TTGTAGGCAGCCAGAGG GAG
<i>pro-IL-1b</i>	CTCCATGAGCTTTGTACAA GG	TGCTGATGTACCAGTTG GGG
<i>pro-IL18</i>	ACTGTACAACCGCAGTAAT ACGG	AGTGAACATTACAGATTT ATCCC
<i>GAPDH</i>	AGGTCGGTGTGAACGGATT TG	TGTAGACCATGTAGTTG AGGTCA
<i>CD80</i>	CCTCAAGTTTCCATGTCCA AGGC	GAGGAGAGTTGTAACGG CAAGG
<i>CD86</i>	ACGTATTGGAAGGAGATTA CAGCT	TCTGTCAGCGTTACTATC CCGC
<i>CD204</i>	CGCACGTTCAATGACAGCA TCC	GCAAACACAAGGAGGTA GAGAGC
<i>CD206</i>	GTTCACCTGGAGTGATGGT TCTC	AGGACATGCCAGGGTCA CCTTT

<i>Il1α</i>	ACGGCTGAGTTTCAGTGAG ACC	CACTCTGGTAGGTGTAA GGTGC
<i>Cxcl1</i>	TCCAGAGCTTGAAGGTGTT GCC	AACCAAGGGAGCTTCAG GGTCA
<i>Cxcl2</i>	CATCCAGAGCTTGAGTGTG ACG	GGCTTCAGGGTCAAGGC AAACT
<i>Ccl2</i>	GCTACAAGAGGATCACCAG CAG	GTCTGGACCCATTCTT CTTGG
<i>Yap</i>	CCAGACGACTTCCTCAACA GTG	GCATCTCCTTCCAGTGT GCCAA
<i>Taz</i>	GTCACCAACAGTAGCTCAG ATCC	GTTGCTGAGGAAGTCTT CTGGAG
<i>Il33</i>	CTACTGCATGAGACTCCGT TCTG	AGAATCCCGTGGATAGG CAGAG
<i>Ccl5</i>	CCTGCTGCTTTGCCTACCT CTC	ACACACTTGCGGTTCC TTCGA

Table S3. List of Primer Pairs Used for Chromatin Immunoprecipitation-Quantitative Polymerase Chain Reaction Analysis in this Study

Gene	Forward	Reverse
<i>Yap1</i>	GCTTGAAGAAGGAGTCGG GC	CTTCAATGCCGTCATGAA CCC
<i>Il18</i>	GTATCACAGGCACAGCTGG A	CTGATGCTGGAGGTTGC AGA
<i>Ccl20</i>	TTGTGGTGACAGGATGAGG C	GGGAATGTACACAAGAA GGCG
<i>Tgfb1</i>	CCTCTTGAGTCCCTCGCAT C	GTGAGGCTCTGACACCA AGG

Data files S1. Gene expression of colonic epithelial cells upon colitis by RNA-Seq analysis.

Data files S2. Gene expression by RNA-seq in small intestinal organoids.