Supplementary Materials & Methods

Tissue preparation & Antibodies staining

Intestine was flushed with cold PBS and Formalin 10%, then arranged into Swiss Roll by rolling from distal to proximal. This ensured that the duodenum and Ileum were located at the outermost and the inner layer of the roll respectively (Figure S1B). Samples were fixed in 10% formalin for 2 hours and then transferred into 15% sucrose/PBS solution and incubated overnight at 4 C°. Next, samples were transferred into 30% sucrose/PBS solution and incubated again overnight at 4 C°. Afterwards, fixed samples were embedded in OCT and kept at -80 C°.

Immunohistochemistry staining was performed with a Dako Autostainer. Antigen retrieval was performed by boiling in citrate solution (pH 6.1) for 20 minutes. Ki67 (Thermo Fisher Scientific, Cat. #RM-9106-S), cleaved caspase 3 (Cell Signaling, Cat. #9664S), and p-ERK1/2 (Cell Signaling, Cat. #4370) antibodies were used at dilution of 1:400, 1:800 and 1:400, respectively.

The IHC samples were scored blinded by a pathologist (AH) based on following criteria. Throughout the analysis, the outermost layer of intestine was considered duodenum, the three innermost layers were considered as ileum, and the part between duodenum and ileum was considered as jejunum. Based on these criteria, more than 70% of intestine was considered as jejunum. The p-ERK1/2 staining was called positive if there was any cytoplasmic, nuclear or both nuclear/cytoplasmic staining between 10 total cells in crypt base. The 10 crypt

base cells were defined based on cell position 0 (Figure S1C). Any cytoplasmic and nuclear staining was measured as positive staining for cleaved-caspase 3 and Ki67 respectively. Overall, 20 crypts were counted for each part of small intestine for individual mouse.

Quantitative RT-PCR

RNAlater (Thermo Fisher, Cat. #AM7021) was used for stabilization of intestinal samples. The RNA extraction was done based on the protocol of the RNeasy Mini Kit from Qiagen (Cat. #74104). Next, iScriptTM cDNA Synthesis Kit (Cat. #1708891) was used to make cDNA from RNA. Quantitative RT-PCR was performed on BioRad CFX384^{TM} Real-Time system with SsoFast™ EvaGreen® Supermix (Cat. #1725202). Analysis of gene expression was also performed with CFX384^{TM} Real Time software. Table S1 contains a list of primers and their sequences.

Table S1) qRT-PCR primers

Primer	Forward Primers	Reverse Primers
Chga	CAGCTCGTCCACTCTTTCCG	CCTCTCGTCTCCTTGGAGGG
Lyz	GGAATGGATGGCTACCGTGG	CATGCCACCCATGCTCGAAT
Alpi	AGGATCCATCTGTCCTTTGG	ACGTTGTATGTCTTGGACAG
Lrig	AAGGGAACTCAACTTGGCGAG	ACGTGAGGCCTTCAATCAGC
HopX	CATCCTTAGTCAGACGCGCA	AGGCAAGCCTTCTGACCGC
Bmi1	AATTAGTCCCAGGGCTTTTCAA	TCTTCTCCTCATCTGCAACTTCTC
Lgr5	ACCCGCCAGTCTCCTACATC	GCATCTAGGCGCAGGGATTG
Muc2	ACAAAAACCCCAGCAACAAG	GAGCAAGGGACTCTGGTCTG
PGK	TCAAAAGCGCACGTCTGCCG	AAGTCCACCCTCATCACGACCC
ASCL2	GCCTACTCGTCGGAGGAA	CCAACTGGAAAAGTCAAGCA
Olfm4	AGTGACCTTGTGCCTGCC	CACGCCACCATGACTACA

Supplemental Figure Legends

Figure S1. C59 induces proliferation in the intestinal crypt base.

A) Representative images of proliferation in duodenum and ileum of mice treated with one dose of vehicle or C59 (100 mg/kg) 24 hours prior to euthanasia. DAPI labeled nuclei of cells and proliferative cells were marked with EdU given 2 h prior to sacrifice. Arrows indicates EdU^+ cells in crypt base. Scale bar: 25 μm. Insets show higher magnification of representative crypts. Scale bar: 10 μm.

B) Schematic image of our Swiss Roll method illustrating how we defined segments of the small intestine for scoring.

C) Illustration of the counting bins used to quantitate EdU^+ cells. The crypt base cells were defined based on cell position 0.

D) Quantification of EdU^+ cells in crypt base after 3 days of treatment with vehicle or C59 (100mg/kg, once daily, QD). Graph represents quantification of $Ki67^+$ cells within total 10 counted cells in the crypt base. 20 crypts were counted for each part of the intestine per mouse (Vehicle, n= 4 mice. C59, n=4 mice), **$P<0.01$, ***$P<0.001$, Mann–Whitney U-test.

E) Quantification of cleaved-caspase 3 IHC in crypt base. Percentage of cleaved-caspase 3 negative cells in the bottom of crypts was graphed for three parts of small intestine. 20 crypts were counted for each part of the intestine per mouse (Vehicle, n=4 mice. C59, n=3 mice).

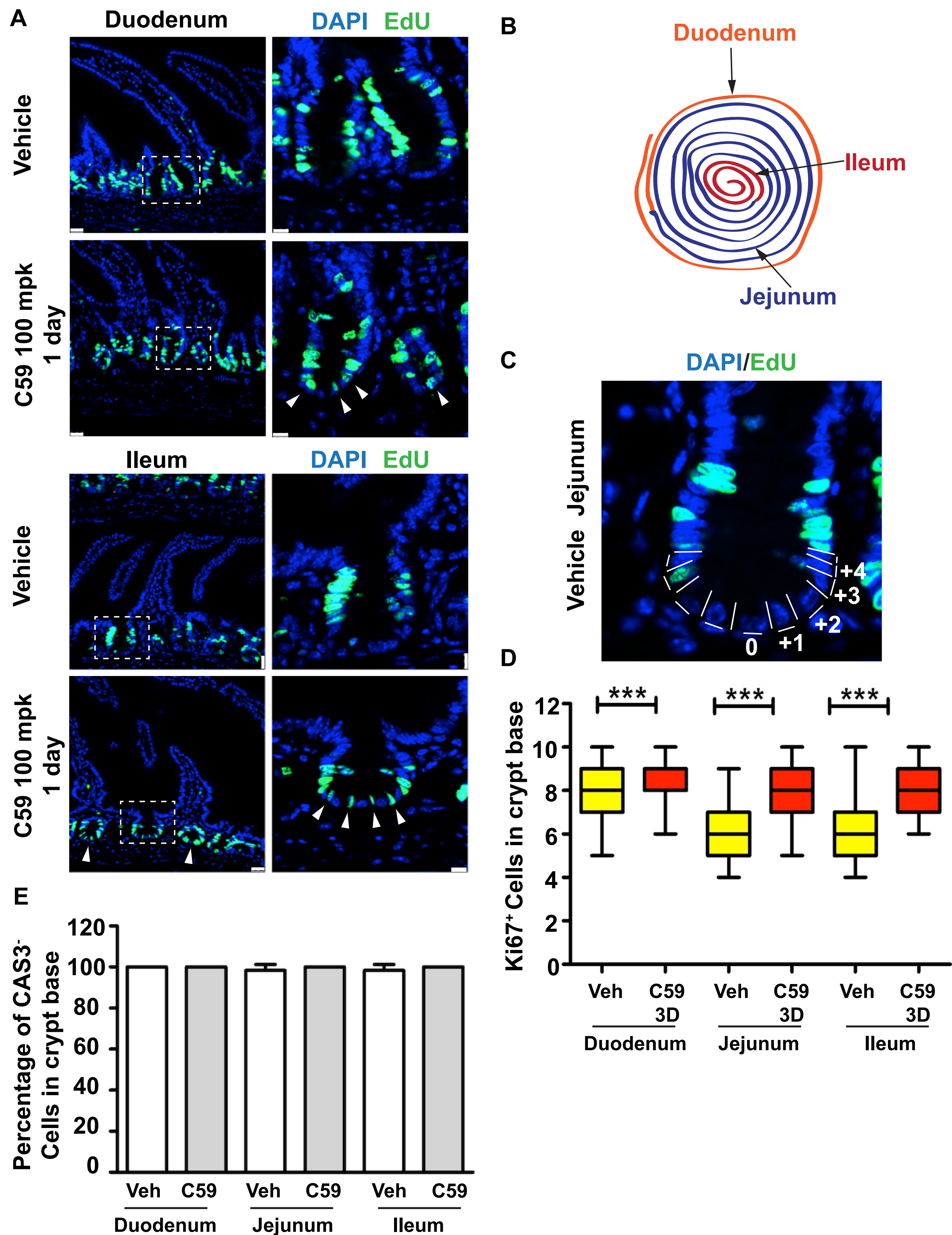

Figure S1

Figure S2. Lower doses of C59 also induce proliferation in the intestinal crypt base.

A) C59 treatment (50 mg/kg dose, BID or twice daily) induced intestinal stem cell proliferation starting the first day after treatment, followed by the disappearance of proliferating cells by the fourth day

B) Left panel shows representative images of jejunal samples after two days of vehicle or C59 administration (50 mg/kg, BID: twice daily). Quantification of EdU^+ cells in crypt base is presented in the right panel (3 mice per group).

C) Intestinal crypt proliferation was induced with lower doses of C59. Representative image of ileum samples is shown.

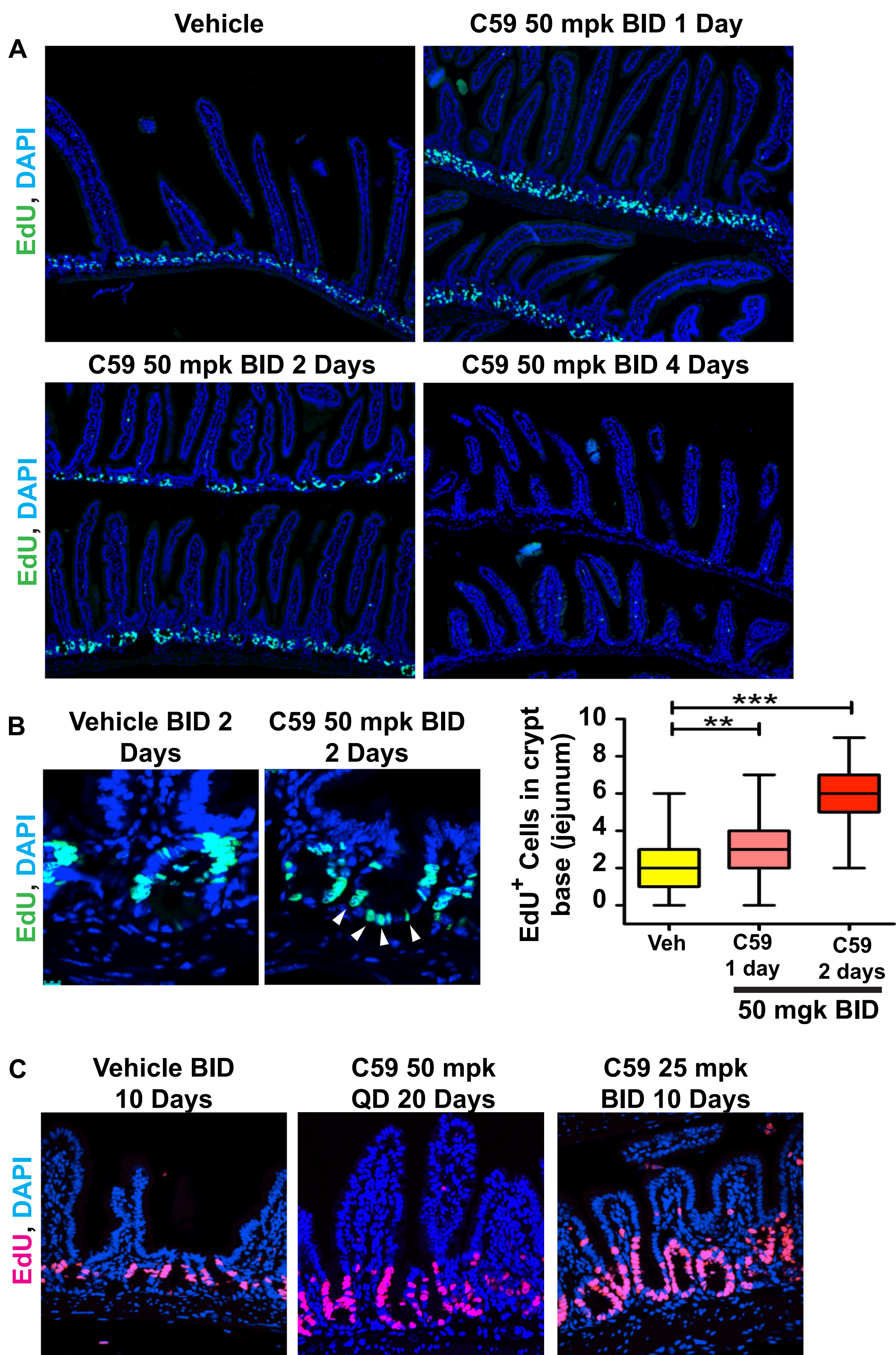

Figure S2

Figure S3. Intestinal stem cells lineage tracing in C59 treated mice.

A) Optimization of timing between tamoxifen injection and C59 gavage for Lgr5-*EGFP-IRES-CreER^{T2}/Rosa-LSL-tdTomato* mice. To label only Lgr5 stem cells, *Lgr5-EGFP-IRES-CreER^{T2}/Rosa-LSL-tdTomato* mouse was injected with one dose of tamoxifen and the intestine was harvested 24 hours later. The left panel shows EGFP color that only marks Lgr5 stem cells and the middle panel shows td-Tomato that demonstrates Cre activation-caused recombination by marking cells red. The right panel shows a merge between lineage traced (red) and stem cell marker (green). The arrow shows one single cell that is only td-tomato positive, but not EGPF positive, indicating the first intestinal progenitor cells were emerging in some crypts within 24 hours after Tamoxifen injection. Scale bar: 10 µm.

B) Optimization of timing between tamoxifen injection and C59 gavage for *Bmi1-CreER^{T2}/Rosa-LSL-tdTomato* mice. To mark *Bmi1* stem cells, *Bmi1-CreER^{T2}/Rosa-LSL-tdTomato* mouse was injected with one dose of tamoxifen and intestine was harvested 24 hours later. *Bmi-CreER^{T2}* not only marked stem cells in Crypts, but also some differentiated cells in villi.

C) Top panel displays quantification of *Lgr5* lineage tracing after 3 days of C59 (100 mg/kg) treatment. The red-labeled cells reaching to any part of villi were considered as a positive lineage trace in this treatment. About 10 crypt-villi were counted per each part of intestine for individual mouse (vehicle, n=4 mice; C59, n=4 mice, three experimental replicates). Lower panel displays a

quantification of *Lgr5* lineage tracing after 2 days of C59 (50 mg/kg BID) treatment. Any red cells reaching the intersection between crypt and villi were considered as a positive lineage trace. About 10 crypt-villi units were counted per each part of intestine for individual mouse (vehicle, n=3 mice; C59, n=4 mice; three experimental replicates).

D) Drug dosing protocol. *Bmi1-CreER^{T2}/Rosa-LSL-tdTomato* mice were treated with tamoxifen and C59 according to the timeline.

E) *Bmi1* lineage tracing was reduced in C59 treated (100 mg/kg QD) mice within 3 days of treatment compared to control mice. Representative images of lineage tracing in vehicle and C59 groups are shown.

F) Quantification of *Bmi1* lineage tracing in vehicle and C59 treated mice. Any crypt with two or more red cells out of 10 cells in crypt base was considered as positive for lineage tracing. At least 20 crypts were counted for each part of intestine per mouse. Graph presents the ratio of positive crypts as percentage from total crypts counted in vehicle- and C59- treated groups. Non-significant (NS), ***P<0.001, Mann–Whitney U-test.

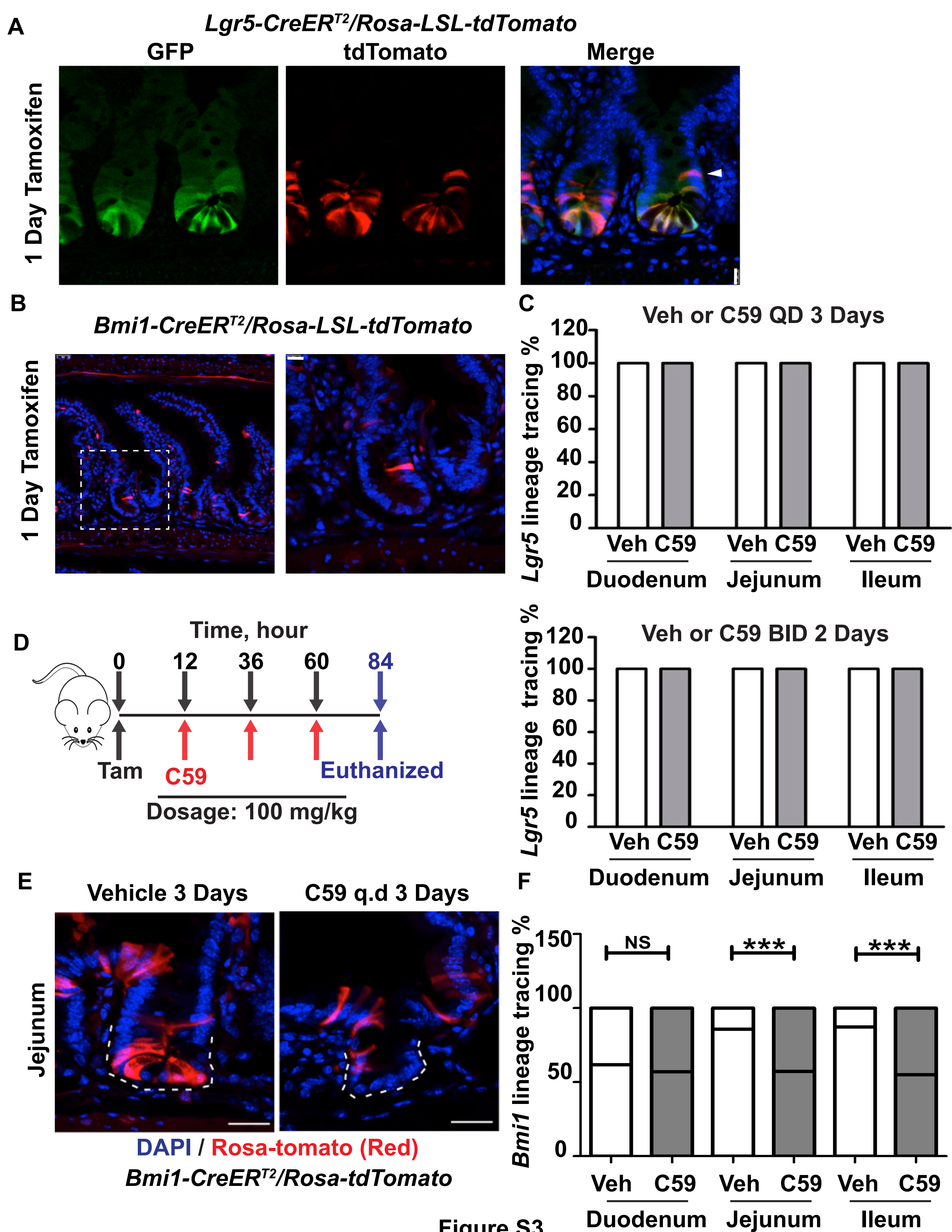

Figure S3

Figure S4. Short term PORCN inhibition does not impact intestinal epithelial cell differentiation.

A) *Lgr5* expression was decreased after C59 treatment, but the rebound of expression was seen around 24 hours after the first dose.

B) *Lgr5*+ intestinal stem cells are lost upon PORCN inhibition. To address the mosaic nature of *Lgr5-EGFP* expression, we used *Lgr5-EGFP-IRES-CreERT2/Rosa-LSL-tdTomato* mice following the twice-daily treatment schema shown in Fig 2C. In this approach, EGFP and EdU staining was examined in tdTomato positive crypts only. EdU staining in lineage traced mice showed that following C59 treatment, tdTomato positive cells no longer expressed EGFP (the marker for *Lgr5*+ intestinal stem cells) and instead their crypt base cells were positive for EdU, the marker for proliferation. Representative images of jejunal samples (vehicle, n=3 mice; C59, n=3 mice, three experimental replicates). Scale bar: 20 μm.

C) Mice were treated following two alternative protocols for C59 (+: 50 mg/kg, BID or ++: 100 mg/kg, QD) and qRT-PCR was performed. Graph shows expression of alkaline phosphatase (*Alpi*), chromogranin A (*Chga*), Mucin2 (*Muc2*), Lysozyme (*Lyz*) and Ki67 markers normalized to *Pgk* and Actin (*Actb*) expression. Each dot represents one mouse. Non-significant (NS), *P<0.05, Mann–Whitney U-test.

D) Increased p-ERK1/2 staining in base of crypts after C59 treatment. Representative images of jejunal samples with p-ERK1/2 staining. The boxed regions are shown in Figure 3B. Scale bar: 50 μm.

E) Trametinib treatment did not affect *Lgr5* stem cell numbers or their lineage tracing ability. EGFP and tdTomato expression was examined in vehicle and trametinib treated *Lgr5-EGFP-IRES-CreER^{T2}/Rosa-LSL-tdTomato* mice. Tamoxifen was injected 12 hours prior to starting trametinib 3 mg/kg/day daily for 4 consecutive days. Mice were euthanized 5 hours after the last treatment. n=3 mice per group. Scale bar: 50 µm.

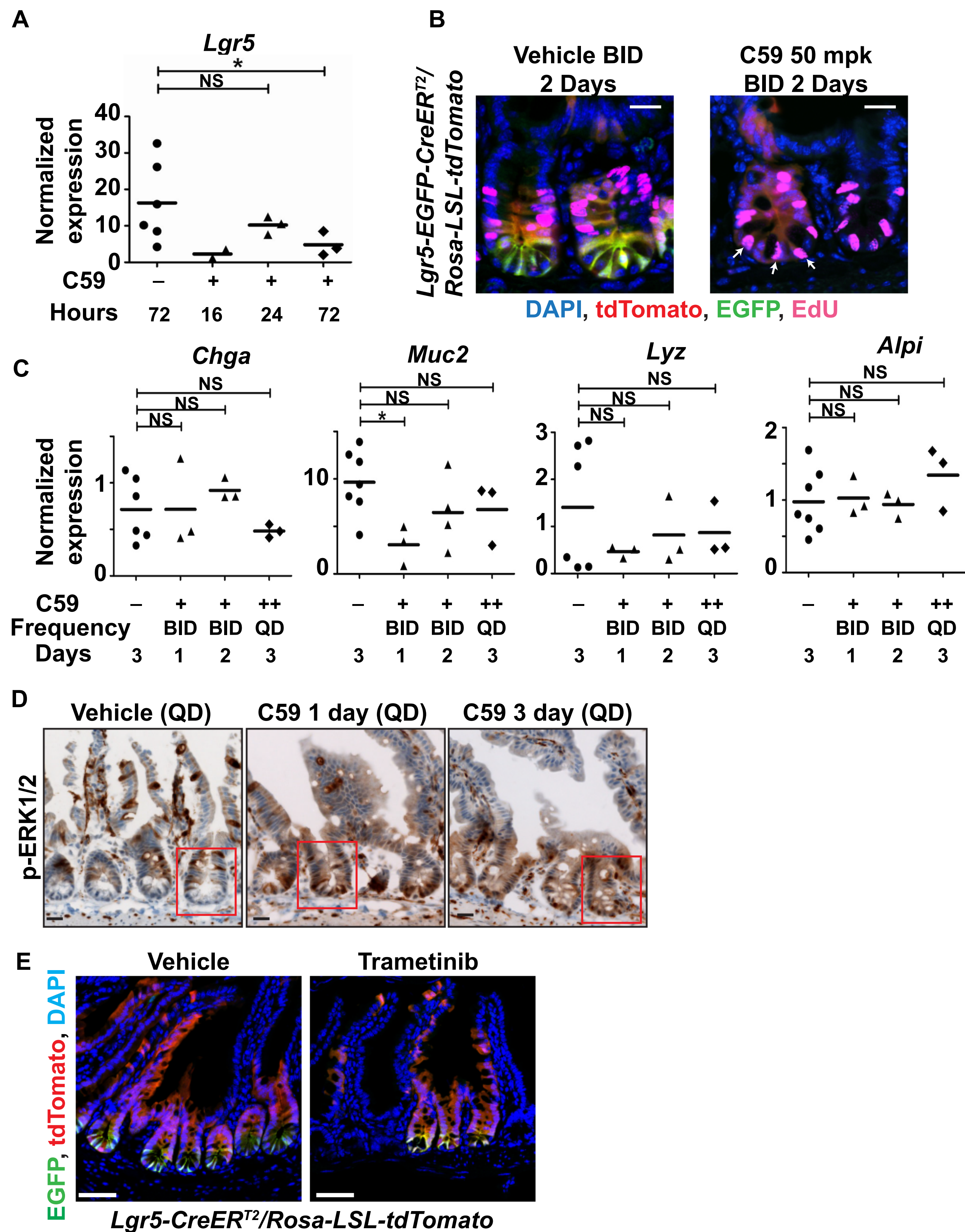

Figure S4

Figure S5. Trametinib suppressed MAPK signaling and blocked proliferation of intestinal stem cells in jejunum without causing toxicity at the crypt base.

A) Proliferation was decreased in trametinib + C59 treated mice. Graph presents total EdU^+ cells in jejunal crypt. NS, non-significant; *, $p<0.05$; ***, $p<0.001$, Kruskal Wallis One-Way ANOVA test.

B) C59 induced proliferation and impact of MAPK signaling inhibition in duodenum and ileum. Top and lower charts show quantification of EdU^+ in duodenum and ileum respectively. (Vehicle, n=7; trametinib plus vehicle, n=6; C59, n=8; trametinib plus C59, n= 6, two experimental replicates). NS, non-significant; **, $p<0.01$; ***, $p<0.001$, Kruskal Wallis One-Way ANOVA test.

C) Inhibition of p-ERK1/2 in intestinal samples after trametinib treatment. Representative images of p-ERK1/2 (left) and cleaved-cas 3 staining (right) in jejunum samples derived from C59 alone, trametinib plus vehicle and trametinib plus C59 treated mice. Red arrows indicate positive staining. Scale bar: 50 µm.

D) Apoptosis was not induced in the crypt base after trametinib + C59 combination therapy. Graph shows quantification of cleaved caspase 3 negative cells in the base of crypts. Trametinib plus vehicle, n=3; C59, n=4, trametinib plus C59, n= 3.

E) PORCN inhibition induced ISC proliferation through activation of MAPK signaling.

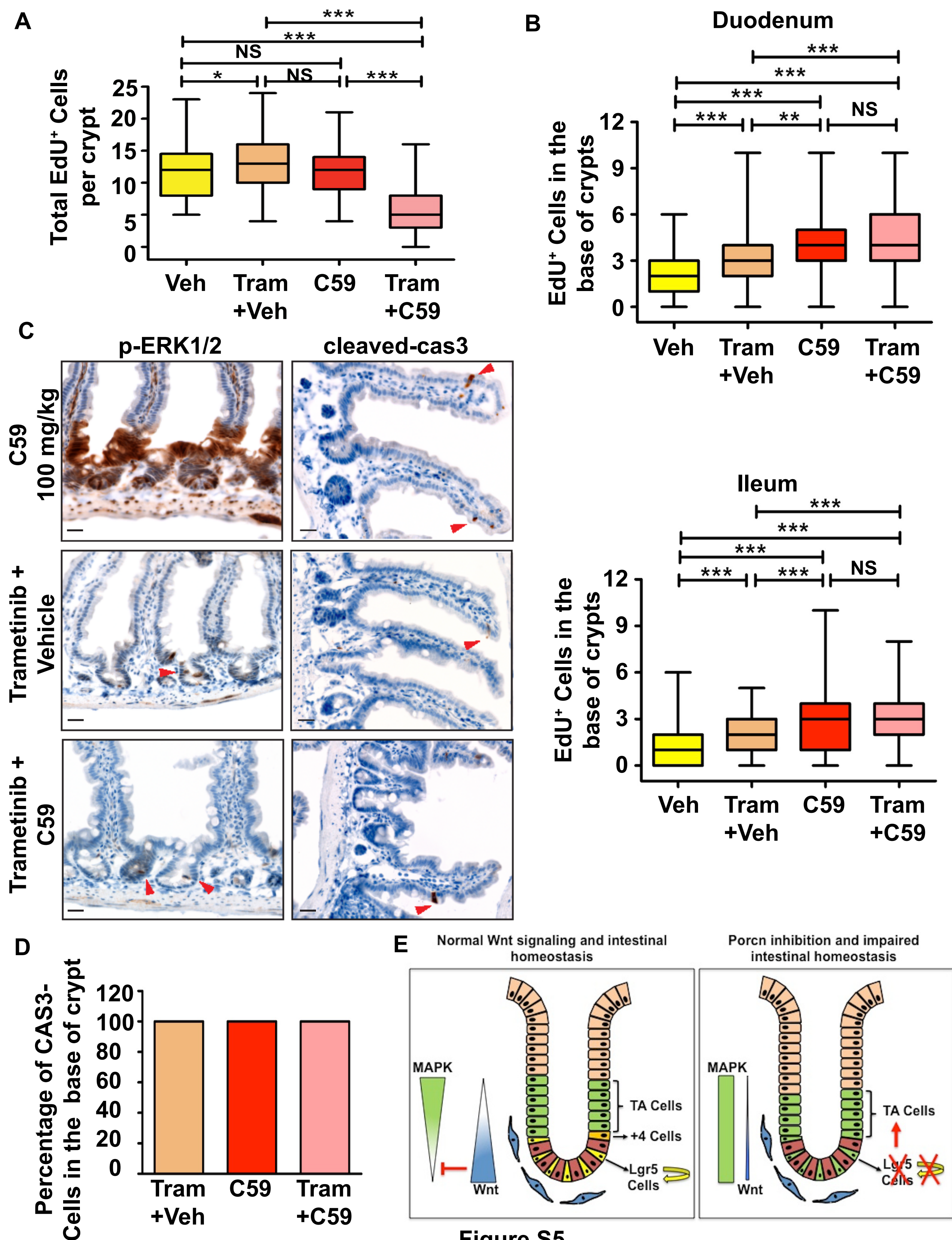

Figure S5