Figure S1. Related to Figure 1
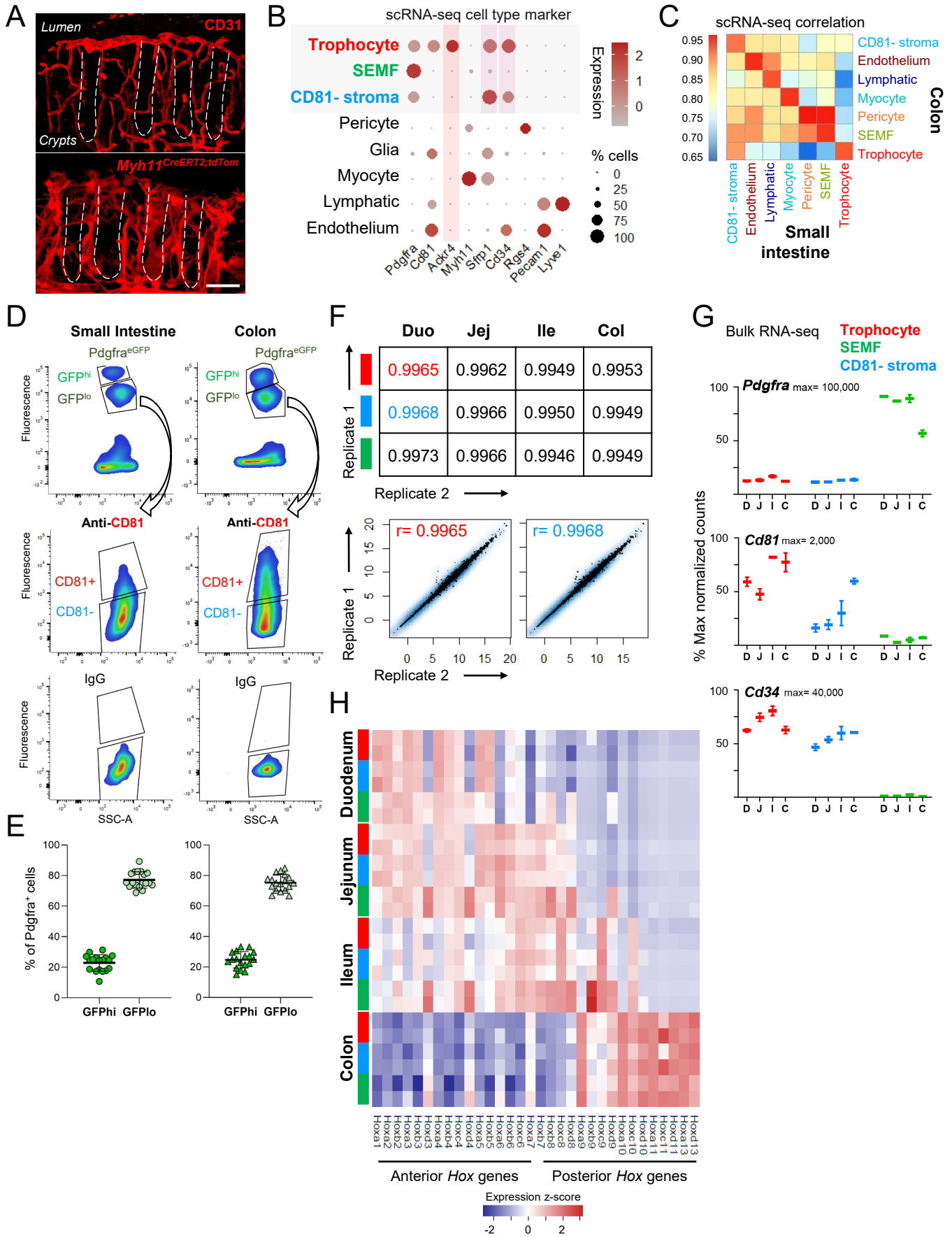
Figure S1. Mesenchymal cell components and marker genes. Related to Figure 1.

A) Top: Cross-section of endothelial cell marker CD31 staining (red) in colon crypt whole-mounts. z-stack 56 µm. Bottom: Cross-section of $Myh11^{Cre(ERT2);tdTom}$ mice (tdTOM) colonic whole-mount 5 days after tamoxifen injection to label myocytes red. Scale bar 50 µm. For orientation, crypts are demarcated with dashed lines.

B) scRNAseq -based expression of selected marker genes that identify cell types in small intestine SI [S1] and colonic (this study) mesenchyme (combined data). *Ackr4* is specific to trophocytes among all mesenchymal cells.

C) Correlation of global scRNA-seq profiles for each SI and colonic cell population (see also Figure 1E). Color scale: Pearson correlation coefficient.

D) Representative FACS plots illustrating the gating strategy to purify Pdgfra^{eGFP} high and low cells and sub-gating of CD81$^+$ or CD81$^-$ fractions among Pdgfra^{eGFP} low cells in SI (left) and colon (right). Gating with isotype IgG was used as a control in each experiment.

E) Percentages of Pdgfra^{eGFP} -high and -low cells captured by flow cytometry, expressed relative to all Pdgfra$^+$ cells (100%). Lines indicate mean ±SD; each datapoint is from an independent experiment (n=18).

F) Pearson correlation values from bulk RNA-seq analysis of replicate samples based on all expressed genes (rlog-transformed read counts). Duo, duodenum; Jej, jejunum; Ile, ileum; Col, colon. Color blocks indicate cell type: red, trophocytes; blue, CD81$^-$ stromal cells; green, SEMFs. Bottom: Representative correlation plots between replicates from duodenal trophocytes (left) and CD81$^-$ stromal cells (right).

G) Expression (normalized counts from bulk RNA-seq) of selected marker genes in FACS-purified cells from different intestinal regions. n=2 mice for each cell isolation. Whiskers: minimum to maximum values.

H) Expression heatmap of *Hox* genes (row z-scores) in FACS-purified cells from different intestinal segments. Each row represents a sample, color blocks indicate cell types: red, trophocytes; blue, CD81$^-$ stromal cells; green, SEMF.

A

Trophocyte | CD81⁻ stroma | SEMF

Clustered genomic regions

-1.5kb center +1.5kb

Signal intensity

0 3 6 | 0 3 6 | 0 3 6

Troph. / CD81⁻ / SEMF — ATAC
Troph. / CD81⁻ / SEMF — RNA [0-8]
[0-1355] Scale
Ackr4

Troph. / CD81⁻ / SEMF — ATAC [0-19]
Troph. / CD81⁻ / SEMF — RNA
[0-539] Scale
Cd81

B

ATAC [0-8]
RNA [0-11201] Scale
Sfrp1

Small Intestine | Colon

Sfrp1

C

% Max normalized counts (bulk RNA-seq)

Sfrp1 max=200,000
Frzb max=4,000
Dkk2 max=16,000

Trophocyte
CD81- stroma
SEMF

Apcdd1 max=10,000
Wif1 max=2,000
Wls max=10,000

D J I C | D J I C | D J I C

D

Bulk RNA-seq (FPKM values)

	Trophocyte		CD81⁻ stroma		SEMF	
	D	C	D	C	D	C
Wnt1	--	--	--	--	--	--
Wnt2	--	10	--	8	--	--
Wnt2b	199	226	189	175	11	36
Wnt3	--	--	--	--	--	--
Wnt3a	--	--	--	--	--	--
Wnt4	10	43	20	44	30	28
Wnt5a	14	13	12	23	77	149
Wnt5b	4	17	4	23	8	24
Wnt6	--	--	--	1	--	3
Wnt7a	--	--	--	--	--	--
Wnt7b	--	--	--	--	--	--
Wnt8a	--	--	--	--	--	--
Wnt8b	--	--	--	--	--	--
Wnt9a	8	3	7	3	2	2
Wnt9b	--	--	--	--	--	--
Wnt10a	--	--	--	--	--	--
Wnt10b	--	--	--	1	--	--
Wnt11	5	2	3	4	--	1
Wnt16	--	1	--	2	--	--

E

scRNA-seq

Small Intestine

Trophocyte
CD81- stroma
SEMF
Pericyte
Glia
Myocyte
Lymphatic
Endothelium

Expression
2
1
0

% cells
0 20 40 60

Colon

Trophocyte
CD81- stroma
SEMF
Pericyte
Glia
Myocyte
Lymphatic
Endothelium

Expression
2
1
0

% cells
0 10 20 30 40 50

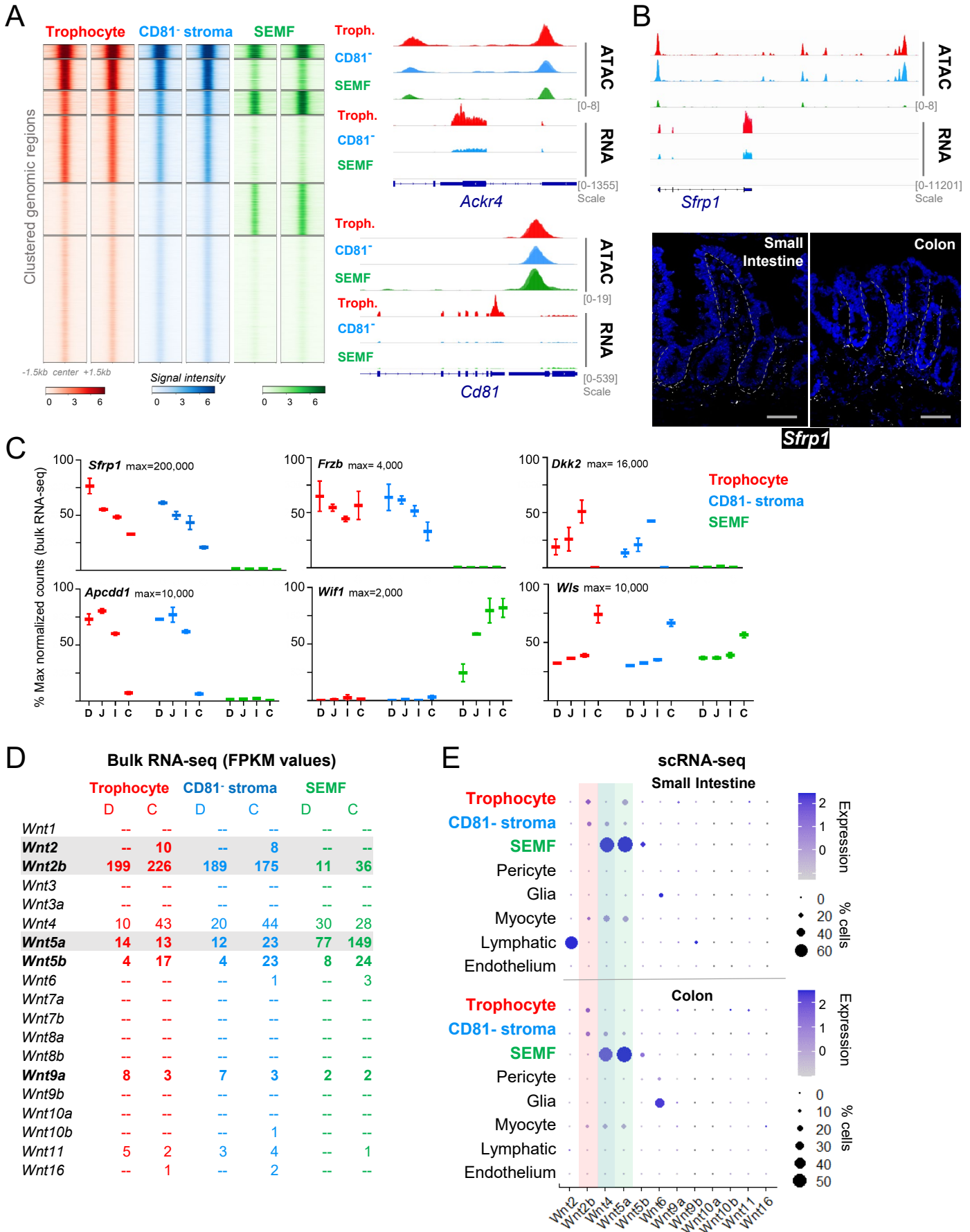Wnt2 Wnt2b Wnt4 Wnt5a Wnt5b Wnt6 Wnt9a Wnt9b Wnt10a Wnt10b Wnt11 Wnt16

Figure S2. Chromatin profiles and expression of Wnt pathway genes in mesenchymal cell types. Related to Figures 1 and 2.

A) *K*-means clustering (*k*=6) of ATAC signal intensity at accessible enhancers, sorted by signal strength in duplicate samples per group. Trophocyte and CD81⁻ stromal landscapes are highly similar. Right, Integrated Genome Viewer (IGV) tracks plot ATAC- and RNA-seq reads (the relative y-axis scales are indicated) for trophocyte-restricted marker genes *Ackr4* and *Cd81* (8-kb regions are shown) in each PDGFRA⁺ cell type.

B) Top, ATAC-seq and RNA-seq reads of accessible chromatin as IGV-sample tracks (duplicate overlay) at the *Sfrp1 locus,* which is expressed in Pdgfra^{lo} cells but not in SEMF. A 110-kb genomic region is shown and relative scales are indicated. Bottom, in situ hybridization (RNAscope) for *Sfrp1* (white) in SI and colonic tissue sections. Nuclei in blue (DAPI). Epithelium is outlined with dashed lines. Scale bars 50 μm.

C) Bulk RNA-seq expression of Wnt-pathway genes across PDGFRA⁺ cell types and intestinal segments: duodenum (D), jejunum (J), ileum (I), and colon (C). Segmental expression is shown for selected negative Wnt regulators (top row) and ancillary pathway components (bottom). Y-axes: percent of maximum normalized counts for each gene. n=2 biological replicates per cell type and per segment. Box: 25th to 75th percentile, whiskers: minimum to maximum values.

D) Wnt ligand expression (mean fragments per kb per million mapped fragments (FPKM) from 2 biological replicates) in bulk PDGFRA⁺ cell types from wild-type mouse duodenum (D) and colon (C). Dashes: FPKM <1.

E) Expression of Wnt ligands across all mesenchymal cell types in scRNA-seq data from SI (top) [S1] and colon (bottom). Cells were identified by known marker genes (Figure S1B). Color scale: mRNA level (normalized counts), circle size: fraction of cells in each population expressing the gene. Only the Wnt genes shown were detected in any cell type.

A

Wnt2 max= 800

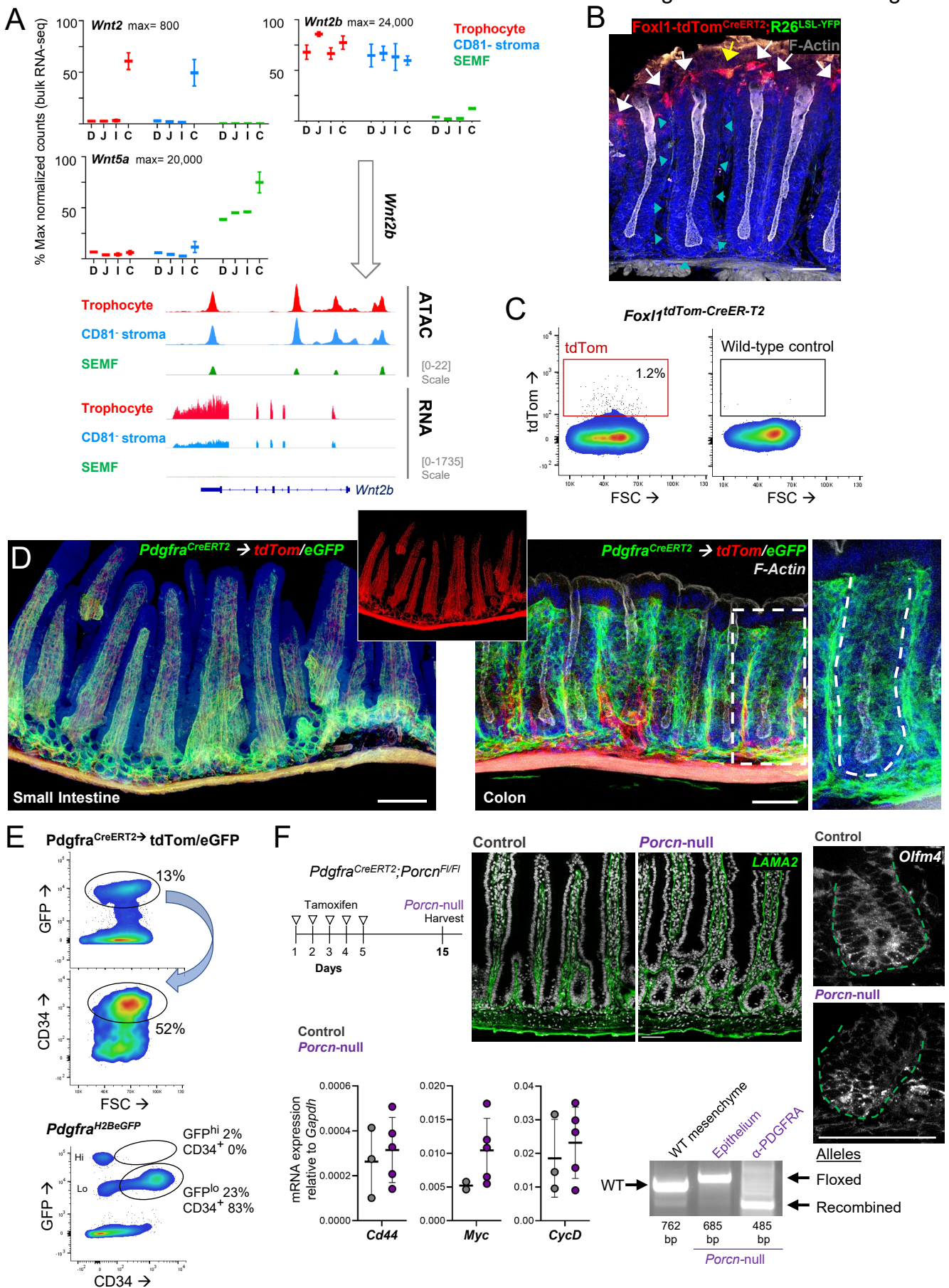**Wnt2b** max= 24,000

Trophocyte
CD81- stroma
SEMF

Wnt5a max= 20,000

% Max normalized counts (bulk RNA-seq)

D J I C

Wnt2b

Trophocyte
CD81⁻ stroma
SEMF
ATAC
[0-22]
Scale

Trophocyte
CD81⁻ stroma
SEMF
RNA
[0-1735]
Scale

Wnt2b

B
Foxl1-tdTom^{CreERT2};R26$^{LSL-YFP}$ F-Actin

C
Foxl1$^{tdTom-CreER-T2}$

tdTom 1.2%   Wild-type control

tdTom →

FSC →   FSC →

D
Pdgfra^{CreERT2} → tdTom/eGFP   Pdgfra^{CreERT2} → tdTom/eGFP   F-Actin

Small Intestine   Colon

E
Pdgfra^{CreERT2}→ tdTom/eGFP

GFP →   13%

CD34 →   52%

FSC →

Pdgfra^{H2BeGFP}

GFP →

Hi   GFP^{hi} 2%
     CD34$^+$ 0%

Lo   GFP^{lo} 23%
     CD34$^+$ 83%

CD34 →

F
Pdgfra^{CreERT2};Porcn$^{Fl/Fl}$

Tamoxifen   Porcn-null
            Harvest
1 2 3 4 5   15
Days

Control   Porcn-null   LAMA2

Control   Olfm4

Porcn-null

Control
Porcn-null

mRNA expression relative to Gapdh

Cd44   **Myc**   **CycD**

WT mesenchyme Epithelium α-PDGFRA

WT   ← Floxed
      ← Recombined

762 bp   685 bp   485 bp
Porcn-null

Alleles

Figure S3. *Wnt* gene expression and loss of Wnt secretion in PDGFRA⁺ intestinal mesenchyme. Related to Figure 2.

A) Top, expression of selected Wnt agonists in mesenchymal cell types across the intestinal length (bulk RNA-seq normalized counts represented as % of indicated maxima, n=2 per group). Bottom, IGV tracks of ATAC-seq (upper tracks) and RNA-seq (lower tracks) reads from SI cell types at the *Wnt2b* locus. A 30-kb genomic region is shown. Y-axis scales are indicated.

B) Whole-mount image of colonic tissue from Foxl1^tdTom-CreERT2 (red) mice crossed with Rosa26^+/YFP (green) mice, treated with 5 daily injections of tamoxifen and counterstained for F-Actin (phalloidin, light grey) and nuclei (DAPI, blue). Overlay of 10 μm z-stacks. White arrows point to Tom⁺ cells, yellow arrows to recombined Tom⁺YFP ⁺ cells, and light blue arrows to SEMFs lacking either label. The epithelial luminal surface displays autofluorescence from the required high excitation intensity. Scale bar 50 μm. The image is representative of n=3 biological replicates.

C) Flow cytometry plots (smoothened scatter plots with pseudo-colored density) for tdTom⁺ cells in *Foxl1^tdTom-CreERT2* (left) and wild-type (right) isolated mesenchyme. Average percentage of gated live single cells from n=3 biological replicates each. FSC, forward scatter area.

D) Whole-mount fluorescence images from *Pdgfra^CreERT2;Rosa26 ^mT/mG* SI (left) and colon (right), where Cre activity converts ubiquitous tdTom (red) to eGFP (green) signal. Counter-stain: F-Actin (Phalloidin, grey), and nuclei (DAPI, blue). Middle inset: tdTom channel only from an SI sample. Right: Enlarged area showing eGFP signal, with crypt epithelium outlined (dashed line). Scale bars: SI 200 μm, colon 50 μm.

E) eGFP signal intensity (smoothened FACS scatter plots with pseudo-colored density) in SI mesenchyme isolated from *Pdgfra^CreERT2;Rosa26 ^mT/mG* mice. Top: 13.5% of live single cells are GFP⁺. Middle (sub-gating for CD34 Ab staining): 52% of GFP⁺ cells express CD34. Bottom (eGFP and CD34 signal intensity in *Pdgfra^H2BeGFP* mice): Among all live mesenchymal cells, 83% of GFP^lo cells and no GFP^hi cells express CD34, confirming bulk RNA-seq data (Figure S1G). Shown are representative plots and percentages averaged from n=3 replicates. FSC, forward scatter area.

F) Top, experimental design for *Pdgfra^CreERT2;Porcn^fl/fl* mice and immunofluorescence for laminin (LAMA2, green) and nuclei (DAPI, grey) in SI tissue sections from control and *Porcn*-null animals. Right, *Olfm4* in-situ hybridization (RNAscope, white) signals in single crypts (dashed outline) representing n=4 (control) and n=6 (*Porcn*-null) mice. Scale bars 50 μm. Bottom left, mRNA levels of ISC-associated genes measured by qRT-PCR in crypts isolated from control (grey) and *Porcn*-null (purple) SI. Results are normalized to *Gapdh levels.* Bottom right, *Porcn* gene disruption confirmed by genotyping PCR of FACS-sorted PDGFRA Ab-stained cells from wild-type (WT) or *Porcn*-null mesenchyme. Unrelated sample lanes were cropped. The expected sizes of PCR products are indicated below each lane of the agarose gel.
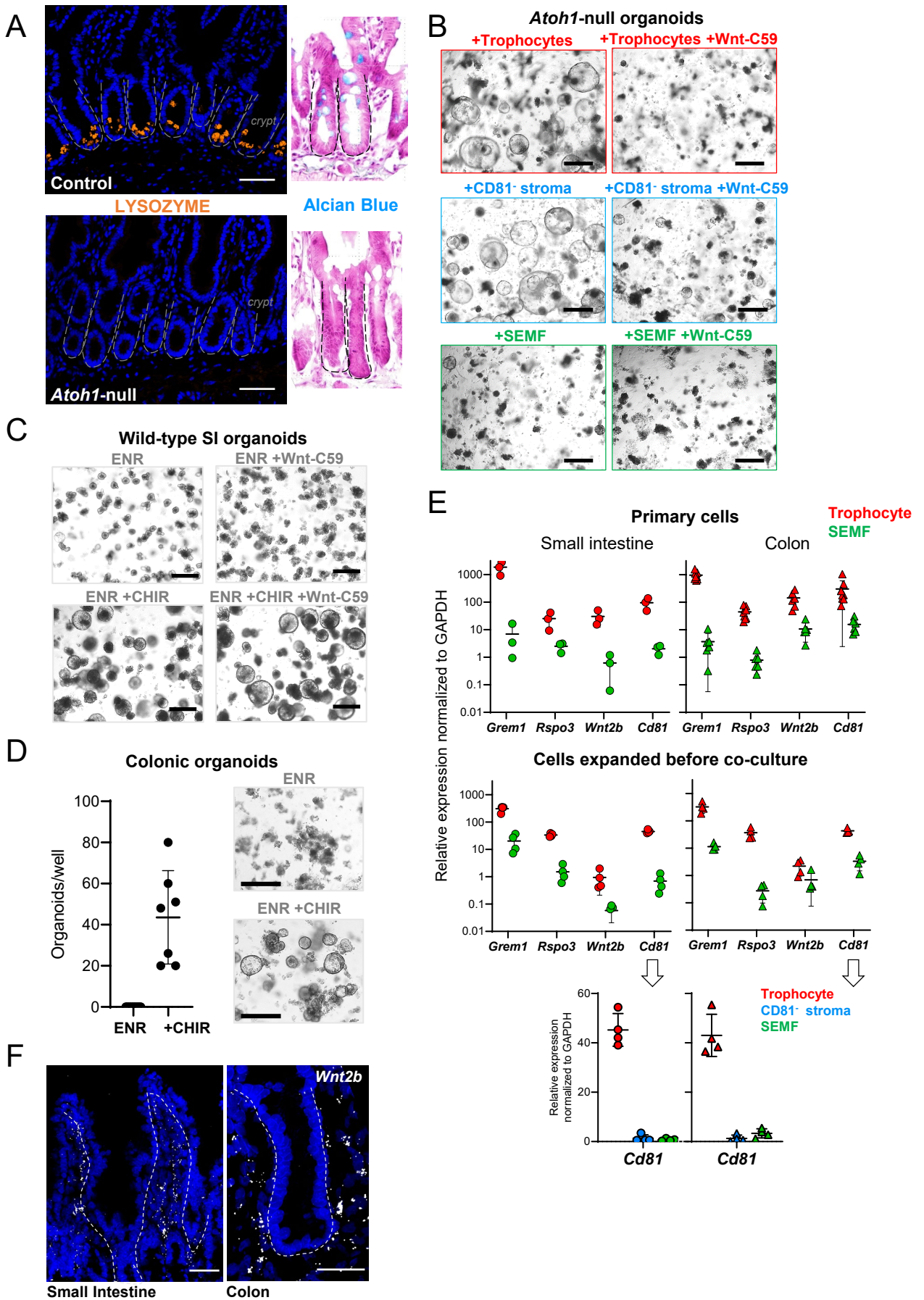
A

LYSOZYME — Control / *Atoh1*-null

Alcian Blue

B ***Atoh1*-null organoids**
+Trophocytes / +Trophocytes +Wnt-C59
+CD81⁻ stroma / +CD81⁻ stroma +Wnt-C59
+SEMF / +SEMF +Wnt-C59

C **Wild-type SI organoids**
ENR / ENR +Wnt-C59
ENR +CHIR / ENR +CHIR +Wnt-C59

D **Colonic organoids**
Organoids/well
ENR / +CHIR
ENR / ENR +CHIR

E **Primary cells**
Trophocyte / SEMF
Small intestine / Colon
Relative expression normalized to GAPDH
Grem1 *Rspo3* *Wnt2b* *Cd81*

Cells expanded before co-culture

Trophocyte / CD81⁻ stroma / SEMF
Relative expression normalized to GAPDH
Cd81 / *Cd81*

F *Wnt2b*
Small Intestine / Colon

Figure S4. Wnt dependency of *Atoh1*-null and colonic organoids and retained expression differences of mesenchymal cells *in vitro*. Related to Figure 2.

A) Immunofluorescence for Paneth-cell marker Lysozyme (LYZ, orange plus DAPI counterstain, blue) and Alcian Blue staining for mucus-producing secretory cells (blue) in SI from control and *Atoh1*-null (*Vil1^{CreERT2};Atoh1$^{fl/fl}$*) mice 3 weeks after tamoxifen treatment. Dashed lines indicate crypts and scale bars represent 50 μm.

B) Representative organoids derived from *Atoh1*-null SI crypts 4 days after co-culture with different colonic mesenchymal cells in the presence or absence of the Porcupine inhibitor Wnt-C59. Representative wells from n=3 independent experiments are shown. Data are quantified in Figure 2E. Scale bars 200 μm.

C) Organoid growth from wild-type duodenal crypts 4 days after culture in ENR medium with or without CHIR99021 (CHIR) and with or without Porcupine inhibitor Wnt-C59. Representative wells from n=3 independent experiments are shown. Scale bars 200 μm

D) Organoid counts from wild-type colonic crypts grown for 4 days in ENR medium with or without CHIR99021 (CHIR). Representative wells from n=3 independent experiments are shown. Lines indicate Mean ±SD. Right, bright-field images of organoids derived under the two culture conditions. Scale bars 200 μm.

E) Top, qRT-PCR analysis of relevant ISC-niche markers in SEMF (green) and trophocytes (red) freshly isolated (upper panel) from SI (circles, left) and colon (triangles, right) or expanded for 3 days in culture as unfractionated mesenchyme before cell sorting by flow cytometry (lower panel). Bottom, *Cd81* expression in SEMF, trophocytes (as above), and CD81$^-$ stromal cells (blue) after *in vitro* expansion and FACS sorting. Each dot represents an independent experiment, lines are Mean ±SD (n= 3-4). Data are normalized to *Gapdh levels as* 2^(-ΔCt)*100.

F) RNAscope in situ hybridization for *Wnt2b* (white) in SI (left) and colonic (right) tissue sections counterstained with DAPI (blue). Scale bars 50 μm; crypts and villi are outlined by dashed lines.
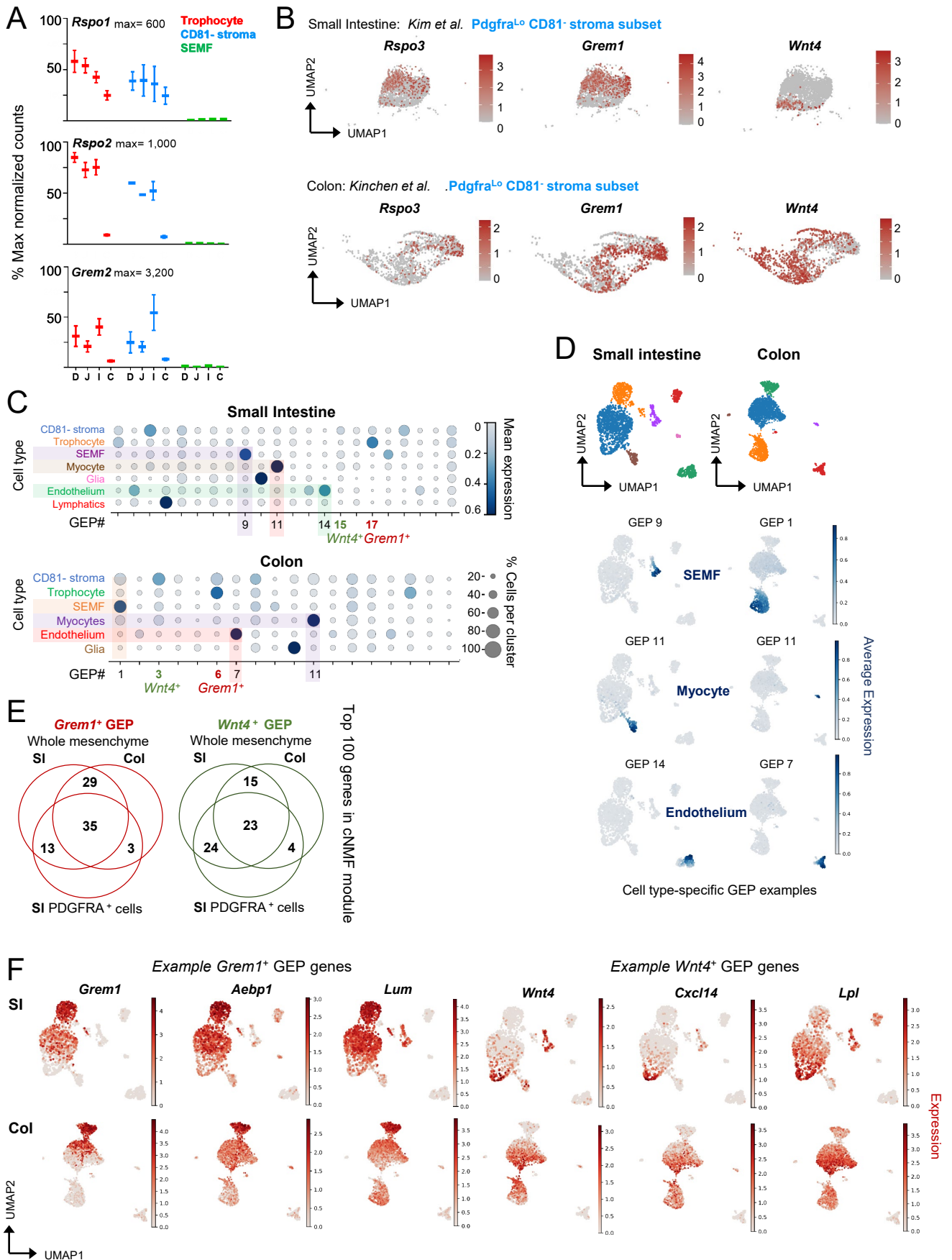
A

Rspo1 max= 600
- Trophocyte (red)
- CD81- stroma (blue)
- SEMF (green)

Rspo2 max= 1,000

Grem2 max= 3,200

% Max normalized counts

D J I C

B Small Intestine: *Kim et al.* Pdgfra^Lo CD81- stroma subset

Rspo3   *Grem1*   *Wnt4*

Colon: *Kinchen et al.* Pdgfra^Lo CD81- stroma subset

Rspo3   *Grem1*   *Wnt4*

C Small Intestine

Cell type: CD81- stroma, Trophocyte, SEMF, Myocyte, Glia, Endothelium, Lymphatics

GEP#   9 11   14 15   17
                    Wnt4+ *Grem1+*

Colon

Cell type: CD81- stroma, Trophocyte, SEMF, Myocytes, Endothelium, Glia

GEP# 1   3   6 7   11
         Wnt4+ *Grem1+*

Mean expression
% Cells per cluster: 20, 40, 60, 80, 100

D Small intestine   Colon

GEP 9   GEP 1   SEMF
GEP 11   GEP 11   Myocyte
GEP 14   GEP 7   Endothelium

Average Expression

Cell type-specific GEP examples

E *Grem1+* GEP   *Wnt4+* GEP
Whole mesenchyme   Whole mesenchyme
SI   Col   SI   Col
29   15
35   23
13   3   24   4
SI PDGFRA+ cells   SI PDGFRA+ cells

Top 100 genes in cNMF module

F *Example Grem1+ GEP genes*   *Example Wnt4+ GEP genes*

Grem1 *Aebp1* *Lum*   *Wnt4* *Cxcl14* *Lpl*

SI

Col

Expression

Figure S5. Expression gradients within and across cell populations.
Related to Figure 3.

A) Normalized bulk RNA-seq counts for *Rspo1, Rspo2* and *Grem2* in purified mesenchymal cell types from mouse duodenum (D), jejunum (J), ileum (I), and colon (C). Data from n=2 mice per group. See also Figure 3B.

B) Marker gene expression in feature plots of whole mesenchyme scRNA-seq data, retrieved and re-analyzed from published studies as indicated [S2, S3]. For clarity, only the *Pdgfra^lo Cd81^-* stromal cell cluster is depicted on the UMAP plots.

C) Percent of cells and average expression of each gene expression program (GEP) derived from cNMF analysis across cell types identified separately by Leiden clustering of scRNA data.

D) UMAP plot from Leiden clustering of scRNA-seq data from SI [S1] and colonic (this study) mesenchyme. Below, SEMF-, myocyte-, and endothelial cell-specific GEP expression scores from cNMF analysis are projected on these UMAP plots.

E) Overlap of top 100 genes in *Grem1^+* or *Wnt4^+* GEPs across independent datasets: Whole mesenchyme from SI [S1] and colon (see Figure 3E) and sorted PDGFRA^+ cells from SI (see Figure 3F).

F) UMAP plots showing examples of genes that feature in the *Grem1^+* and *Wnt4^+* GEPs depicted in Figure 3E. Distribution of these genes in both the SI (top row) [S1] and colon (bottom row) overlaps but is not identical with *Grem1* or *Wnt4*.
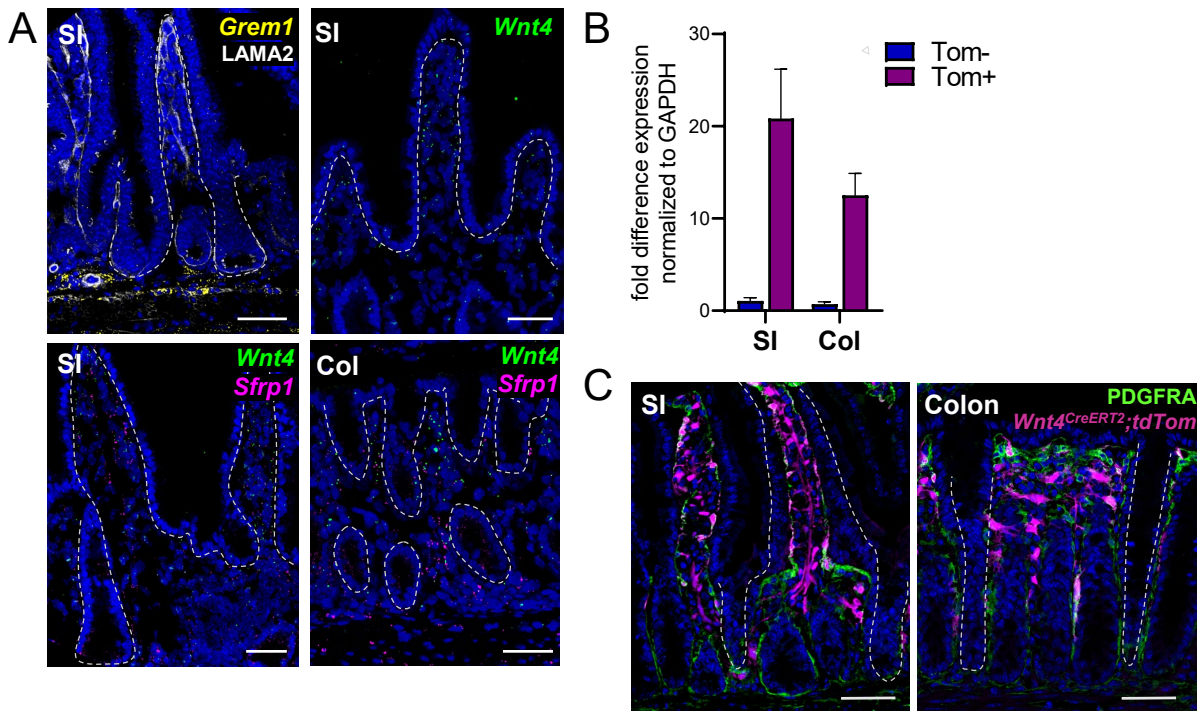
Figure S6. Distribution of signaling molecules in relation to tissue anatomy. Related to Figure 4.

A) RNAscope in situ hybridization for *Grem1* (yellow), *Wnt4* (green), or *Sfrp1* (magenta) as indicated, counterstained for nuclei (DAPI, blue) in SI and colon. Crypts and villi are outlined with dashed lines. Scale bars 50 μm.

B) Expression of *Wnt4* in tdTom$^+$ and tdTom$^-$ FACS-isolated mesenchymal cells from *Wnt4^{CreERT2};R26R^{tdTom}* small intestine (SI) and colon (Col), measured by qRT-PCR. Mean $\pm$SD from n=3 animals.

C) Tissue sections of *Wnt4^{CreERT2};R26R^{tdTom}* SI crypt-villus units and colonic crypts. tdTom signal (magenta) is counterstained with PDGFRA Ab (green, to highlight dense SEMF aggregates) and DAPI (blue). Scale bars 50 μm. Dashed lines indicate outline of epithelium.
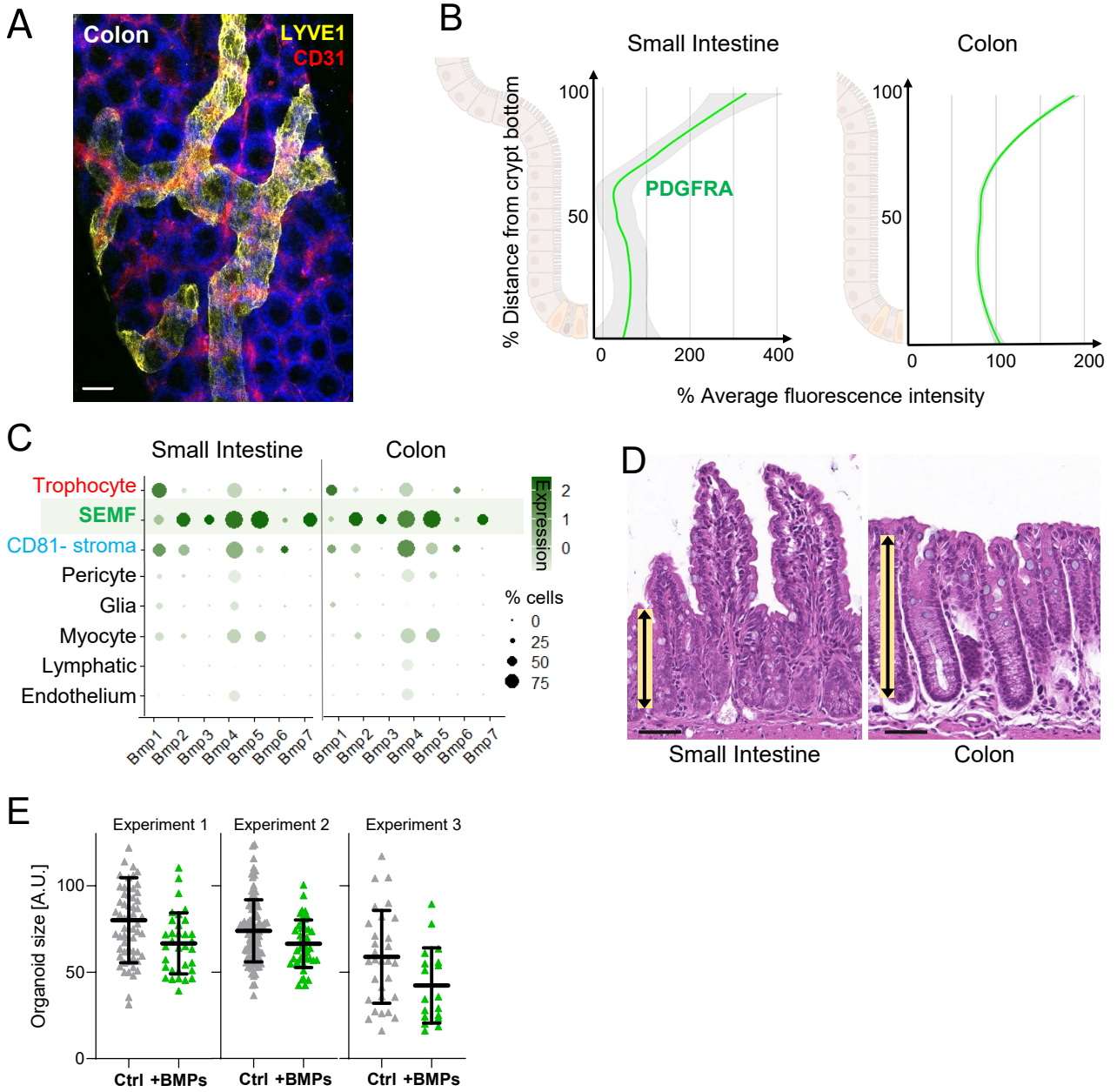
A

Colon LYVE1 CD31

B

Small Intestine

Colon

% Distance from crypt bottom

PDGFRA

% Average fluorescence intensity

C

Small Intestine   Colon

Trophocyte
SEMF
CD81- stroma
Pericyte
Glia
Myocyte
Lymphatic
Endothelium

Bmp1 Bmp2 Bmp3 Bmp4 Bmp5 Bmp6 Bmp7   Bmp1 Bmp2 Bmp3 Bmp4 Bmp5 Bmp6 Bmp7

Expression
2
1
0

% cells
0
25
50
75

D

Small Intestine   Colon

E

Experiment 1   Experiment 2   Experiment 3

Organoid size [A.U.]

Ctrl +BMPs   Ctrl +BMPs   Ctrl +BMPs

Figure S7. Distribution of signaling molecules in relation to tissue architecture.
Related to Figures 6 and 7.

A) Wild-type colonic tissue stained for lymphatic vessel (LYVE1, yellow), endothelial cell (CD31, red) and nuclear (DAPI, blue) markers and photographed from the serosal side. Crypts far from lymphatic vessels outnumber those that lie close. Scale bar 50 µm.

B) Fluorescence intensity of anti-PDGFRA Ab staining in SI and colonic tissue sections, expressed as the percent of average fluorescence intensity from the crypt base upward including the villus base (SI) or the top of the colonic crypt (Col). Smoothened average signals (green) are graphed across n=3 samples, with 95% confidence intervals shaded in grey.

C) scRNA-seq expression of BMP ligands across SI [S1] and colonic mesenchymal cell types (see Figure 1E for the corresponding UMAP plots).

D) Hematoxylin and eosin (H&E)-stained section of mouse SI (ileum, left) and colonic (right) tissue to illustrate differences in crypt height (double arrows). Scale bars 50 µm.

E) Individual experiments for data collated in Figure 7E. Organoid size 4 days after SI crypts were co-cultured with PDGFRA^{lo} CD81^{-} CD55^{hi} stromal cells pre-treated for 5 days with rBMP2/4/7 (+BMPs) or untreated (Ctrl, serum-free medium only). Values in arbitrary units (A.U.), each representing one organoid. Lines indicate mean ±SD.

Supplemental References

[S1] McCarthy, N., Manieri, E., Storm, E.E., Saadatpour, A., Luoma, A.M., Kapoor, V.N., Madha, S., Gaynor, L.T., Cox, C., Keerthivasan, S., et al. (2020). Distinct Mesenchymal Cell Populations Generate the Essential Intestinal BMP Signaling Gradient. Cell Stem Cell 26, 391-402.e5. 10.1016/j.stem.2020.01.008.

[S2] Kim, J.E., Fei, L., Yin, W.C., Coquenlorge, S., Rao-Bhatia, A., Zhang, X., Shi, S.S.W., Lee, J.H., Hahn, N.A., Rizvi, W., et al. (2020). Single cell and genetic analyses reveal conserved populations and signaling mechanisms of gastrointestinal stromal niches. Nat Commun 11.

[S3] Kinchen, J., Chen, H.H., Parikh, K., Antanaviciute, A., Jagielowicz, M., Fawkner-Corbett, D., Ashley, N., Cubitt, L., Mellado-Gomez, E., Attar, M., et al. (2018). Structural Remodeling of the Human Colonic Mesenchyme in Inflammatory Bowel Disease. Cell 175, 372-386.e17.