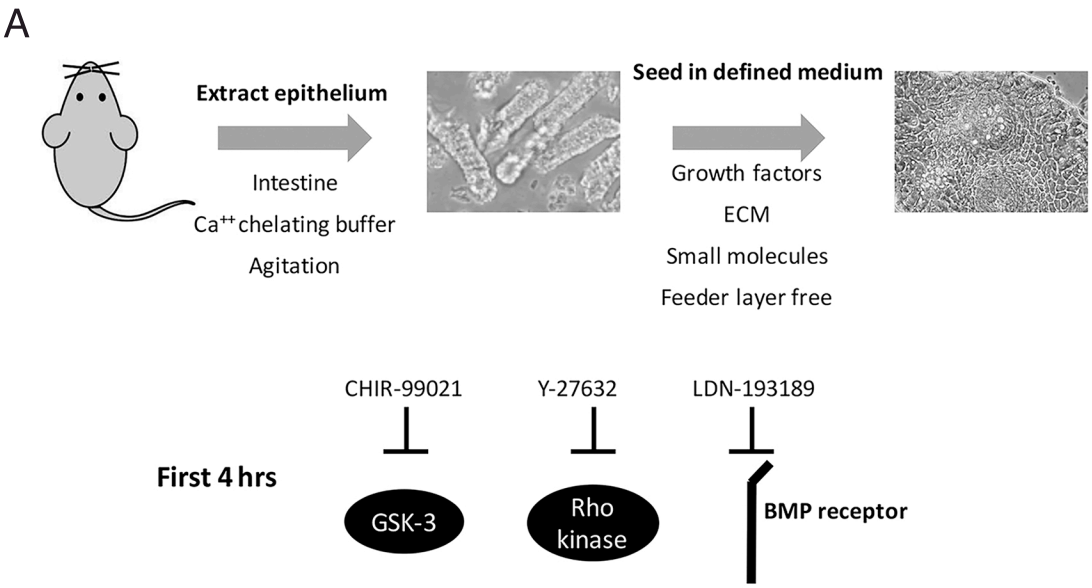
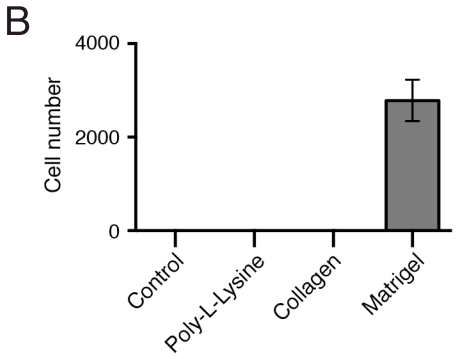
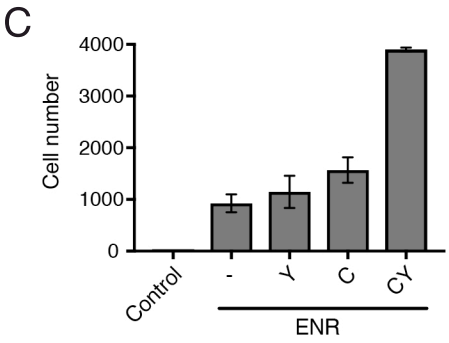
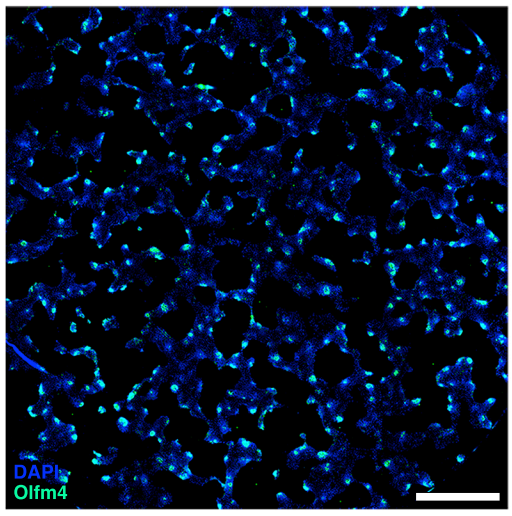
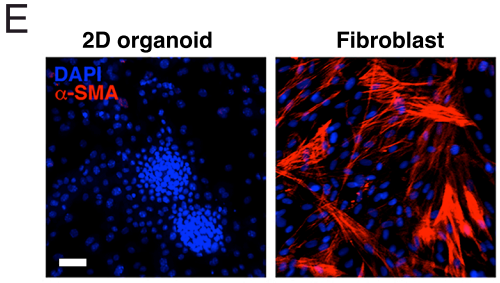
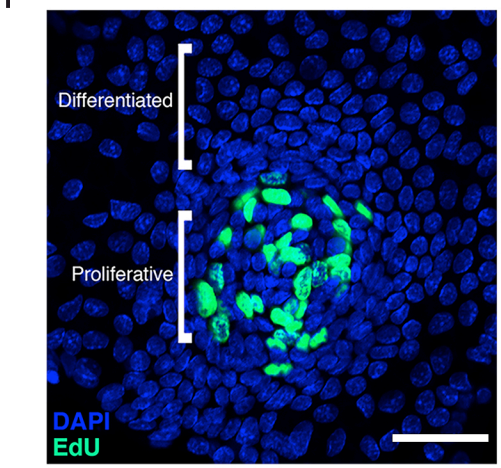

A
Extract epithelium
Intestine
Ca++ chelating buffer
Agitation
Seed in defined medium
Growth factors
ECM
Small molecules
Feeder layer free
First 4 hrs
CHIR-99021
Y-27632
LDN-193189
GSK-3
Rho kinase
BMP receptor


B
Cell number
4000
2000
0
Control
Poly-L-Lysine
Collagen
Matrigel


C
Cell number
4000
3000
2000
1000
0
Control
-
Y
C
CY
ENR


D
DAPI
Olfm4


E
2D organoid
Fibroblast
DAPI
α-SMA


F
Differentiated
Proliferative
DAPI
EdU

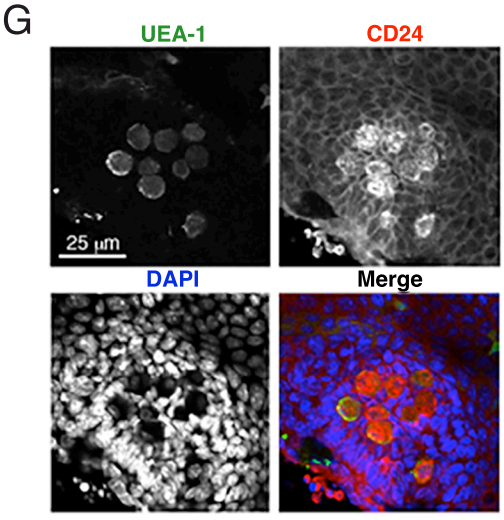

G
UEA-1
CD24
25 μm
DAPI
Merge

Figure S1. Related to Figure 1. Enteroid monolayers establish proliferative focal patterning and major differentiated cell types. (**A**) Steps in seeding intestinal crypt explants. Mice jejunums were washed and incubated in Ca^{++} chelating buffer to promote epithelial release from the mesenchyme. Extracted crypts were suspended in the growth factors supplemented with GSK-3, ROCK, and BMP receptor inhibitors CHIR-99021, Y-27632, LDN-193189, respectively. Crypt suspensions were seeded into 96-well imaging plates pre-coated with laminin-rich extracellular matrix. Kinase inhibitors were removed from the media after four hours. In 48 hours, sheets of epithelium form from initial crypt fragments. (**B**) Purified mouse small intestine crypts were seeded (100 crypts/well) onto 96-well plates coated with the indicated substrates. At 48 hrs post-seeding, cells were fixed, stained with DAPI and counted. Error bars represent mean ± SEM of triplicate wells. (**C**) Crypts assayed as in (B) with indicated treatment: ENR = EGF (50 ng/ml), Noggin (100 ng/ml), R-spondin (500 ng/ml); Y = Y-27632 (10 μM); C = CHIR 99021 (3 μM). Error bars represent mean ± SEM of triplicate wells. (**D**) Image of an entire well from a 96-well plate of enteroid monolayers stained for OLFM4 (green) and DAPI (blue). Note foci of OLFM4 staining. Scale bar = 1 mm. (**E**) Enteroid monolayers do not contain mesenchymal cells. Mesenchymal cells were not detected via staining of enteroid monolayers with alpha-smooth muscle actin (α-SMA). Fibroblast cultures were stained and imaged in parallel as a positive control for the α-SMA+ cell type. Scale bar = 25 μm. (**F**) High magnification of crypt foci and surrounding villus region. Confocal image of enteroid monolayers. Cultures were treated with EdU for two hours, fixed and stained. EdU, green; DNA, blue. Scale bar = 25 μm. (**G**) Staining of crypt foci with Paneth cell specific marker CD24 and the lectin UEA-1 demonstrating co-label of the Paneth cell subpopulation of cells. Granularity of Paneth cells is observed in DIC image.

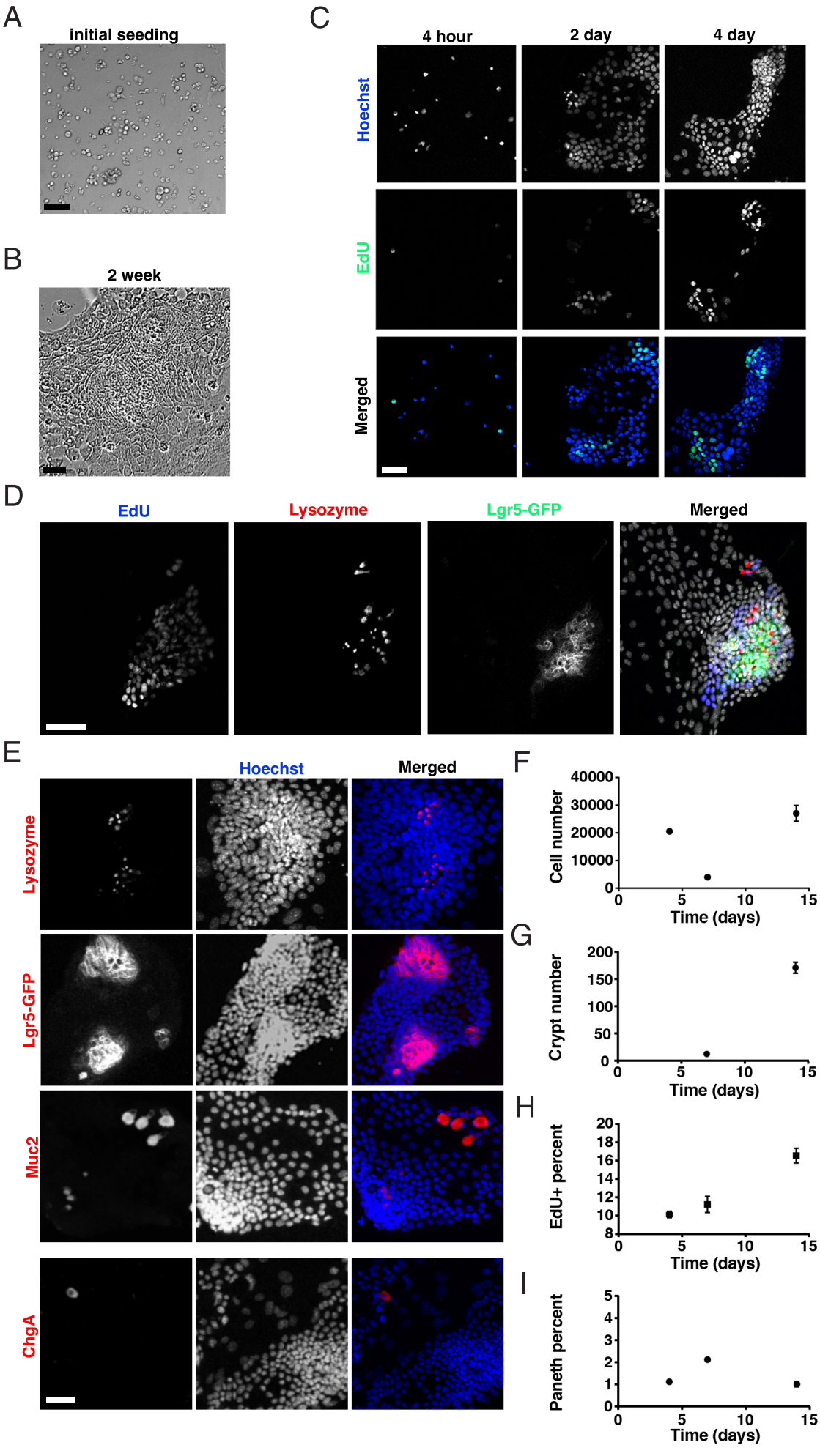

A
initial seeding
B
2 week
C
4 hour
2 day
4 day
Hoechst
EdU
Merged
D
EdU
Lysozyme
Lgr5-GFP
Merged
E
Hoechst
Merged
Lysozyme
Lgr5-GFP
Muc2
ChgA
F
Cell number
40000
30000
20000
10000
0
0
5
10
15
Time (days)
G
Crypt number
200
150
100
50
0
0
5
10
15
Time (days)
H
EdU+ percent
20
18
16
14
12
10
8
0
5
10
15
Time (days)
I
Paneth percent
5
4
3
2
1
0
0
5
10
15
Time (days)

Figure S2. Related to Figure 1. Enteroid monolayers are capable of self-organization. (**A**) Brightfield image taken after 3D organoids were sheared, enzymatically dissociated, and plated on a Matrigel-coated plate, demonstrating that cultures were initially seeded as single cells and small clumps. Scale bar = 25 μm. (**B**) Brightfield image of crypt-like foci formed 2 weeks after seeding from dissociated 3D organoids. Scal bar = 50 μm (**C**) Enteroid monolayers form crypt-like foci when seeded from single cells and small cell clusters. After seeding, cells were allowed to incubate for the indicated amounts of time, pulsed with EdU, fixed, and stained. Scale bar = 25 μm. (**D**) Enteroid monolayers form a stem cell niche when seeded from dissociated 3D organoids. 2 weeks after seeding, enteroids derived from $Lgr5^{eGFP\text{-}DTR}$ mice were pulsed with EdU, fixed, and stained for the indicated markers. Scale bar = 25 μm. (**E**) Enteroid monolayers produce mature intestinal cell types when seeded from dissociated 3D organoids. 2 weeks (for Lyz, Lgr5, and Muc2 stain) or 4 days (for ChgA stain) after seeding, enteroid monolayers from $Lgr5^{eGFP\text{-}DTR}$ mice were fixed and stained for the indicated markers. Scale bar = 15 μm. (**F-I**) At the indicated time points, enteroid monolayers were fixed, stained for markers of the indicated cell types (Hoechst for nuclei, Lgr5 for crypts, EdU for EdU+ cells, Lysozyme for Paneth cells), and the number of each cell type quantified using customized algorithms. Error bars represent mean ± SEM of triplicate wells, each well containing 24 fields of view.

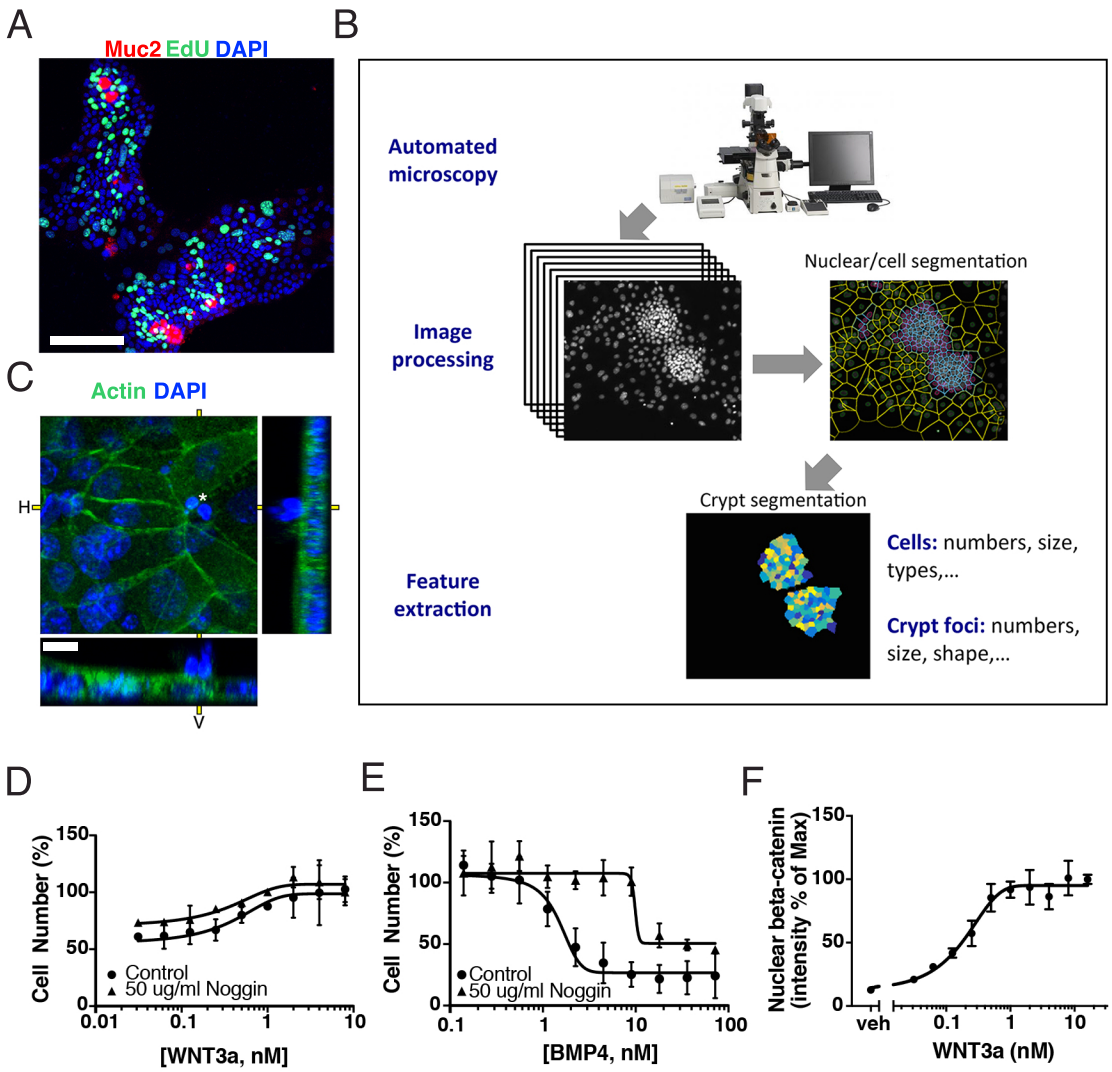

A
Muc2 EdU DAPI
B
Automated microscopy
Image processing
Nuclear/cell segmentation
Crypt segmentation
Feature extraction
Cells: numbers, size, types,...
Crypt foci: numbers, size, shape,...
C
Actin DAPI
H
V
D
Cell Number (%)
Control
50 ug/ml Noggin
[WNT3a, nM]
E
Cell Number (%)
Control
50 ug/ml Noggin
[BMP4, nM]
F
Nuclear beta-catenin (intensity % of Max)
veh
WNT3a (nM)

Figure S3. Related to Figure 1, Figure 2, and Figure 3. The maintenance of growth and pattern formation for crypt foci is regulated by WNT and BMP. (**A**) Example image of mouse colon monolayer cultures stained for DNA (DAPI, blue), proliferative cells (EdU, green), and Goblet cells (Muc2, red). Scale bar = 50 μm. (**B**) Image processing steps of enteroid monolayers. Using high-content microscopy, large collections of images of enteroid cultures under various treatments were collected. Next, images were computationally processed to identify nuclei and cell boundaries. Using a custom algorithm to search for regions of high cell density, individual crypt foci were identified. Finally, single-cell and crypt-level features were extracted. (**C**) Confocal image of enteroid monolayers stained for actin (green) and DNA (DAPI, blue). Asterisk mark an extruding cell that can be seen right above the tissue plane. Right and bottom panels indicate horizontal (H) and vertical (V) projections. Scale bar = 10 μm. (**D,E**) Effect of WNT3a and BMP4 concentration response curves on cell number. Enteroid monolayers were treated for 48 hours with WNT3a (**D**) or BMP4 (**E**) then fixed, imaged and cell numbers were counted by automated image processing (plotted as percent of largest value). Error bars represent mean ± SEM of triplicate wells. (**F**) β-Catenin response to WNT3a treatment in Human Colonic Epithelial Cells (HCEC) demonstrating activation of the WNT pathway across a broad range of concentrations. Error bars represent mean ± SEM of triplicate wells.

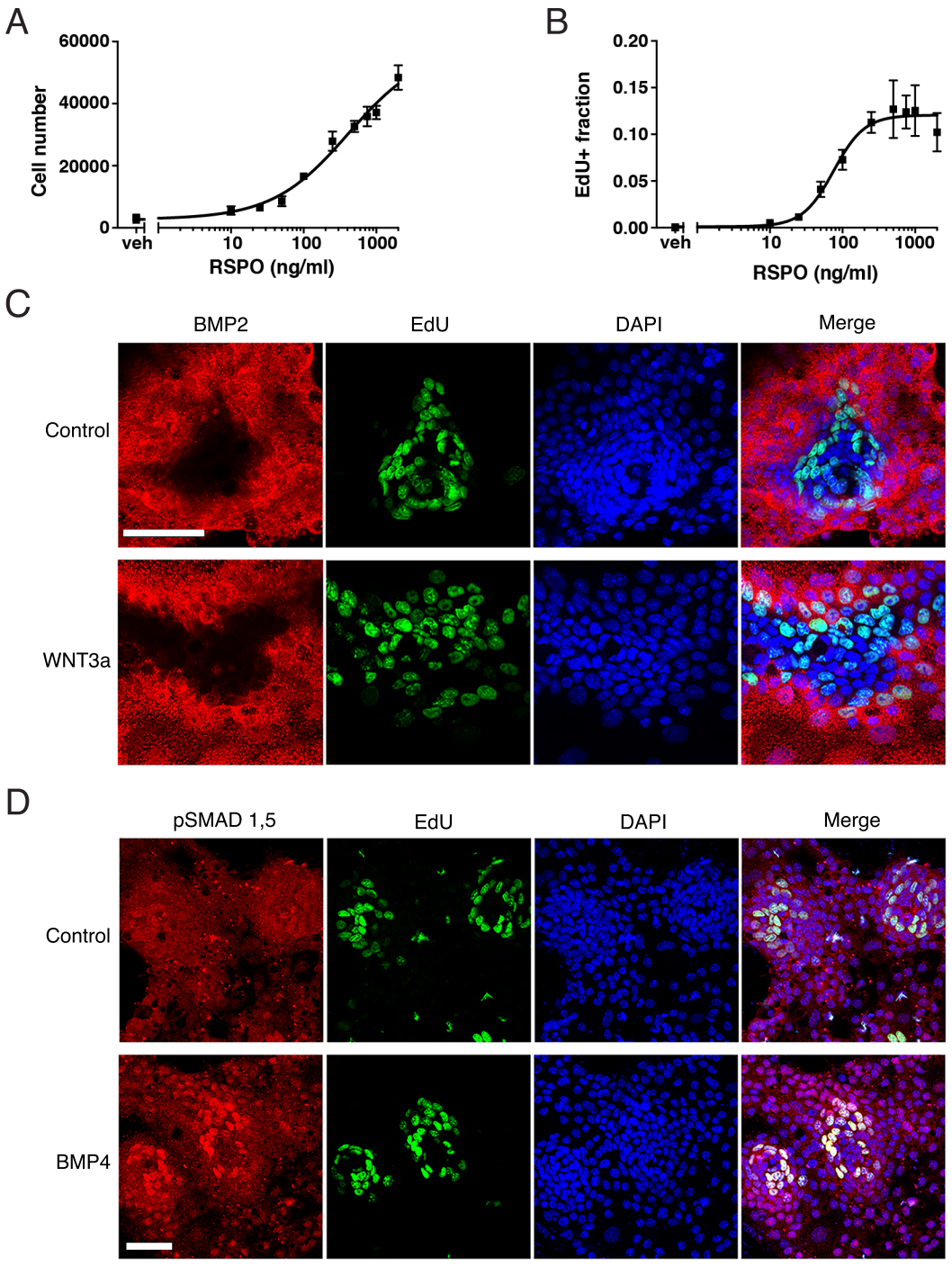

A
Cell number
60000
40000
20000
0
veh
10
100
1000
RSPO (ng/ml)
B
EdU+ fraction
0.20
0.15
0.10
0.05
0.00
veh
10
100
1000
RSPO (ng/ml)
C
BMP2
EdU
DAPI
Merge
Control
WNT3a
D
pSMAD 1,5
EdU
DAPI
Merge
Control
BMP4
E
Control
IWP-2
EC50 = ~1 nM
EdU+ cells (%)
40
30
20
10
0
0.01
0.1
1
10
100
[WNT3a, nM]
F
Density
0.15
0.10
0.05
0.00
0
20
40
60
Distance (µm)
G
Paneth cells (% of initial)
1.5
1.0
0.5
0.0
Control
WNT3a
H
Paneth cells (number)
150
100
50
0
Control
WNT3a

Figure S4. Related to Figure 4. The establishment of crypt foci requires intrinsic WNT and BMP signaling to regulate growth and patterning. (**A,B**) Cell number and fraction EdU+ cells RSPO response curves. Enteroid monolayers were treated for five days as indicated then fixed, stained, and imaged. Cell counts were acquired by automated image processing. Error bars represent mean ± SEM of 4 replicate wells. (**C**) BMP2 is expressed surrounding the proliferative regions in control or WNT3a treated cultures. Enteroid monolayers were stained as indicated and imaged by confocal microscopy. Note lack of BMP2 staining in proliferative crypt foci as marked by EdU. Scale bar = 25 μm. (**D**) Enteroid monolayers were stimulated with buffer control or BMP4 for two hours. Note nuclear accumulation of pSMAD1,5 throughout epithelium in BMP4-treated cells. Scale bar = 25 μm. (**E**) Addition of WNT3a rescues proliferation of enteroids treated with IWP-2. Enteroid monolayers were treated for 48 hours as indicated then fixed, stained for DNA and EdU markers, imaged and cell numbers counted by automated image processing. Error bars represent mean ± SEM of triplicate wells. (**F**) Distribution of nearest EdU+ neighbor distance in untreated enteroid monolayer cultures. Each line represents a replicate well (n = 4). (**G, H**) Loss of Paneth cells under WNT3a treatment. Enteroid monolayers were treated for 48 hours as indicated then fixed, stained for DNA (DAPI) and Paneth marker (UEA-1). Paneth cell numbers were quantified and graphed as percent of initial number of Paneth cells seeded at 48 hours (**G**) or absolute Paneth cell numbers at 72 hours (**H**). Error bars represent mean ± SEM of triplicate wells.