PNAS
www.pnas.org

Supplementary Information for

A population of gut epithelial enterochromaffin cells is mechanosensitive and requires Piezo2 to convert force into serotonin release

Constanza Alcaino,[1] Kaitlyn Knutson,[1] Anthony J. Treichel,[1] Gülcan Yildiz,[1] Peter R. Strege,[1] David R. Linden,[1] Joyce H. Li, [2] Andrew Leiter,[2] Joseph H. Szurszewski,[1] Gianrico Farrugia,[1] Arthur Beyder[1]

[1]Enteric Neuroscience Program, Division of Gastroenterology & Hepatology, Department of Physiology & Biomedical Engineering, Mayo Clinic, Rochester, Minnesota.

[2]Division of Gastroenterology, Department of Medicine, University of Massachusetts Medical School, Worcester, Massachusetts.

Corresponding Author:
Arthur Beyder, M.D., Ph.D.
Email: beyder.arthur@mayo.edu

www.pnas.org/cgi/doi/10.1073/pnas.1804938115

This PDF file includes:

Supplementary text
Figs. S1 to S9
Tables S1 to S2
Movie captions S1 to S3
References for SI reference citations

Other supplementary materials for this manuscript include the following:

Movies S1 to S3

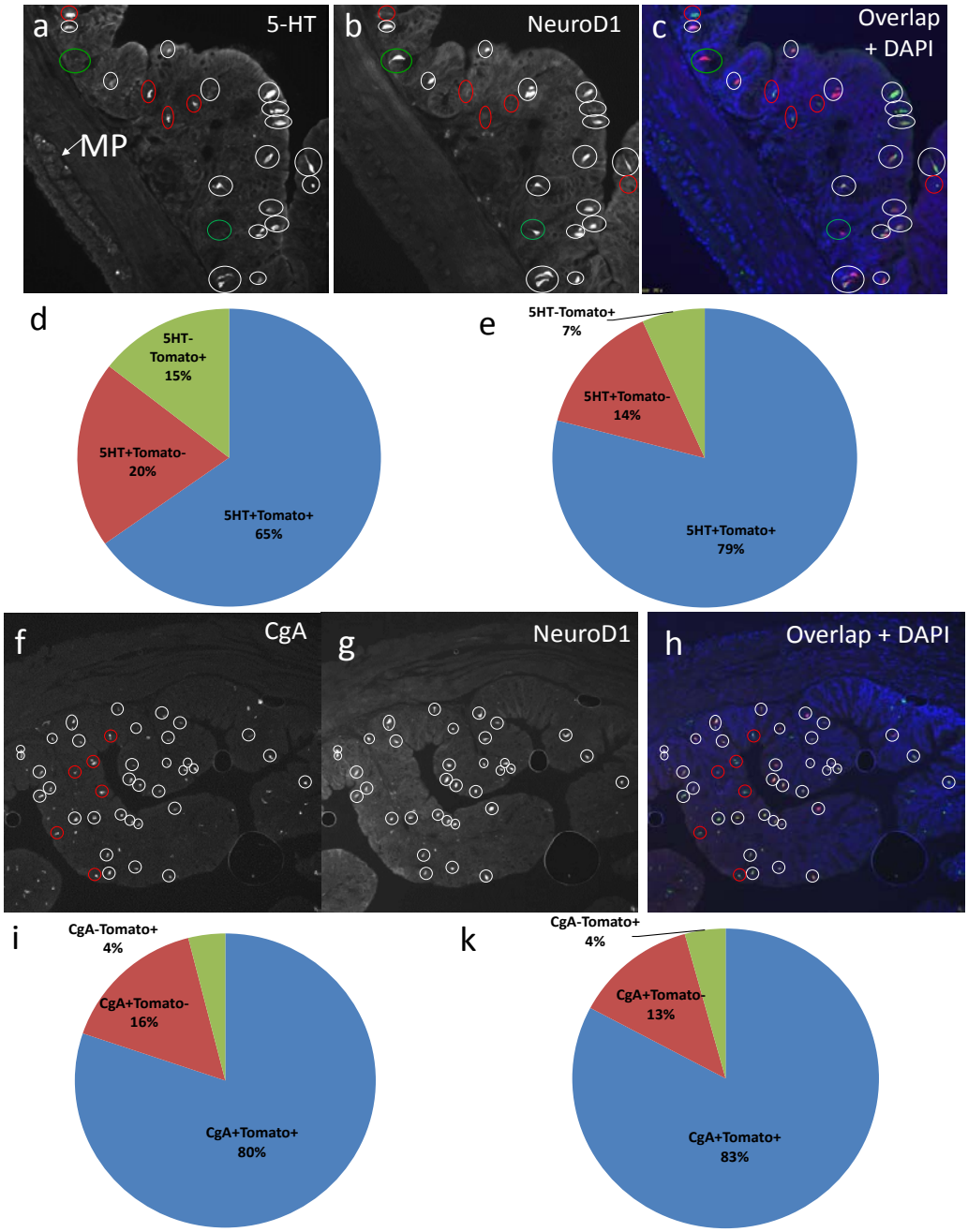


Fig. S1. NeuroD1 is a specific marker of enterochromaffin (EC) cells and enteroendocrine (EE) cells. a-c, **f-h** *NeuroD1-cre;GCaMP5-tdTomato* (*NeuroD1-GCaMP5*) mouse model epifluorescence images of **a-c**, transgenic tdTomato (NeuroD1) and immunolabeled serotonin (5-HT), or **f-h**, tdTomato (NeuroD1) and chromogranin A (CgA) from proximal colon of (10x magnification). Representative images of **a**, 5-HT and **b**, NeuroD1 cells (tdTomato+), and **c**, co-localization with nuclear labeling by DAPI. Serotonergic neurons in the myenteric plexus (MP) are tdTomato-. Quantification of 5-HT and tdTomato co-localization in **d**, small bowel and **e**, colon. Representative images of **f**, immunolabeled CgA and **g**, NeuroD1 cells (tdTomato+) and **h**, co-localization with nuclear labeling by DAPI. Quantification of overlap in **i**, small bowel and **k**, colon. White circles are around cells that are CgA and tdTomato co-localization (white circles), CgA or 5-HT only (red circles), NeuroD1 only (green circle).

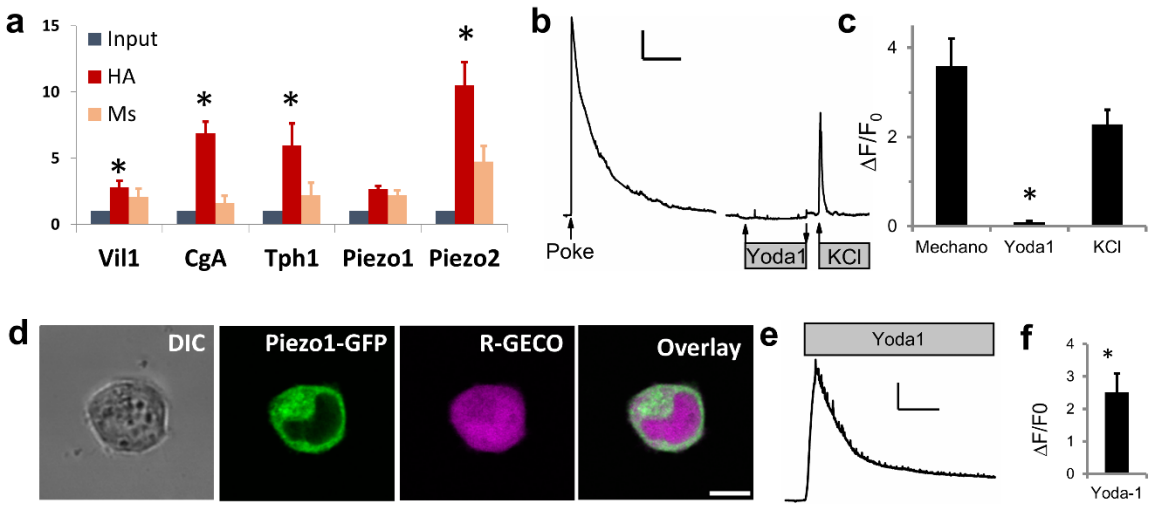


Fig. S2. Piezo1 is not expressed and not functionally relevant in NeuroD1+ cells. a, Expression (mRNA) profiles in *NeuroD1-cre;RiboTag* mouse model using qRT-PCR of epithelium (Input, blue), HA affinity purification (HA, red), and non-targeted mouse IgG control (Ms, brown). Based on expression, the HA-purified samples are enriched (HA/Input) for epithelial marker *Villin1* (*Vil1*) (HA/Input 2.8±0.5, **P*<0.05), for EC cell genes Chromogranin A (*CgA*) (HA/Input 6.9±0.9 **P*<0.05) and Tryptophan hydroxylase 1 (*Tph1*) (HA 6.0±1.6, **P*<0.05), and for *Piezo2* (HA/Input 10.5±1.8, **P*<0.05), but not for *Piezo1* (HA/Input 2.7±0.2, P>0.05) (N=3 mice). **b**, Representative NeuroD1+ cell Ca^{2+} response to direct Mechano stimulation (Poke), followed by 10 µM Yoda1 (1), and 50 mM KCl (scale bars 100 sec, $\Delta F/F_0$ 0.5). **c**, Mean±SEM $\Delta F/F_0$ responses in the same cells to mechanical stimulation (3.60±0.62), 10 µM Yoda1 (0.10±0.03), 50 mM KCl (2.29±0.32) (n=5, **P*<0.05 for Yoda1 compared to Mechano by paired t-test). **d**, Live, confocal image of a HEK cell (DIC) transiently transfected with Piezo1-GFP (courtesy Dr. Philip Gottlieb, SUNY Buffalo), red calcium indicator R-GECO (courtesy Dr. Fouad Chebib, Mayo Clinic) and overlaid images. Scale bar, 10 µm. **e**, Representative Piezo1-GFP HEK cell $\Delta F/F_0$ R-GECO response to 10 µM Yoda1 (scale bars 100 sec, $\Delta F/F_0$ 0.5). **f**, Mean±SEM $\Delta F/F_0$ R-GECO responses to Yoda1 in Piezo1-GFP HEK cells (n=14, 2.51±0.57, **P*<0.05 compared to bath exchange by paired t-test).

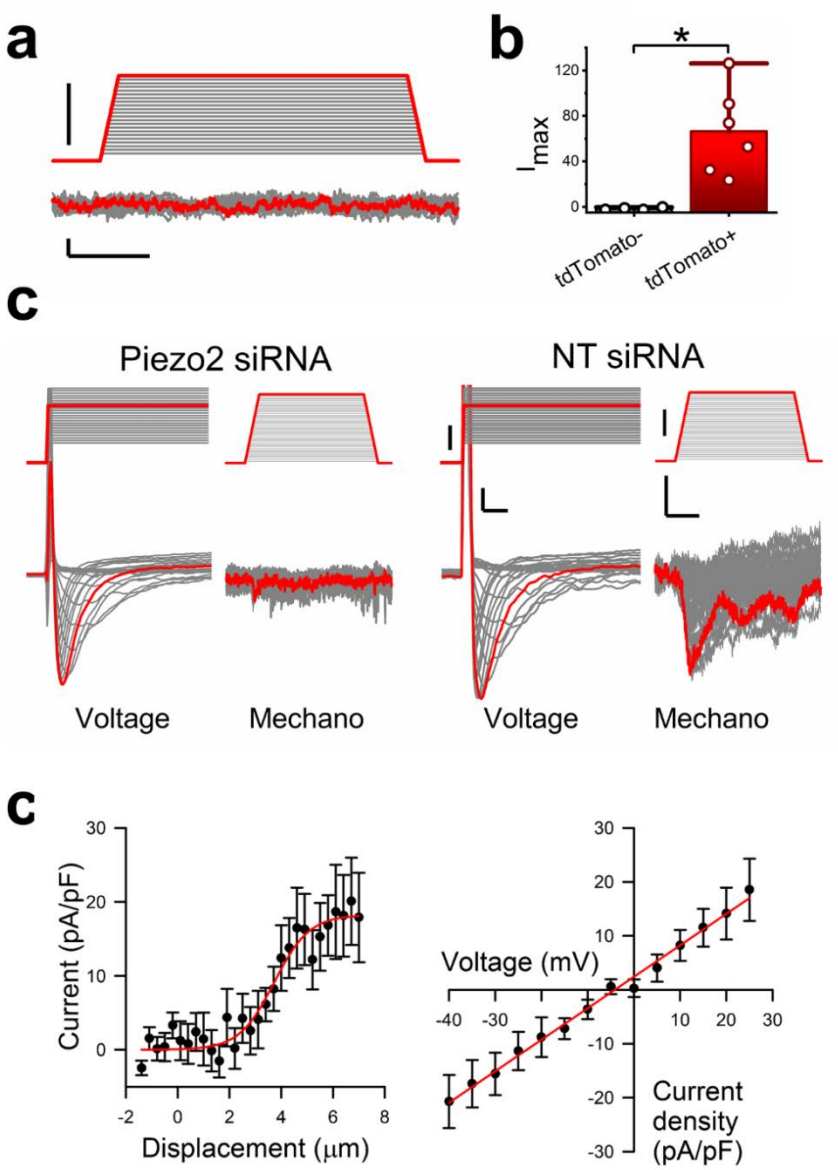


Fig. S3. Non-fluorescent and NeuroD1+ cell electrophysiology controls and analyses. The experiments were performed in primary cultures from *NeuroD1-cre*;*GCaMP5-tdTomato* mouse. **a**, on the left is a representative whole cell voltage-clamp (holding potential -70 mV) mechanostimulation experiment in tdTomato negative non-fluorescent cell. Graded increase in cell membrane deformation (top, 0.3 μm/step) did not result in mechanosensitive currents in these cells (Scale bars 5 μm [top], 20 msec, 10 pA [bottom]). **b**, Mean±SEM peak mechanosensitive whole cell current in non-fluorescent (tdTomato-) cells (-1.6±0.6 pA, n=4) is significantly smaller than NeuroD1+ cells (tdTomato+) (data from Figure 3, 66.5±15.7 pA, n=6) (**P*<0.05 by non-paired t-test). **c** and **d** show that Piezo2 siRNA-treated NeuroD1+ EE cells maintain voltage-dependent Na_V currents(2, 3) but lose mechanosensitive currents. Representative NeuroD1+ tdTomato EE cell voltage-dependent Na_V currents (left panels) and mechanosensitive currents (right panels) for **c**, Piezo2 siRNA, and **d**, NT siRNA. Scale bars are 1 msec, 50 pA for left panels and 20 msec, 20 pA for right panels. **e**-**f**, Capacitance normalized **e**, current-displacement relationships and **f**, voltage-dependence of mechano-sensitive currents at max membrane displacement.

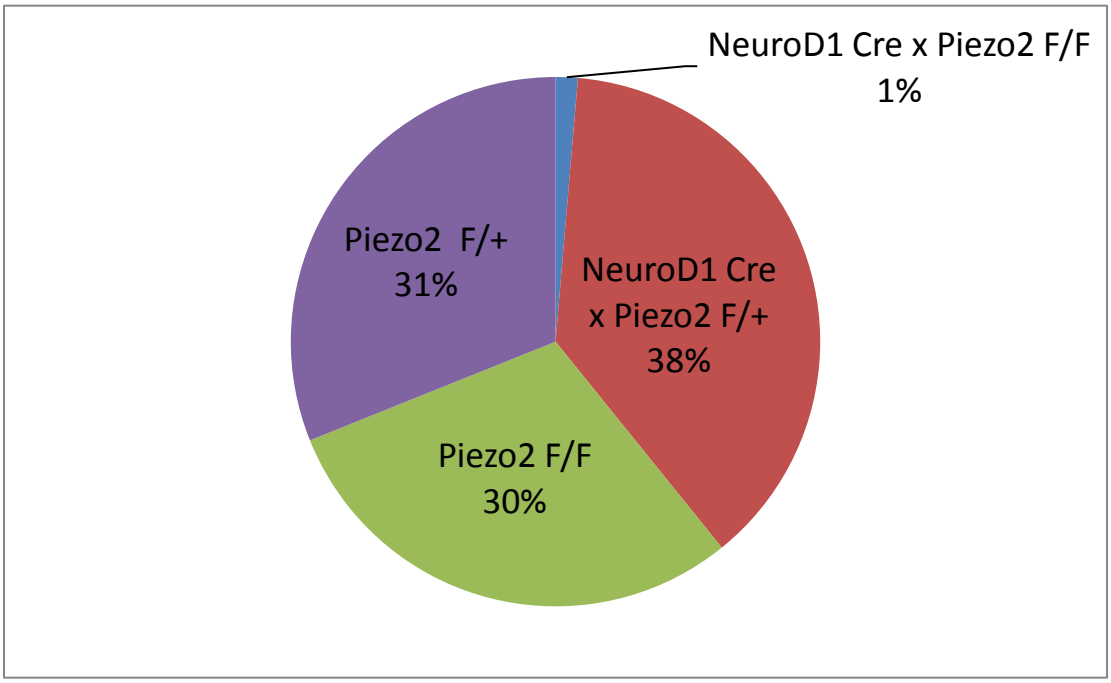


Fig. S4. *NeuroD1-cre;Piezo2$^{f/f}$* is a lethal phenotype (n=74 mice). In an attempt to create cell-specific knockout, we mated the *Piezo2$^{f/f}$* mice with *NeuroD1-Cre;Piezo2$^{f/+}$* mice. Pie chart showing the offspring had the following genotypes: purple *Piezo2$^{f/+}$* (31%, purple), *Piezo2$^{f/f}$* (30%, green), red *NeuroD1-Cre;Piezo2$^{f/+}$* (38%, red) and *NeuroD1-Cre;Piezo2$^{f/f}$* (1%, blue).

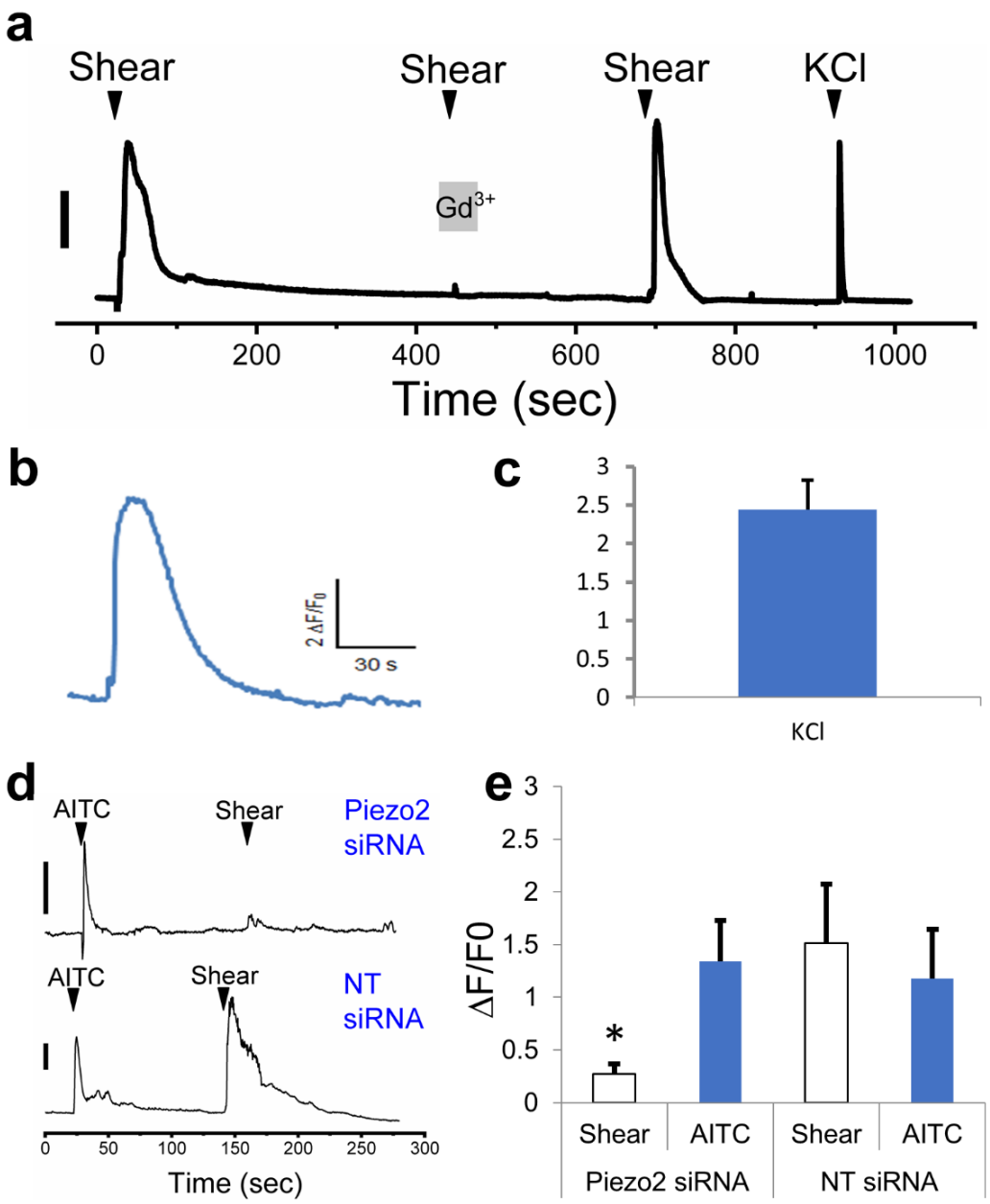


Fig. S5. Calcium imaging NeuroD1+ controls in primary cultures from *NeuroD1-cre;GCaMP5-tdTomato* mouse. a, Representative trace showing full experiment for $\Delta F/F_0$ with Shear, followed by Gd^{3+} (grey bar) with Shear, washout (Shear) and 50 mM KCl. Scale bar (1 $\Delta F/F_0$). **b**, Representative control $\Delta F/F_0$ response to 50 mM KCl, and **c**, Mean±SEM $\Delta F/F_0$ responses for application of 50 mM KCl ($\Delta F/F_0$ =2.6±0.7, n=4). **d** & **e**, TRPA1 agonist (150 µM AITC) and Shear responses in Piezo2 siRNA and NT siRNA NeuroD1+ cells, with **d**, representative responses (Scale bars 0.5 $\Delta F/F_0$) and **e**, Mean±SEM $\Delta F/F_0$ responses for Piezo2 siRNA treated cells ($\Delta F/F_0$ Shear: 0.3±0.1, n=6; $\Delta F/F_0$ and AITC: 1.34±0.38, n=6; **P*<0.05 for shear vs AITC) and NT siRNA treated cells ($\Delta F/F_0$ Shear: 1.67±0.7, n=5 and AITC: 1.2±0.46, n=5; *P*>0.05 for shear vs AITC).

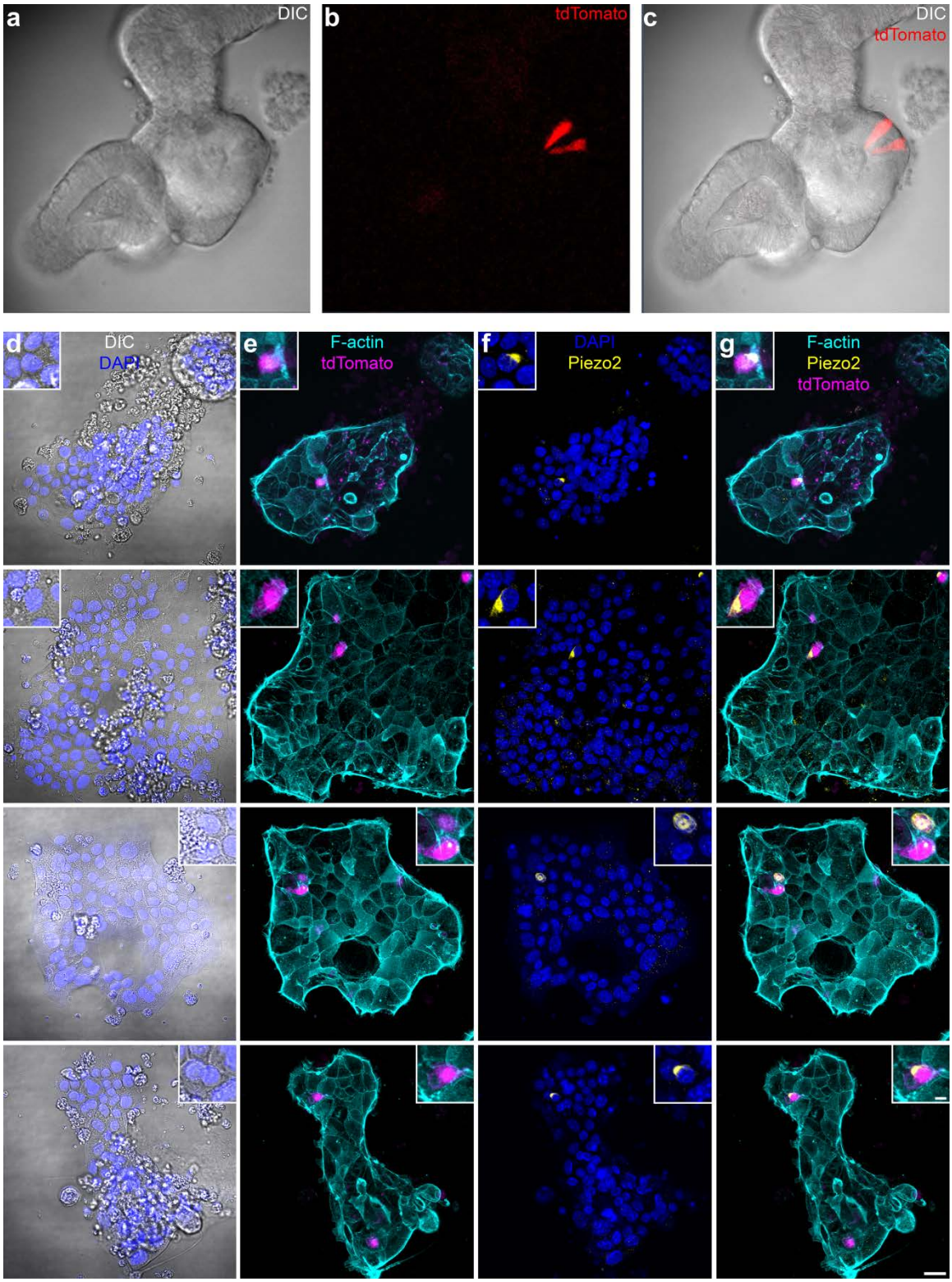


Fig. S6. Planarization of *NeuroD1-cre;GCaMP5-tdTomato* mouse intestinal organoids. a, DIC image of a 3-day old organoid with **b,** tdTomato+ EC cells, and **c**, co-localization DIC and tdTomato. Four representative planarized organoids columns in **d**, DIC with DAPI to label nuclei, **e,** tdTomato and phalloidin to label f-actin, **f**, Piezo2 immunolabeling, and **g**, co-localization. Scale bars = 20 μm (**d-g**) and 5 μm (insets).

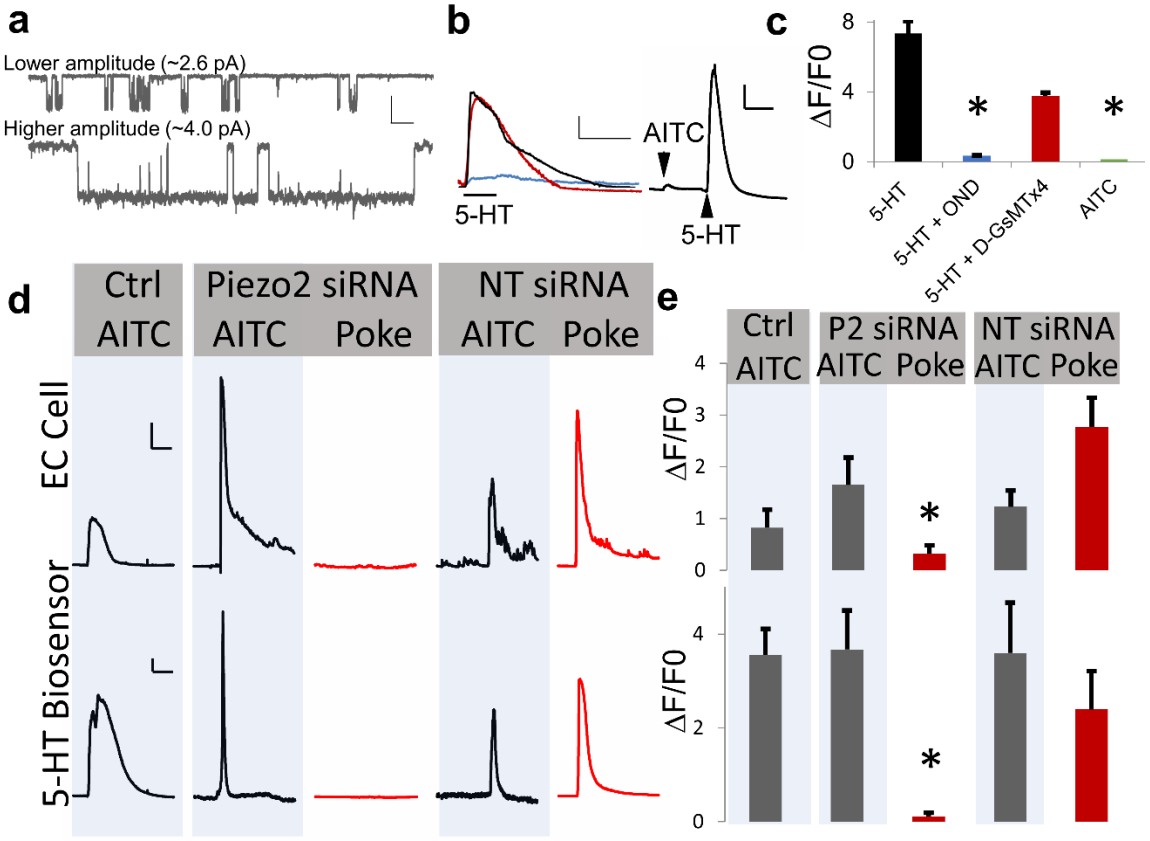


Fig. S7. Serotonin (5-HT) biosensor controls. a, Representative traces of cell-attached patches of HEK293 cells expressing genetically modified high-conductance non-inactivating 5-HT_3 receptors.(4) Pipettes contained 1 µM 5-HT and two amplitudes (~2.6 pA and ~4.0 pA) were found, as previously described.(4)(Scale bars 2pA, 100 msec) **b-c**, Calcium imaging controls from 5-HT_3R biosensor cells. **b**, Representative traces showing Ca^{2+} (GCaMP5) $\Delta F/F_0$ control responses to (left) 20 sec application of 10 µM 5-HT in absence (black) and presence of 10 µM D-GsMTx4 (red) or 0.1 µM Ondansetron (OND, blue trace). Scale bars, 2 $\Delta F/F_0$, 30 sec. On right, 150 µM AITC followed by 10 µM 5-HT (scale bars 100 sec, 0.5 $\Delta F/F_0$). **c**, Mean±SEM $\Delta F/F_0$ responses to 5-HT (black, 7.3±0.7 $\Delta F/F_0$, n=9), 5-HT+Ondansetron (blue, 0.4±0.03 $\Delta F/F_0$, n=11, **P*<0.05 compared to 5-HT, unpaired t-test), 5-HT+D-GsMTx4 (red, 3.8±0.2 $\Delta F/F_0$, n=11, *P*>0.05 compared to 5-HT, unpaired t-test), and AITC (green, 0.1±0.02 $\Delta F/F_0$, n=6, **P*<0.05 compared to 5-HT by paired t-test). **d-e**, siRNA control Ca2+ responses in NeuroD1+ EC cells (top traces and bars) from *NeuroD1-cre;GCaMP5-tdTomato* mouse primary cultures and 5-HT biosensor cells (bottom traces and bars). **d**, Representative NeuroD1+ EC cell and 5-HT biosensor cell responses, showing that chemical activation of TRPA1 by 150 µM AITC results in NeuroD1+ EC cell intracellular Ca^{2+} increase ($\Delta F/F_0$) and 5-HT release from NeuroD1+ EC control (untreated) cells, Piezo2 siRNA treated, and NT siRNA treated NeuroD1+ EC cells. In the same NeuroD1+ EC cells, poke responses and 5-HT release occur only from NeuroD1+ EC cell controls and NT siRNA but not Piezo2 siRNA. Scale bars for top and bottom traces, 0.5 $\Delta F/F_0$, 3 sec. **e**, Mean±SEM for NeuroD1+ EC and 5-HT biosensor cells for control (AITC: $\Delta F/F_0$ EC cell: 0.8±0.4, n=7; $\Delta F/F_0$ biosensor: 3.6±0.6, n=13), and AITC and poke in the same NeuroD1+ EC Piezo2 siRNA treated cells (AITC: $\Delta F/F_0$ EC cell: 1.7±0.5, n=5; $\Delta F/F_0$ biosensor: 3.7±0.8, n=5; Poke: $\Delta F/F_0$ EC cell: 0.3±0.2, n=5; $\Delta F/F_0$ biosensor: 0.1±0.1, n=5), and NeuroD1+ EC NT siRNA treated cells (AITC: $\Delta F/F_0$ EC cell: 1.2±0.3, n=5; $\Delta F/F_0$ biosensor: 3.6±1.1, n=5; Poke: $\Delta F/F_0$ EC cell: 2.8±0.6, n=5; $\Delta F/F_0$ biosensor: 2.4±0.8, n=5). AITC: P>0.05 for Piezo2 siRNA vs NT siRNA. Poke:**P<0.05* for Piezo2 siRNA vs. NT siRNA for EC and biosensor cells.

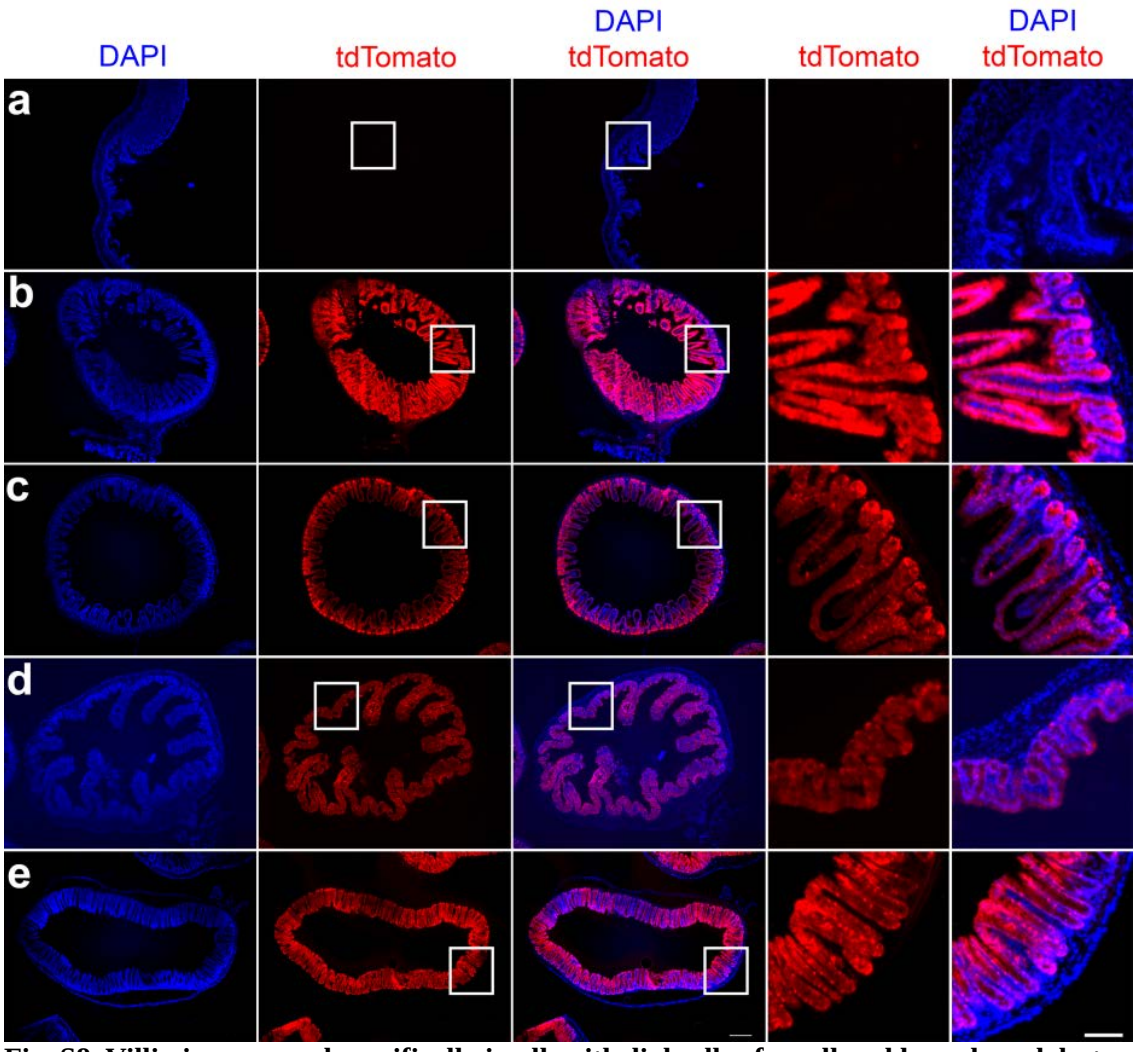


Fig. S8. Villin is expressed specifically in all epithelial cells of small and large bowel, but not stomach. Validation of the Villin-cre (Vil-cre) model using *Vil-cre;Ai9*(*tdTomato*) mouse model. Villin was **a**, not expressed in the stomach, but expressed in **b**, duodenum, **c**, jejunum, **d**, proximal colon and **e**, distal colon. Right two columns are enlarged sections in the white rectangles. Scale bars = 200 μm (**a-e**) and 75 μm (insets).

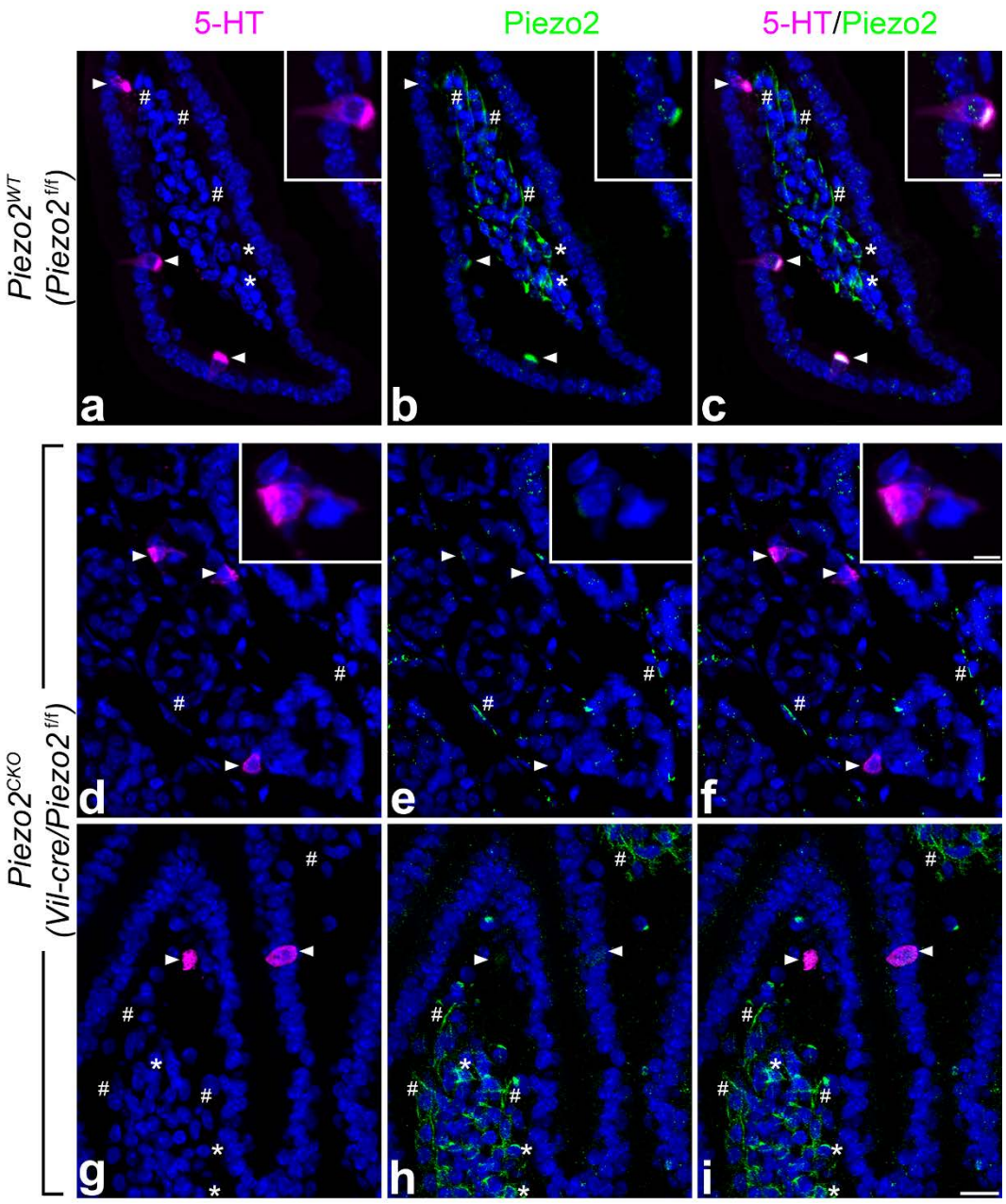


Fig. S9. Validation of gut epithelium conditional Piezo2 knock out knockout mouse model *Piezo2*^{CKO} (*Vil-cre;Piezo2*$^{f/f}$) using immunolabeling and confocal microscopy. In control *Piezo2*^{WT} (*Piezo2*$^{f/f}$) small bowel, DAPI (blue, nuclei), and immunolabeling of **a**, 5-HT (magenta), **b**, Piezo2 (green) and **c**, Co-localization of 5-HT and Piezo2 in 2 of 3 EC cells (arrows). Inset shows a representative 5-HT+ Piezo2+ EC cell. In the subepithelial space (lamina propria), there are Piezo2+ 5-HT- cell bodies (*) and fibers (#) that may represent neurons and their processes, respectively. **d-i,** In *Piezo2*^{CKO} (*Vil-cre;Piezo*$^{f/f}$), with DAPI (blue, nuclei) and immunolabeling of **d**,**g**, 5-HT (magenta), **e**,**h**, Piezo2 (green) and **f**,**i**, Co-localizatoin of 5-HT and Piezo2. These images show that in 5-HT+ EC cells (arrows) Piezo2 is absent. Typical 5-HT+ Piezo2- EC cell shown in inset. In the subepithelial space (lamina propria), there remain Piezo2+ 5-HT- cell bodies (*) and fibers (#) that may represent neurons and their processes, respectively. Scale bars = 20 μm (**a-i**) and 5 μm (insets).

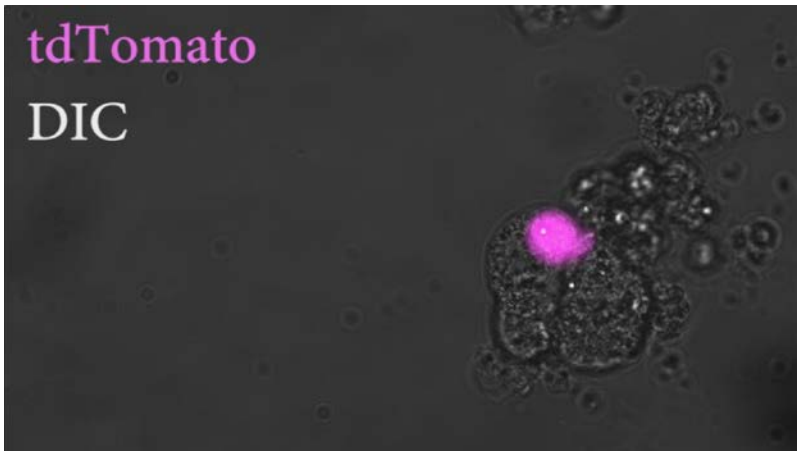
Table S1. PCR primers used in this study.

	Primer Sequence (5'-3')				
	Forward	**Reverse**	**Amplicon Length (bp)**	**Paper or Company**	**Species**
GAPDH	-	-	140	Qiagen	*Mus musculus*
HPRT1	TGGATACAGGCCAGACTTTGTT	CAGATTCAACTTGCGCTCATC	124	(5)	*Mus musculus*
Vil1	TGGAGGAGGAGGATGTGTTC	GGGTCTCAAGGTCTCGGTTT	150	(6)	*Mus musculus*
CgA	CCAAGGTGATGAAGTGCGTC	GGTGTCGCAGGATAGAGAGGA	129	(7)	*Mus musculus*
NeuroD1	AGGAATTCGCCCACGCAGAA	TGGTCATGTTTCCACTTCCTGTTGT	101	Joyce Li, University of Massachusetts.	*Mus musculus*
Tph1	TGTTGACTGCGACATCAGCCGA	GGAAACCAAGGGACAGTCTCCA	138	Origene	*Mus musculus*
Piezo2	GCACTCTACCTCAGGAAGACTG	CAAAGCTGTGCCACCAGGTTCT	140	Origene	*Mus musculus*
Piezo2[#]	CTTGTGAGGTCGGGTGGT	ATGAGGGGATGGGGAGAG	198	(8)	*Mus musculus*
Piezo1	-	-	83	Qiagen	*Mus musculus*

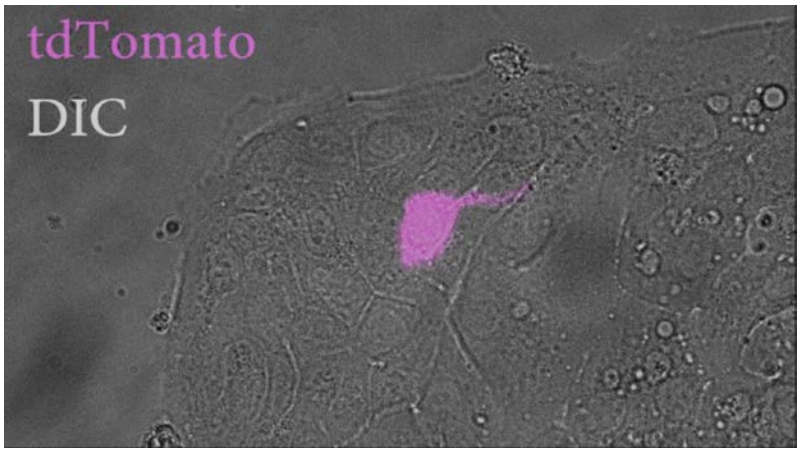
Table S2. Antibodies used in this study.

Antibody	Type	Titre/Concentration	Source	Catalog #	Lot #
Rbt α Piezo2	polyclonal	1:2000 (cryosections), 1:500 (organoids)	A. Patapoutian	Not commercially available	Not commercially available
Alexa Fluor 488-Phalloidin	N/A	1:40/5 Units/mL	Life Technologies	A12379	1785486
Gt α 5-HT (serotonin)	polyclonal	1:2000 (manufacturers stock)	abcam	Ab66047	GR12110-19
Gt α Chromogranin A	polyclonal	1:500/0.4 µg/mL	Santa Cruz	Sc-1488	F0716
Alexa Fluor 647-Ms α HA	monoclonal	1:50/10 µg/mL	BioLegend	682404	B218119
Alexa Fluor 568-Dk α Gt	polyclonal	1:800/2.5 µg/mL	Invitrogen	A11057	1711491
Cy5-Dk α Gt	polyclonal	1:500/3 µg/mL	Jackson	705-175-147	117079
Cy5 Dk α Rbt	polyclonal	1:500/3 µg/mL	Jackson	711-175-152	120907

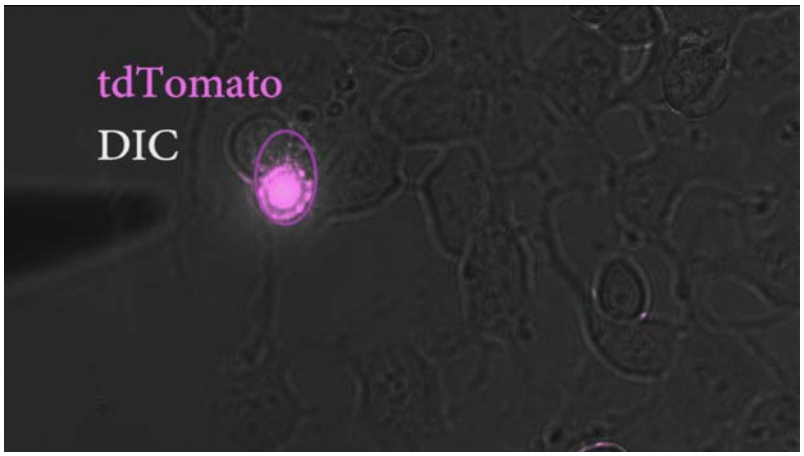
Movie S1. Shear stress increases EC cell intracellular Ca^{2+}.



Movie S2. EC cells within planarized intestinal organoids are mechanosensitive.



Movie S3. Mechanosensitive EC cells release 5-HT in response to membrane displacement.



References

1. Syeda R, *et al.* (2015) Chemical activation of the mechanotransduction channel Piezo1. *eLife* 4.
2. Strege PR, *et al.* (2017) Sodium channel NaV1.3 is important for enterochromaffin cell excitability and serotonin release. *Sci. Rep.* 7(15650).
3. Bellono NW, *et al.* (2017) Enterochromaffin Cells Are Gut Chemosensors that Couple to Sensory Neural Pathways. *Cell* 170(1):185-198 e116.
4. Corradi J, Gumilar F, & Bouzat C (2009) Single-channel kinetic analysis for activation and desensitization of homomeric 5-HT(3)A receptors. *Biophys. J.* 97(5):1335-1345.
5. Wang F, Wang J, Liu D, & Su Y (2010) Normalizing genes for real-time polymerase chain reaction in epithelial and nonepithelial cells of mouse small intestine. *Anal. Biochem.* 399(2):211-217.
6. Sirakov M, Borra M, Cambuli FM, & Plateroti M (2013) Defining suitable reference genes for RT-qPCR analysis on intestinal epithelial cells. *Mol. Biotechnol.* 54(3):930-938.
7. Santana MM, *et al.* (2015) Impaired adrenal medullary function in a mouse model of depression induced by unpredictable chronic stress. *Eur. Neuropsychopharmacol.* 25(10):1753-1766.
8. Maksimovic S, *et al.* (2014) Epidermal Merkel cells are mechanosensory cells that tune mammalian touch receptors. *Nature* 509(7502):617-621.