# Mechanical regulation of stem cell differentiation through stretch-activated Piezo channel 

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#### Abstract

Somatic stem cells constantly adjust their self-renewal and lineage commitment by integrating various environmental cues to maintain tissue homeostasis. While numerous chemical and biological signals have been identified to regulate stem cell behaviors, whether stem cells can directly sense mechanical signals in vivo remains unclear ${ }^{1}$. Here, we show that mechanical stress regulates stem cell differentiation in the adult Drosophila midgut through the stretch-activated ion channel Piezo. We find that Piezo is specifically expressed in previously unidentified enteroendocrine precursor (EP) cells which have reduced proliferation ability and are destined to become enteroendocrine cells (EEs). Loss of Piezo activity reduces EE generation in the adult midgut. Meanwhile, ectopic expression of Piezo in all stem cells triggers both cell proliferation and EE differentiation. Both Piezo mutant and overexpression phenotypes can be rescued by manipulation of cytosolic $\mathrm{Ca}^{2+}$ levels, and increase of cytosolic $\mathrm{Ca}^{2+}$ resembles the Piezo over-expression phenotype, suggesting that Piezo functions through $\mathrm{Ca}^{2+}$ signaling. Further studies suggest that $\mathrm{Ca}^{2+}$ signaling promotes stem cell proliferation and differentiation through separate pathways. Finally, Piezo is required for both mechanical activation of stem cells in a gut expansion assay and the increase of cytosolic $\mathrm{Ca}^{2+}$ in response to direct mechanical stimulus in a gut compression assay. Altogether, our study demonstrates the existence of a special group of stem cells in the fly midgut that can directly sense mechanical signals through Piezo.

Drosophila midgut stem cells have emerged as an attractive in vivo model for understanding adult stem cell behaviors ${ }^{2-4}$. Like their mammalian counterparts, fly intestinal stem cells


[^0](ISCs) produce two major classes of cells that compose the adult intestinal epithelium: absorptive enterocytes (ECs) and secretory enteroendocrine cells (EEs) ${ }^{4}$. Many extrinsic signals, including chemicals, nutrition, pathogens, and cytokines, have been shown to regulate ISCs proliferation and differentiation ${ }^{4,5}$. However, whether midgut stem cells can sense biomechanical signal remains unknown.

From a screen for Gal4 lines with midgut expression, we identified Piezo-Ga4 (BL59266) ${ }^{6}$, a Gal4 under control of a cloned enhancer of Piezo, that was expressed in a subpopulation of escargot (esg) positive stem cells in the adult fly midgut (Extended Data Fig. 1a). Piezo is a cation ion channel that directly senses mechanical tension in lipid bilayers ${ }^{7}$. It was initially identified in mammalian cells as a touching sensor ${ }^{8}$, and further found responsible for mechanoreception in different kind of cell types ${ }^{9}$. The Drosophila genome encodes a single Piezo homolog, which has been characterized previously as a receptor for mechanotransduction in sensory neurons ${ }^{6,10}$.

To faithfully represents the expression pattern of Piezo, we knocked-in a Gal4, PiezoGal4[KI] (we use Piezo-Gal4[KI] as Piezo-Gal4 thereafter), after the first start codon of Piezo through homologous recombination (Extended Data Fig. 1b). UAS-RFP driven by Piezo-Gal4[KI] showed a pattern similar to BL59266 in esg ${ }^{+}$cells, but was also detected in some ECs located in the cardia and copper and iron regions (Fig. 1a, Extended Data Fig. 1c$\mathrm{f}, \mathrm{h}$ ), which is consistent with published Piezo mRNA profiles along the midgut (Extended Data Fig. 1g ) ${ }^{11}$. Because esg is expressed in both ISCs and enteroblast cells (EBs, a progeny of ISCs that is destined to ECs), we used the ISC specific marker Delta-lacZ and the EB marker $\mathrm{Su}(\mathrm{H}) \mathrm{Gbe}$-lacZ to precisely identify Piezo $^{+}$cells. Strikingly, Piezo is expressed in a subpopulation $(\sim 40 \%)$ of $\mathrm{Dl}^{+}$cells, and is absent from EBs (Fig. 1a, Extended Data Fig. 1i). We also noticed that all "newborn" EEs - esg and Prospero (Pros, the EE specific marker) double positive cells - are also Piezo ${ }^{+}$, suggesting that Piezo $^{+}$cells may represent EE cell precursors (Fig. 1c, Extended Data Fig. 1k,1). Indeed, G-TRACE ${ }^{12}$ labeled progenies of Piezo+ cells are primarily EEs $(\sim 90 \%)$, compared with ISCs $\left(\mathrm{Dl}^{+}\right)$and EBs $\left(\mathrm{Su}(\mathrm{H}) \mathrm{Gbe}^{+}\right)$ (Fig 1d,e, Extended Data Fig. 1m-o). Additionally, Bleomycin damage ${ }^{13}$ or inhibition of Notch by the $\gamma$-secretase inhibitor DAPT ${ }^{14}$ promotes both EE and Piezo ${ }^{+}$cell generation (Fig. 1f, Extended Data Fig. 2a). Finally, ablation of $\mathrm{Piezo}^{+}$cells using the pro-apoptotic protein Reaper (Rpr) significantly reduced not only $\mathrm{Piezo}^{+}$cells but also EE cells number after 4 weeks (Fig. 1g,h), and both cell types are recovered after one-week of suppression of Rpr expression (Fig. 1g,h), suggesting that Piezo ${ }^{+}$cells are an important source for EE generation. We further investigated whether $\mathrm{Piezo}^{+}$cells are self-regenerative or primarily derived from ISCs. First, mitotic Piezo ${ }^{+}$cells (marked by anti-phospho-Histone3 staining) only represent a small portion ( $\sim 10 \%$ ) of the total mitotic cells (Fig. 1i, Extended Data Fig. 2c-f), suggesting that Piezo ${ }^{+}$cells have reduced proliferation abilities compared to Piezo ${ }^{-}$ stem cells. Bleomycin damage promotes the mitosis of both $\mathrm{Piezo}^{+}$and $\mathrm{Piezo}^{-}$cells without increasing the percentage of Piezo ${ }^{+}$mitotic cells, suggesting that an intrinsic mechanism limits the proliferation ability of $\mathrm{Piezo}^{+}$cells (Extended Data Fig. 2d,e). Finally, random GFP-marked clone generated from ISCs contains $\mathrm{Piezo}^{+}$cells, supporting that $\mathrm{Piezo}^{+}$cells are generated from ISCs (Extended Data Fig. 2g).

Altogether, our data suggest that previously considered $\mathrm{Dl}^{+}$ISCs are heterogeneous and composed of $\sim 60 \%$ mitotic active multipotent ISCs $\left(\mathrm{Piezo}^{-}\right)$and $\sim 40 \%$ less mitotic unipotent Piezo ${ }^{+}$cells that mainly generate EEs. To avoid confusion with true ISCs (mitotic active and multipotent) and EBs (occasionally referred as Notch active EC progenitors), we refer to these Piezo ${ }^{+}$population as "enteroendocrine precursors" (EPs).

To investigate the function of Piezo, we analyzed the phenotype of Piezo ${ }^{K O}$, a null allele with a complete deletion of the Piezo coding sequence ${ }^{6}$. Midguts from Piezo ${ }^{K O}$ homozygous flies showed no obvious phenotypes as compared to control flies during the early developmental and young adult stages, albeit Piezo is expressed in some stem cells during the larval and pupal stages (Extended Data Fig. 3). In wildtype (WT) flies, the number of esg $^{+}$and EE cells increases as flies age ${ }^{15}$. However, in Piezo ${ }^{K O}$ mutants, the number of EEs, but not esg ${ }^{+}$cells, fails to increase (Fig. 2a,b), suggesting that the generation of EEs after adulthood is affected. Additionally, Piezo mutant clones generate $80 \%$ fewer EEs than controls, which can be rescued by expressing GFP-tagged full-length Piezo (Fig. 2c,d). These data suggest that the reduced EE generation is an autonomous defect.

Previous studies have shown that Piezo functions through increase of cytosolic $\mathrm{Ca}^{2+16-19}$. Consistently, knocking down Stromal interaction molecule (Stim), previously used as an effective target to decrease cytosolic $\mathrm{Ca}^{2+20}$, also led to the production of fewer EEs (Fig $2 \mathrm{c}, \mathrm{d})$. Further, elevating cytosolic $\mathrm{Ca}^{2+}$ by knocking down Plasma membrane calcium ATPase (PMCA) or Sarco-endoplasmic reticulum calcium ATPase (SERCA) rescued and even reversed the reduction of EEs in the Piezo mutant (Fig 2c,d). Meanwhile, overexpressing Piezo in esg ${ }^{+}$cells caused an increase of both esg ${ }^{+}$cells and EEs, which phenocopied the increase of $\mathrm{Ca}^{2+}$ through SERCA reduction, inositol-1,4,5-trisphosphate receptors (IP3R), Stim, and Orai over-expression, as well as PMCA knockdown (Fig 2e, Extended Data Fig. 4a-c, 5). Calcium imaging shows that cytosolic $\mathrm{Ca}^{2+}$ is significantly increased by Piezo over-expression in the stem cells (Extended Data Fig. 6a-d, Extended Data Video 1,2). Meanwhile, the Piezo over-expression phenotype is suppressed by reducing cytosolic $\mathrm{Ca}^{2+}$ using either Stim-RNAi or InsP3R-RNAi (Fig 2e, Extended Data Fig. 4a-c). Finally, Bleomycin damage triggers an up-regulation of $\mathrm{Ca}^{2+}$ and cell number increase of esg $^{+}$and EE cells in both WT and Piezo ${ }^{K O}$ midguts, supporting that $\mathrm{Ca}^{2+}$ is the downstream effector of Piezo (Extended Data Fig. 5d-e, 6e-h).

Inhibition of Notch signaling has been shown to promote both ISCs renewal and EEs differentiation ${ }^{14,21}$, even in EE progenitors that already have low Notch activity ${ }^{22}$. Meanwhile, increase of cytosolic $\mathrm{Ca}^{2+}$ has been found to inhibit Notch activity in both cultured mammalian cells and flies ${ }^{23,24}$. Therefore, we tested whether Piezo functions through Notch inhibition by increasing cytosolic $\mathrm{Ca}^{2+}$. Indeed, blocking Notch activation by knocking-down a fucosyltransferase ( $O$-fut) essential for Notch processing reverses the Piezo ${ }^{\text {KO }}$ phenotype (Fig. 2c,d, Extended Data Fig. 4h), and increasing Notch activity by expression of the Notch intracellular domain ( $\mathrm{N}^{\mathrm{ICD}}$ ) blocks the phenotype of both Piezo over-expression and SERCA knockdown (Fig. 2e, Extended Data Fig. 4a-g). Further, overexpressing Piezo in $\mathrm{esg}^{+}$cells produced more $\mathrm{Dl}^{+}$stem cells, consistent with a reduction in Notch activity (Extended Data Fig. 6i,j). Finally, neither Piezo overexpression nor SERCA knockdown had any effects in EB cells (in which Notch has already been activated),
supporting that Notch signaling is the primary target (Extended Data Fig. 6k,l). Altogether, our data suggest that Piezo promotes EE differentiation by elevating cytosolic $\mathrm{Ca}^{2+}$ and inhibition of Notch.

To further dissect the function of $\mathrm{Ca}^{2+}$, we used channelrhodopsin (ChR) to optogenetically increase cytosolic $\mathrm{Ca}^{2+}$ levels. Activation of ChR in $\mathrm{Dl}^{+}$cells promotes both ISCs proliferation and EEs production, resembling the Piezo over-expression phenotype (Fig. 3a,b; Extended Data Fig. 7a-d). This ChR-induced phenotype is blocked by knockdown of both Stim and InsP3R, suggesting that this effect is $\mathrm{Ca}^{2+}$ dependent (Extended Data Fig. 7e,f). In addition, activation of ChR in Piezo ${ }^{+}$EP cells significantly increased EE cells at the expense of EP cells, suggesting an increase of differentiation from EPs to EEs (Fig. 3a,b). A recent study showed that Piezo activation promotes cell proliferation through $\mathrm{Ca}^{2+}$ induced ERK (extracellular signal-regulated kinase) phosphorylation ${ }^{19}$. Consistently, over-expression of Piezo in $\mathrm{esg}^{+}$cells increases phospho-ERK staining (Extended Fig. 7g). However, reducing ERK signaling through Ras knockdown, or blocking cell proliferation by Yorkie$R N A i$ only affect cell proliferation but not EE differentiation in Piezo over-expressing cells (Extended Fig. 7h-k), supporting that Piezo promotes EE differentiation independently of proliferation. Consistently, increasing cytosolic $\mathrm{Ca}^{2+}$ by Thapsigargin (Thap), a SERCA inhibitor, significantly increased stem cell proliferation and EEs generation (Fig. 3c,d). Further blocking mitosis using the MEK (mitogen-activated protein kinase kinase) inhibitor Trametinib (Tram), only reduced Thap-trigged proliferation, but not the increase in EEs differentiation (Fig. 3c,d, Extended Data Fig. 71-n). $\mathrm{Ca}^{2+}$ imaging showed that $\mathrm{Ca}^{2+}$ is increased in stem cells treated by Thap, which is not blocked by Tram (Extended Data Fig. $70-q$, Extended Data Video 3). All together, these data suggest that cytosolic $\mathrm{Ca}^{2+}$ increase promote cell proliferation (through ERK phosphorylation) and cell differentiation (though Notch inhibition) in a cell context dependent manner.

To test if mechanical challenges from food digestion can activate Piezo, we increased the mechanical load in the GI track by feeding flies with food containing indigestible fiber, methylcellulose (MC), a widely used food thickener and ingredient for cell culture. This MC food induce an "over-full" phenotype, as fly midguts, from~10-15\% flies after 4-5 days of MC feeding, showed a significant increase in diameter (Fig 4a, Extended Data Fig. 8). Interestingly, midguts with increased diameter showed a significant increase in the number of $\mathrm{esg}^{+}$cells and EEs (Fig 4b,c), as well as Piezo ${ }^{+}$EP cells (Extended Data Fig. 8g-j). This effect is blocked by either Piezo knock-down or null mutant (Fig 3b,c, Extended Data Fig. $8 \mathrm{k}, \mathrm{l}$ ). Live-cell imaging of $\mathrm{Ca}^{2+}$ activities shows an increase of average $\mathrm{Ca}^{2+}$ level in MC fed flies, suggesting that the phenotype is related to increased $\mathrm{Ca}^{2+}$ level (Extended Data Fig. 8n-q, Extended Data Video 4). Indeed, this over-full phenotype is blocked by reducing cytosolic $\mathrm{Ca}^{2+}$ (Fig. 4b,c, Extended Data Fig. 8n-q, Extended Data Video 4), suggesting that the mechanical stress generated by the indigestible food promotes EEs generation through Piezo activation and subsequent increase in cytosolic $\mathrm{Ca}^{2+}$. As Piezo is mainly enriched in EP cells, the increase of stem proliferation may be caused by either a feedback signal from the increased EE generation ${ }^{25}$ or by low level of Piezo present in the ISCs.

To test directly whether mechanical forces can activate EP cells, we engineered a microfluidic chip that can hold a dissected fly midgut and generate a mechanical
compression through controlled air pressure (Fig. 4d, Extended Data Fig. 9a-d). Using this device, we recorded the calcium signal in $\mathrm{Dl}^{+}$stem cells of the fly midguts (Piezo-Gal4 was tested initially but was not used due to the low GCAMP6s expression.) Interestingly, significantly more stem cells showed high cytosolic $\mathrm{Ca}^{2+}$ upon mechanical compression, and this activation was only triggered transiently by the change in tissue shape, as $\mathrm{Ca}^{2+}$ activity returned to normal within $\sim 20 \mathrm{~s}$ even in the presence of constant compression ( Fig 4 e ; Extended Data Video 6). Importantly, this mechanically triggered $\mathrm{Ca}^{2+}$ activity is significantly reduced in either Piezo ${ }^{K O}$ or Piezo ${ }^{R N A i}$ midguts (Fig 4e, Extended Data Fig. $9 \mathrm{e}-\mathrm{g}$; Extended Data Video 7, 8). Finally, either increase of cytosolic $\mathrm{Ca}^{2+}$ through $\operatorname{SERCA}$ knockdown or decrease of cytosolic $\mathrm{Ca}^{2+}$ through Stim and IP3R knockdown render the cells irresponsive to the mechanical stimulus (Fig. 4f, Extended Data Fig. 9h-1, Extended Data Video 9,10). These data suggest that $\mathrm{Ca}^{2+}$ levels in $\mathrm{Piezo}^{+}$cells can be regulated by a transient mechanical stimulus, which may be generated by repeated vascular muscle contractions during digestion.

In conclusion, we have demonstrated that a new population of unipotent stem cells (EPs) can directly sense mechanical signals in vivo to adjust their differentiation accordingly, and that this mechanosensing is mediated through Piezo activation and cytosolic $\mathrm{Ca}^{2+}$ increase. Our findings suggest a potential direct linkage between food digestion with generation of EEs, which regulate various physiological functions, including stem cell proliferation, intestinal motility, digestion, and appetite ${ }^{25,26}$. Such mechanism may provide the midgut ability to response to particular mechanical challenges to maintain tissue homeostasis.

## METHODS

Drosophila stocks and culture
The following strains were obtained from the Bloomington Drosophila Stock Center: UASmtdTomato3XHA (BL30124), UAS-tdTomato (BL3321, BL3322), UAS-IVS-NES-jRGECO (BL63795), UAS-IVS-GCaMP6s (BL42746), UAS-mCherry.CAAX (BL59021), UASmCherry.nls (BL 38424), UAS-CsChrimson (BL55134), UASp-Act5C-mRFP((BL24778); UAS-mCD8-GFP(BL32185), G-Trace fly: UAS-RedStinger, UAS-Flp1.D, Ubi(FRT.Stop)Stinger/CyO (BL28280); Act-(FRT.Stop)lacZ, Ubi-(FRT.Stop)Stinger/CyO (isolated from BL51308); hsFLP; Sco/CyO (BL1929); Piezo-Gal4 (with cloned promoter, BL59266); RNAi lines as previously reported ${ }^{20}$ : UAS-Serca ${ }^{R N A i}$ (BL25928), UASStim $^{R N A i}$ (BL27263, BL52911), UAS-Stim (BL41757), tub-Gal80ts (BL7016), UAS-Ofut1 ${ }^{R N A i}$ (BL9377), UAS-InsP3R ${ }^{R N A i}$ (BL25937, BL51686), UAS-Notch ${ }^{I C D}$ (BL52008), $U A S-t t k 69^{R N A i}(\mathrm{BL} 26315, \mathrm{BL} 36748), U A S-I n s P 3 R$ (BL30742) and UAS-PmcaRNAi (BL31572); UAS-Rpr (BL5823), Piezo ${ }^{K O}$ (BL58770), UAS-GFP-Piezo/CyO (BL58772), UAS-GFP-Piezo/TM6B (BL58773) ${ }^{6}$. UAS-Ras $1^{R N A i}$ (106642), UAS-YkiRNAi (104523), $U A S$-Piezo ${ }^{R N A i}$ (2796), $U A S$-ase ${ }^{R N A i}$ (108511), UAS-ttk69 ${ }^{R N A i}$ (101980) as previously reported ${ }^{10,29}$, was from the Vienna Drosophila RNAi Center. esg-GFP was from David Doupe. $S u(H)-l a c Z$ was from Pedro Saavedra; hsFlp, tub-Gal4, UAS-nlsGFP; FRT40, tubGal80 was from Kevin Kim; Su(H)-Gal4 and Dl-Gal4 was from Steven X. Hou ${ }^{30}$, UAS-Orai was from Gaiti Hasan, esg-Gal4, UAS-nlsGFP, and Dl-lacZ were from lab stocks. Flies were
reared on standard cornmeal/agar medium supplemented with yeast. Adult flies were entrained in 12:12 light-dark cycles at $25^{\circ} \mathrm{C}$ unless specifically stated otherwise.

To prepare Methylcellulose (MC) food, $10 \%$ w/w MC (sigma, 274429) was added to $5 \%$ sucrose solution and stirred until fully dissolved. Adult flies 5-7 days after hatching were water-starved (soaked filter paper) for one day at $29^{\circ} \mathrm{C}$, and transferred to vials with MC food or control food ( $5 \%$ sucrose soaked filter paper). Food was changed every other day. Fly midguts with a significantly enlarged diameter ( $>50 \%$ increase compared with the normal section of the same midgut) were counted as enlarged MC feed gut ( $\sim 10-15 \%$ of total dissected midguts).
$4 \mu$ M DAPT (Sigma, D5942), $10 \mu \mathrm{~g} / \mathrm{ml}$ Bleomycin (Calbiochem \#203408), $0.5 \mu \mathrm{M}$ Thapsigargin (Tocris, 1138), and 10 $\mu \mathrm{M}$ Trametinib (Selleckchem, S2673) were used for chemical treatment. All feeding experiments were done using 5\% sucrose saturated filter paper unless specifically stated otherwise.

For the lineage tracing experiments ${ }^{12}, 4-5$-day-old flies were incubated at $32{ }^{\circ} \mathrm{C}$ for one day to activate Gal4 and then maintained at $25^{\circ} \mathrm{C}$ for 7 days. For Piezo-Gal4, flies were incubated at $32{ }^{\circ} \mathrm{C}$ for 4 days and then maintained at $25^{\circ} \mathrm{C}$ for 3 days because of its low activity. Lineage tracing of MC feed fly was done by induction of flies for 4-5 days under $32^{\circ} \mathrm{C}$ and when feeding the fly on $5 \%$ sucrose $+10 \% \mathrm{MC}$ food for 4 days at $25^{\circ} \mathrm{C}$. To visualize the Gal4 expressing cells, flies were shifted to $32{ }^{\circ} \mathrm{C}$ overnight before analysis. To create random clones using hsFLp, Ubi-(FRT.Stop)Stinger, we heat shocked the 3-4 days old adult flies at $37^{\circ} \mathrm{C}$ for 30 min and then kept them at $25^{\circ} \mathrm{C}$ for 2 weeks.

For the MARCM experiments ${ }^{31}$, 4-5-day-old flies were heat-shocked three times at $37{ }^{\circ} \mathrm{C}$ for 1 hour within one day. Then flies were maintained at $25^{\circ} \mathrm{C}$, except for the flies containing RNAi which were maintained at $32{ }^{\circ} \mathrm{C}$ to increase the expression of the dsRNAs. Temperature has no significant effect on the ratio of EEs in the progenies (data not shown). Midguts from female flies were analyzed after 14 days. (GFP positive clones were induced by transient incubation at $32^{\circ} \mathrm{C}$, then flies were kept at $25^{\circ} \mathrm{C}$ for 10 days and $32^{\circ} \mathrm{C}$ overnight before analysis)

## Immunofluorescence imaging

Immunostainings of Drosophila midguts were performed as previously described ${ }^{32}$. The following primary antibodies were used: mouse anti-Prospero (1:50, Developmental Studies Hybridoma Bank, MR1A), rabbit anti-phospho-Histone H3 (Millipore \#06-570; 1:1000); mouse anti-HA (Abcam, ab18181), rabbit anti-dpErk1/2 (Cell Signaling \#4370; 1:500), mouse anti-Delta (1:50, Developmental Studies Hybridoma Bank, C594.9B), mouse anti- $\beta$ galactosidase (1/400, Promega, Z3781), rabbit anti-Tachykinin (1/5000, Veenstra et al. ${ }^{33}$ ). Secondary antibodies were goat anti-rabbit and anti-mouse IgGs conjugated to Alexa 555 and Alexa 647 (used at 1:500, Thermofisher, A-21428, A-21244, A-21235, A-21422). Fly guts were mounted in Vectashield with DAPI (Vector Laboratories). In all micrographs, blue staining shows the nuclear marker DAPI. Fluorescence micrographs were acquired with a Zeiss LSM 780 confocal microscope. All images were adjusted and assembled in NIH ImageJ.

## CRISPR/Cas9 genome editing

Guide RNAs (gRNAs) targeting the start codon of Piezo were designed using the "Find CRISPRs" online tool (http://www.flyrnai.org/crispr2/() ${ }^{34,35}$. The genome editing efficiency of different candidate gRNAs was tested in tissue culture using T7 endonuclease assay ${ }^{36}$, and the following sequence with highest cutting efficiency was used:

CTGGAGGAGAACGGCGCCGG.
$\sim 1 \mathrm{~kb}$ genomic fragments from the upstream and downstream of the start codon were amplified from fly genome using following primers:

Up F: CTTCGGTACCGGATCACTGTGCATGTGAGGCATTA
Up R: GCTTCATTTTGGATCACTCAGACTCCGACTCCAAC

## Dn F: CGGCGGCCGCTCTAGTCAGCTATGCGTGCATGGT

Dn R: AAGCTGGGTGTCTAGGGGAATGTGGTAGGCAAACTA

Genomic fragments were cloned into the up- and down-stream of Gal4-SV40 in pENTR vector by In-fusion (Clontech) to make the donor construct.

For CRISPR/Cas9-mediated homologous recombination, gRNA in pCFD3 (0.2ug/ul) and donor DNA ( $0.5 \mathrm{ug} / \mathrm{ul}$ ), were co-injected into the embryos of nos-Cas9/attP2 flies ${ }^{37}$. Knockin flies were selected by genomic PCR using following primers from insertion and Piezo gene:

## Upstream: F. CCCACAATTTCGCACTCTTT

## R. GTCTTCACGGGGAAAAATGA

## Downstream: F. GTGGTTTGTCCAAACTCATCAATG

## R. CGGACAGCAGGAAAATGAGA

Piezo-Gal4 knock in homozygous flies are viable and fertile. qPCR of whole adult flies showed that Piezo mRNA from homozygous Piezo-Gal4 knock-in flies was reduced by $\sim 50 \%$ compared to Piezo-Gal4/CyO. (The mRNA of Piezo from Piezo-Gal4/CyO was not significantly different from WT flies.) Also, qPCR of Piezo ${ }^{K O}$ (BL58770) is consistent with this allele being a complete null ${ }^{6}$ as it showed a $>95 \%$ reduction of Piezo mRNA.

## Optogenetic activation of CsChrimson in fly midgut

Red-shifted channelrhodopsin CsChrimson ${ }^{38}$ was used to increase cytosolic $\mathrm{Ca}^{2+}$ in stem cells by light. UAS-CsChrimson was expressed using either Dl-Gal4 or Piezo-Gal4. All crosses and the early development of flies were under dark conditions at $18{ }^{\circ} \mathrm{C}$. Experiment was done at $25^{\circ} \mathrm{C}$. Adult flies were kept either on $2 \%$ Agarose food containing 5\% sucrose $+1 \%$ yeast extract under dark or on $2 \%$ Agarose food containing 5\% sucrose $+1 \%$ yeast extract +50 mM all-trans-retinal (ATR) in presence of orange-red light from LED. 2X1 meter SMD5050 RGB LED strip (total power ~2 X 4 Watt, eTopxizu) was attached to the
inner wall of a cylinder chamber ( $\sim 10 \mathrm{~cm}$ in diameter and 15 cm in height) covered by aluminum foil to enhance the light intensity Extended Data Fig. 7a. The RGB LED strip was set at constant maximal brightness with green ( $500 \sim 560 \mathrm{~nm}$ ) and red ( $600 \sim 650 \mathrm{~nm}$ ) LED units on (estimated light intensity $\sim 2.5 \mathrm{~mW} / \mathrm{cm}^{2}$ ). The power of the LED is controlled manually to maintain $12 / 12$ on/off circadian rhythm. Flies were kept under indicated condition for 2 weeks before analysis.

## Calcium imaging

Cytosolic $\mathrm{Ca}^{2+}$ was monitored in ISCs using the red fluorescent indicator RGECO $^{39}$. GFP was used as an internal control and an indicator of stem cells and EBs. Young adult flies (45 days after eclosion) were first incubated at $32{ }^{\circ} \mathrm{C}$ for $5-7$ days before the experiment. For live-cell imaging experiment, dissected intact midgut was cultured in adult-hemolymph-like (AHL) media plus $2 \%$ fetal bovine serum (FBS). Addition of FBS into the AHL moderately increases the average cytosolic $\mathrm{Ca}^{2+}$ level and reduced the oscillation frequency, but allow a longer maintenance of dissected midgut under normal condition up to 5-6hr. Air-permeable lummox dish (SARSTEDT, 94.6077.331) was used as the imaging device as previously described ${ }^{40}$. Images of anterior midgut area were captured on Zeiss LSM 780 confocal microscope equipped with definite focus using Plan-Neofluar 25x/oil N.A. 0.8 lens. A zstack of dual-color image ( 488 nm excitation/500-550nm detection for GFP, and 561 nm excitation/580-650nm detection for RGECO) was recorded every 20 sec . Both color channels were recorded simultaneously with line-based scanning. Images were manually analyzed in NIH ImageJ.

## Microfluidic chip design and operation

The fly gut was immobilized and force stimuli applied in a microfluidic chip. The design took advantage of the pressure sensitivity of the poly material (PDMS, building materials of the microfluidics), and had been applied in previous studies of C. elegans ${ }^{41}$. The chip was designed using the software of Tanner L-Edit and fabricated following standard microfluidics fabrication procedures ${ }^{42}$. The layout of the design is shown in Extended Data Fig. 9. The middle channel was designed for loading and holding the gut, with the size of 6 mm long and $200 \mu \mathrm{~m}$ wide. The two-side channels were for delivering the pressure, with the size of 1 mm long and $450 \mu \mathrm{~m}$ width. The membrane in between is $70 \mu \mathrm{~m}$ wide, which was used for squeezing the guts when pressures were applied. The pattern was transferred onto a silicon wafer via photoresist with the height of $200 \mu \mathrm{~m}$, which was then transferred to PDMS and bonded with glass. To achieve the desired softness, the PDMS was mixed 20:1 with the cross-linker.

Freshly dissected fly midguts were loaded in the channel inlet with the anterior part of the gut located in the middle between the two membranes. In the device, compressed air is connected to the side channels via a bidirectional switch. In the off state, the side channels are at the atmospheric pressure, and no pressure is applied on the gut. When switched to the on state, compressed air presses the PDMS membrane and squeezes the gut. The ratio of the channel width reduction was $\sim 30 \%$ during the compression and the relaxation time of the PDMS membrane was $\sim 1 \mathrm{sec} . \mathrm{Ca}^{2+}$ signals were indicated by GCAMP $6 \mathrm{~s}^{43}$ and captured using a Zeiss LSM 780 confocal microscope equipped with a definite focus using Plan-

Neofluar 10x/0.30 lens. The anterior midgut area was recorded as time-lapse of z-stacks capturing the whole depth of the midgut every 2 sec . GCAMP6s emission was excited at 448 nm and recorded at 500-550 nm and tdTomato was excited 561 nm at and recorded at 580$610 \mathrm{~nm} . \mathrm{Ca}^{2+}$ imaging experiments were done with identical acquisition parameters for consistency. Images from the experiment were projected using maximum intensity projection and analyzed using a macro in ImageJ to automatically detect the number of GFP-positive cells in each frame. Tracing of $\mathrm{Ca}^{2+}$ signals in individual stem cells was done using Z-axis profiling function of NIH ImageJ. $\mathrm{Ca}^{2+}$ signal in individual stem cells during mechanical compression was tacked manually.

## RT-qPCR

Total RNA was extracted from 5-7 days old female by TRIZOL reagent (Thermo Fisher), converted to cDNA template after DNase I treatment and purification by QIAGEN RNeasy kit. Real-time PCR was performed using SYBR Green with GAPDH and alpha-tubulin as an internal control. Piezo mRNA was detected by two pairs of independent primers (Supplementary Table 2).

## Statistics and Reproducibility

All the images presented and used for quantification are from the anterior region of adult female fly midgut for consistency. 2-3 square areas ( $10,000 \mu \mathrm{~m}^{2}$ unless specified otherwise) were randomly selected from each midgut and quantified automatically using cell counting function of NIH ImageJ. All experiments were independently biologically repeated at twice (unless specified otherwise) with similar results presented in the figures. No randomization or blind test was used. Statistical analysis was performed using Microsoft Excel. All pvalues were determined by two-tailed Student's t-test with unequal variances. Sample sizes were chosen empirically based on the observed effects and listed in the figure legends.

## Data availability

All relevant data have been included in the paper and the supplementary files. Original quantifications of different cell numbers were listed in the Supplementary Dataset file. Complete genotypes information is provided in Supplementary Table 1. Original data that support the findings of this study are available from the corresponding author upon request.

## Extended Data



Extended Data Figure 1. Piezo expression pattern and Piezo ${ }^{+}$cell lineage in the fly midgut. a. Expression pattern of Gal4 (BL59266) driven by the Piezo promoter ${ }^{6}$. b. Schematic of Drosophila Piezo gene structure. Gal4 together with poly-A tail was knocked in after the first start codon of Piezo. The ten predicted Piezo isoforms share the same N-terminus. We refer to this knock-in Gal4 line as Piezo-Gal4[KI]. All Piezo-Gal4 lines used in the manuscript are Piezo-Gal4[KII]. c-f. Piezo expression pattern in the midgut (Piezo-Gal4,UAS-tdTomato3XHA). Tissue was stained with anti-HA antibody to enhance the original signal. In addition to the small diploid stem cells, Piezo is also expressed in ECs after the cardia and around the $\mathrm{Cu} / \mathrm{Fe}$ region of the midgut. Gal4 activity outside the intestinal epithelium from tracheal cells can also be detected. g. Expression pattern of Piezo
mRNA along different sections of the midgut. h. Drosophila midgut with $\mathrm{Piezo}^{+}$cells labeled by PiezoGal4, UAS-mCherryCAAX (Piezo>mCherry) and esg ${ }^{+}$cells labeled by esg$n l s G F P$. i. Midgut with Piezo ${ }^{+}$cells labeled by Piezo-Gal4, UAS-Piezo-GFP. $\mathrm{Dl}^{+}$stem cells were stained by anti-Delta antibody. Cells positive for Piezo ${ }^{\text {GFP }}$ are indicated by arrowheads. j. Midgut expressing $U A S$-Piezo- $G F P$ in esg ${ }^{+}$cells with F-actin labeled by $U A S-A c t 5 C-R F P$. A recent study has shown that Piezo may form large cytoplasmic aggregates under stressed condition ${ }^{19}$, however, in the fly midgut, the GFP-tagged Piezo protein is localized primarily on the plasma membrane under both quiescent or overproliferation conditions (i,j). k. esg>GFP is used as an indicator of "newborn" EEs. Under normal physiological condition, around $2-3 \%$ of esg $^{+}$cells stain for Pros, suggesting that they are either differentiating or have just differentiated into EEs (indicated by arrowheads). In addition, all the newborn EEs are also positive for Piezo. Piezo and Pros double positive but esg negative cells can be found occasionally (indicated by yellow arrowhead), most likely reflecting their late stage of differentiation. l. If the newborn EEs are restricted to any specific EE subtype was tested. Tachykinin (Tk) is stained with antibody. Piezo ${ }^{+}$newborn EEs are composed of both $\mathrm{Tk}^{+}$and $\mathrm{Tk}^{-}$cells, suggesting that Piezo ${ }^{+}$cells are precursors for different types of EEs. Cells positive for both Piezo and Pros (left panel), Piezo and Tk (middle panel), Pros and Tk (right panel) were indicated by arrowheads. m. $\mathrm{Dl}^{+}, \mathrm{Su}(\mathrm{H})^{+}$and Piezo $^{+}$cells were traced using Dl-Gal4, $\mathrm{Su}(\mathrm{H})$-Gal4, and Piezo-Gal4 together with UAS-Flp, Act>FRT>Stop>FRT>nlsGFP. TubGAL8OTS. $G A L 8 O^{T S}$ was used to suppress the early activity of Gal4 before adulthood. $\mathrm{GFP}^{+}$clones were induced by transient incubation at $32^{\circ} \mathrm{C}$. Pros (Red) and GFP double positive cells are indicated by arrowheads. n. Compared with $\mathrm{Dl}-\mathrm{Gal} 4^{\mathrm{TS}}$, which generate large $\mathrm{GFP}^{+}$EC clones, Piezo-Gal4 ${ }^{\mathrm{TS}}$ primarily generates individual $\mathrm{GFP}^{+}$cells with occasional $\mathrm{GFP}^{+} \mathrm{EC}$ cell clone (indicated by arrowhead). o. To visualize the cells with Gal4 activity, which is repressed by the presence of tubGal80 ${ }^{\mathrm{TS}}$, we incubated the flies at $32^{\circ} \mathrm{C}$ overnight before analysis. In this figure, two Pros ${ }^{+}$cells are GFP positive but RFP negative (indicated by arrowheads), suggesting that they are derived from Piezo $^{+}$cells and stop expressing Piezo. All experiments were independently repeated at least twice with similar results presented in the figures. Scale bar: a, $50 \mu \mathrm{~m} ; \mathbf{c}, 500 \mu \mathrm{~m}$.; d-f, 100 $\mu \mathrm{m} . \mathbf{h}, 50 \mu \mathrm{~m} ; \mathbf{i}, \mathbf{j}, 25 \mu \mathrm{~m} ; \mathbf{k}, \mathbf{l}, \mathbf{m}, 20 \mu \mathrm{~m} ; \mathbf{n}, 50 \mu \mathrm{~m} ; \mathbf{0}, 10 \mu \mathrm{~m}$.


Extended Data Figure 2. Piezo ${ }^{+} / \mathbf{E P}$ cells are ISC-derived EE precursors with reduced mitotic ability.
a. Midguts from flies treated with Bleomycin ( $10 \mu \mathrm{~g} / \mathrm{ml}$ Bleo in 5\% sucrose) or the $\gamma$ secretase inhibitor DAPT ( 4 mM DAPT in $5 \%$ sucrose). Cells that are positive for both Piezo and Pros are indicated by arrowheads. Note that the majority ( $>95 \%$ ) of Piezo and Pros double positive cells are also positive for esg, suggesting that these cells are "newborn" EEs that still retain esg-GFP signal. b. Percentage of the newborn EEs (Piezo and Pros double positive cells vs. total Pros $^{+}$EEs) in fly midguts under control, Bleomycin, and DAPT treatments. Cells within $200 \mu \mathrm{~m}$ X $200 \mu \mathrm{~m}$ areas, $\mathrm{n}=27$ (control), $\mathrm{n}=25$ (Bleo), and $\mathrm{n}=22$ (DAPT), were analyzed. c. Midgut with stem cells labeled by esg-GFP(Green), Piezo ${ }^{+}$
cells labeled by Piezo-Gal4>nlsRFP (Red), and mitotic cells labeled by anti-phosphoHistone H3 ( pH 3 ) (Magenta). $\mathrm{pH} 3^{+}$mitotic cell is indicated by arrowhead. d,e. Representative images of midguts from flies feed on either control ( $5 \%$ sucrose) or Bleomycin (5\% sucrose plus $10 \mathrm{ug} / \mathrm{ml}$ Bleomycin) food. Piezo ${ }^{+}$EP cells are labeled by Piezo-Gal4>nlsGFP (Green), mitotic cells are labeled by pH3 staining (Red). Mitotic Piezo ${ }^{+}$ cells are indicated by arrowheads. Since all $\mathrm{pH} 3^{+}$cells are $\mathrm{Dl}^{+}$cells (according to Dl-lacZ labeled midgut), we counted all Piezo negative $\mathrm{pH}^{+}$cells as $\mathrm{pH}^{+}$ISCs. Under both control ( $5 \%$ sucrose) and damage ( $5 \%$ sucrose $+10 \mu \mathrm{~g} / \mathrm{ml}$ Bleomycin) conditions, only around 8 $10 \%$ of the $\mathrm{pH}^{+}$cells are $\mathrm{Piezo}^{+}\left(\sim 40 \%\right.$ of total $\mathrm{Dl}^{+}$cells), suggesting that $\mathrm{Piezo}^{+}$cells are significantly less mitotically active compared to Piezo- $\mathrm{Dl}^{+}$cells. f. If the $\mathrm{pH} 3^{+} \mathrm{Piezo}^{+}$cells are also Pros" as previously described "enteroendocrine mother cell (EMC)" was tested ${ }^{21}$. Around $50 \%$ of these $\mathrm{pH}^{+} \mathrm{Piezo}^{+}$cells show low levels of Pros staining. Meanwhile, all the $\mathrm{pH} 3^{+} \mathrm{Pros}^{+}$cells are positive for Piezo, suggesting that $\mathrm{Piezo}^{+}$EP cells represent more general EE precursor cells compared to EMCs. Mitotic Piezo ${ }^{+}$cells are indicated by arrowheads. All experiments were independently repeated at least twice with similar results presented in the figures. g. Random GFP ${ }^{+}$clones were generated using hsFlp; Ubi-(FRT.Stop)GFP/Piezo-Gal4; UAS-nlsRFP. 3-4 days old flies were heat shocked at $37^{\circ} \mathrm{C}$ for 30 min once to induce clones in ISCs. Then these flies were kept at $25^{\circ} \mathrm{C}$ for 2 weeks before analysis. Within each $\mathrm{GFP}^{+}$clone, which is derived from ISCs, there are typically 1-2 Piezo ${ }^{+}$cells in the cluster (indicated by arrowheads), suggesting that Piezo ${ }^{+}$cells are generated from ISCs after adulthood. All experiments were independently repeated at least twice with similar results presented in the figures. Data are expressed as mean + s.e.m. P-values are calculated from two-tailed Student t-test with unequal variance. Scale bar: a,c, $50 \mu \mathrm{~m} ; \mathbf{d}, \mathbf{f} 20$ $\mu \mathrm{m} ; \mathbf{g}, 25 \mu \mathrm{~m}$.


Extended Data Figure 3. Expression and function of Piezo in larval and pupal midguts.
a. Expression pattern of Piezo in larval and pupal midguts. $\mathrm{Piezo}^{+}$cells are labeled by PiezoGal4>nlsGFP. Piezo is enriched in adult midgut precursor cells (AMPs) during larval stages. Strong expression of Piezo is also detected in tracheal cells associated with the midgut (the nucleus of tracheal cells are indicated by yellow arrowhead). After pupariation, the GFP signal can be detected at low level in most midgut cells (including ECs), but enriched in a few stem cells and EEs, which presumably are newborn EEs. Pupal gut 72 hours after pupae formation (APF) is shown with cells positive for both Piezo and Pros are indicated by arrowheads in Zoom1 and Zoom 2. Importantly, high Piezo level is detected in a large number of EEs present in the mid-section of the pupal gut, suggesting that the association of Piezo expression and EE differentiation is probably conserved during the pupal stage. $\mathbf{b}$. Live imaging of larval and pupal midguts expressing GCAMP and mcd8RFP by Dl-Gal4. Cells with high GCAMP activity are indicated by arrowheads. c,d. Midguts from Piezo null flies show no significant EE generation defects during larval, pupal, or early adult stages (12 Days after eclosion). Number of midgut areas quantified: $\mathrm{n}=24$ (WT, larva), $\mathrm{n}=23$ (WT, pupa), $\mathrm{n}=28$ (WT, young adult), $\mathrm{n}=23$ (Piezo ${ }^{\mathrm{KO}}$, larva), $\mathrm{n}=23$ (Piezo ${ }^{\mathrm{KO}}$, pupa), $\mathrm{n}=28$ (Piezo ${ }^{\text {KO }}$, young adult). These results indicate that mechanical controlled Piezo activation is not the major mechanism for EE production during early development. Unlike the adult midgut, the larval midgut does not regenerate through mitosis and only grow through increases in cell size. It is only during late stages of 3rd instar larval development that the
quiescent AMPs start to proliferate and generate both new ECs and EEs for pupal gut formation, and the majority of new EEs ( $\sim$ several hundred) are created within a very narrow time window $\sim 72-96 \mathrm{hr}$ APF (after pupae formation) ${ }^{27}$. Therefore, the generation of EEs is 15-30 times faster at that stage than during the adult stage under physiological condition, suggesting that a different mechanism that stimulates strong acute EE differentiation is probably involved during developmental stages. e,f. Knocking down SERCA using esg-Gal4 during larval stages significantly increases EE cell number. Meanwhile, overexpression of Piezo-GFP has no significant phenotype. A cluster of extra EE cells are indicated by write circle. Number of midgut areas quantified: $\mathrm{n}=26$ (WT), $\mathrm{n}=28$ (Serca ${ }^{\text {RNAi }}$ ), $\mathrm{n}=26$ (Piezo ${ }^{\text {GFP }}$ ). All experiments were independently repeated at least twice with similar results presented in the figures. Data are expressed as mean + s.e.m. P-values are calculated from two-tailed Student t-test with unequal variance. Scale bar: $50 \mu \mathrm{~m}$.


Extended Data Figure 4. Piezo regulate stem cell differentiation primarily through $\mathbf{C a}^{2+}$ signaling, which is upstream of Notch, Ttk69, and the Achaete-Scute complex (AS-C).
a. Phenotypes associated with $U A S-G F P$ (at $25^{\circ} \mathrm{C}$ or $32^{\circ} \mathrm{C}$ ), $U A S$-Piezo ${ }^{G F P}$ together with Stim ${ }^{R N A i}$, InsP3R ${ }^{R N A i}$ and $N^{I C D}$, and UAS-GFP together with Stim ${ }^{R N A i}$, Stim ${ }^{R N A i}+$ Piezo ${ }^{R N A i}$, InsP3R ${ }^{R N A i}, N^{I C D}$, InsP3R over-expression (InsP3R ${ }^{O E}$ ), and Orai overexpression (Orai ${ }^{O E}$ ) (at $32^{\circ} \mathrm{C}$ ). Overexpression of PiezoG ${ }^{\mathrm{FP}}$ using esg-Gal4 did not show a significant phenotype at $25^{\circ} \mathrm{C}$. By contrast, incubation at $32^{\circ} \mathrm{C}$ for 4 days showed an increased in the number of both esg $^{+}$cells and Pros ${ }^{+}$EEs. Moderate over-expression of Piezo at $25^{\circ} \mathrm{C}$ had no significant effects. However, strong over-expression at $32^{\circ} \mathrm{C}$ caused an increase in both esg+ cells and EEs, which phenocopied the increase of cytosolic $\mathrm{Ca} 2+$ through SERCA reduction. All flies were incubated at the indicated temperature for 4-5 days before analysis. b. Statistics of the number of $\mathrm{esg}^{+}$and Pros $^{+}$cells within $10,000 ~ \mu \mathrm{~m}^{2}$ area. Number of midgut areas quantified: $\mathrm{n}=30\left(\mathrm{GFP} 25^{\circ} \mathrm{C}\right), \mathrm{n}=31\left(\mathrm{GFP} 32^{\circ} \mathrm{C}\right), \mathrm{n}=25$ (InsP3R $\left.{ }^{\mathrm{OE}} 32^{\circ} \mathrm{C}\right), \mathrm{n}=27\left(\right.$ Orai $\left.^{\mathrm{OE}} 32^{\circ} \mathrm{C}\right)$, $\mathrm{n}=31$ (Stim-i $32^{\circ} \mathrm{C}$ ), $\mathrm{n}=27$ (Stim-i, Piezo-i $32^{\circ} \mathrm{C}$ ), $\mathrm{n}=29\left(\operatorname{InsP3R}-\mathrm{i} 32^{\circ} \mathrm{C}\right), \mathrm{n}=29\left(\mathrm{~N}-\mathrm{ICD} 32^{\circ} \mathrm{C}\right)$. c. Average number of mitotic cells within the fly midgut from indicated genotypes were quantified. Number of midguts analyzed: $\mathrm{n}=20$ $\left(\right.$ GFP $\left.25^{\circ} \mathrm{C}\right), \mathrm{n}=19\left(\operatorname{GFP} 32^{\circ} \mathrm{C}\right), \mathrm{n}=20\left(\right.$ Piezo $\left.{ }^{\text {GFP }} 25^{\circ} \mathrm{C}\right)$, $\mathrm{n}=19\left(\right.$ Piezo $\left.^{\text {GFP }} 32^{\circ} \mathrm{C}\right)$, $\mathrm{n}=18$ (SercaI, $32^{\circ} \mathrm{C}$ ), $\mathrm{n}=18\left(\operatorname{InsP} 3 \mathrm{R}^{\mathrm{OE}} 32^{\circ} \mathrm{C}\right), \mathrm{n}=24\left(\mathrm{Orai}{ }^{\mathrm{OE}} 32^{\circ} \mathrm{C}\right)$, $\mathrm{n}=19\left(\right.$ Stim-i $\left.32^{\circ} \mathrm{C}\right), \mathrm{n}=19(\mathrm{Stim}-\mathrm{i}$, Piezo-i $32^{\circ} \mathrm{C}$ ), $\mathrm{n}=19$ (Piezo ${ }^{\text {GFP }}$, Stim-i $32^{\circ} \mathrm{C}$ ), $\mathrm{n}=18$ (InsP3R-i $32^{\circ} \mathrm{C}$ ), $\mathrm{n}=18$ (Piezo ${ }^{\mathrm{GFP}}$, InsP3R-i $32^{\circ} \mathrm{C}$ ), $\mathrm{n}=17\left(\mathrm{~N}-\mathrm{ICD} 32^{\circ} \mathrm{C}\right), \mathrm{n}=17$ (Piezo ${ }^{\mathrm{GFP}}, \mathrm{N}-\mathrm{ICD} 32^{\circ} \mathrm{C}$ ). d,e. EE production induced by overexpression of Piezo ${ }^{\text {GFP }}$ is blocked by ase ${ }^{R N A i}$ (Acheate-Scute complex component asense). Number of midgut areas quantified: $\mathrm{n}=29(\mathrm{Ctl}), \mathrm{n}=30$ (ase-i). $\mathbf{f}, \mathbf{g}$. Expression of $N^{I C D}$ in the presence of $\operatorname{Serca}{ }^{R N A i}$ significantly reduced both stem cell proliferation and EE production. Meanwhile, knocking-down ase specifically blocks EE differentiation but not proliferation. Number of midgut areas quantified: $n=27(\mathrm{Ctl}), \mathrm{n}=24$ (NICD), n=25 (ase-i). Even though ttk69 and $A S$ - $C$ knock down affect Piezo and Serca related phenotypes, we think that $\mathrm{Ca}^{2+}$ signaling probably does not directly affects Ttk69 or $A S$ - $C$ since previous studies have shown that Ttk69 and AS-C reduction can convert Notchhigh EB cell into EEs ${ }^{28}$, but neither Piezo over-expression nor Serca knockdown has any effect in EBs. h. MARCM clones of cells homozygous for FRT (Ctl), Piezo null allele $\left(\right.$ Piezo $\left.^{K O}\right)$, Stim-RNAi, Piezo ${ }^{K O}+$ Piezo $^{G F P}$, Piezo ${ }^{K O}+$ Pmca-RNAi, Piezo ${ }^{K O}+$ SercaRNAi, Piezo ${ }^{K O}+$ O-fut-RNAi, and Piezo ${ }^{K O}+$ ttk69-RNAi. Rescue/reversion of the reduction of EEs in Piezo null clones by increasing cytosolic $\mathrm{Ca}^{2+}$ (by knocking down the $\mathrm{Ca}^{2+}$ export pump Pmca or endoplasmic reticulum $\mathrm{Ca}^{2+}$ ATPase Serca) or by reducing Notch activity (by knocking down its key processing enzyme O-fut, and knocking down EE cell fate repressor $t t k 69$ ). All data are collected from at least two independent replicates and are expressed as mean + s.e.m.. P-values are calculated from two-tailed Student $t$-test with unequal variance. Scale bar, $50 \mu \mathrm{~m}$.


Extended Data Figure 5. Prolonged increase of stem cell proliferation may reduce EE cell number.
a. Fly midguts of each indicated genotype/condition were analyzed after 5 and 10 days incubations at $32^{\circ} \mathrm{C}$. esg ${ }^{+}$cells and EE cells were labeled by esg>GFP and anti-Pros staining. Representative images from two independent replicates were shown. $\mathbf{b}$. Quantification of mitosis ( $\mathrm{pH} 3+$ cell number) of midguts from flies expressing GFP only (control, $\mathrm{n}=16 / 5$ days, $\mathrm{n}=16 / 10$ days), full-length $\operatorname{Stim}$ ( $\operatorname{Stim}^{O E}$, $\mathrm{n}=15 / 5$ days, $\mathrm{n}=17 / 10$ days), Serca ${ }^{R N A i}$ (Serca-i, $\mathrm{n}=18 / 5$ days, $\mathrm{n}=16 / 10$ days), $\operatorname{Piezo}^{G F P}$ ( $\mathrm{n}=17 / 5$ days, $\mathrm{n}=18 / 10$ days), $P M C A^{R N A i}$ (PMCA-i, $\mathrm{n}=15 / 5$ days, $\mathrm{n}=15 / 10$ days), and flies fed $\mathrm{Bleo}^{+}$containing food (regular food $+10 \mathrm{ug} / \mathrm{ml}$ Bleomycin, $\mathrm{n}=15 / 5$ days, $\mathrm{n}=13 / 10$ days). $\mathbf{c}$. Quantification of Pros ${ }^{+}$ EE cell number from $10,000 \mu \mathrm{~m}^{2}$ regions: $\mathrm{n}=31 / 5$ days, $\mathrm{n}=30 / 10$ days (control); $\mathrm{n}=30 / 5$ days, $\mathrm{n}=32 / 10$ days $\left(\right.$ Stim $\left.^{O E}\right)$; $\mathrm{n}=30 / 5$ days, $\mathrm{n}=30 / 10$ days ( Serca $^{R N A \dot{I}}$ ); $\mathrm{n}=31 / 5$ days, $\mathrm{n}=32 / 10$ days ( Piezo $^{G F P}$ ); $\mathrm{n}=32 / 5$ days, $\mathrm{n}=31 / 10$ days ( $P_{M C A}{ }^{R N A i}$ ); $\mathrm{n}=29 / 5$ days, $\mathrm{n}=28 / 10$ days ( $\mathrm{Bleo}^{+}$). Bleomycin treatment or $P M C A^{R N A i}$ significantly reduced the number of EEs. This reduction is primarily due to increased turn-over of EEs, since blocking cell mitosis for 5 days had no significant effect on EE cell number (Extended Data Figure 7). The differences between stem cell proliferation and EE differentiation may due to a different level of cytosolic $\mathrm{Ca}^{2+}$ increase and the $\mathrm{Ca}^{2+}$ depletion in the ER store. d. Change of Piezo ${ }^{+}$ cells and EEs after 5 and 10 days of control ( $5 \%$ sucrose) or Bleomycin ( $5 \%$ sucrose plus $10 \mathrm{ug} / \mathrm{ml}$ Bleomycin) treatment. Representative images from two independent replicates were shown. e. Quantification of Piezo ${ }^{+}$cells and EEs from 10-15 midguts for each condition. Both Piezo ${ }^{+}$cells and EEs number increased after 5 days of Bleomycin treatment and significantly decreased after 10 days of treatment. Cell numbers were quantified within $10,000 \mu \mathrm{~m}^{2}$ area, except for pH 3 , which is quantified from the whole midgut. All data are
expressed as mean + s.e.m. values. P-values are calculated from two-tailed Student t-test with unequal variance. Scale bar, $50 \mu \mathrm{~m}$.


Extended Data Figure 6. Piezo over-expression increases cytosolic $\mathrm{Ca}^{\mathbf{2 +}}$ level which further triggers proliferation of ISCs but not EBs.
a. Overexpression of Piezo ${ }^{G F P}$ in esg+ cells (esg>Gal4/UAS-Piezo ${ }^{G F P} ; U A S-R G E C O$ ) at $32^{\circ} \mathrm{C}$ causes an increase in cytosolic $\mathrm{Ca}^{2+}$ (indicated by the calcium reporter RGECO) compared to control (esg>Gal4/UAS-GFP; UAS-RGECO). Representative images from three short time-lapse imaging of cultured fly midguts were shown. Scale bar: $50 \mu \mathrm{~m}$. $\mathbf{b}$. Typical traces of $\mathrm{Ca}^{2+}$ oscillations in $\mathrm{esg}^{+}$cells of midgut from either control or Piezo ${ }^{\text {GFP }}$ flies from three independent replicates. c. $\mathrm{Ca}^{2+}$ oscillation frequency of esg ${ }^{+}$cells from either control or Piezo ${ }^{\text {GFP }}$ midguts. Data of 27 cells from three replicates for each condition were shown. d. Statistics for average RGECO signal intensity in all GFP ${ }^{+}$cells (blue) and percentage of $\mathrm{Ca}^{2+}$ positive cells (signal higher than 3 X s.d. of background) compared to total $\mathrm{GFP}^{+}$cells (orange). Signal intensities were calculated from $10,000 \mu \mathrm{~m}^{2}$ regions: $\mathrm{n}=17$ (control), $\mathrm{n}=22\left(\right.$ Piezo $\left.^{\mathrm{GFP}}\right)$ from three independent experiments. e, Bleomycin (Bleo, $10 \mathrm{ug} / \mathrm{ml}$ ) (5 days treatment) triggers a significant increase of esg ${ }^{+}$cell and EE cells in both WT and Piezo ${ }^{K O}$ flies. Represented images from three independent replicates were shown. f, Images of live midguts from WT and Piezo ${ }^{K O}$ flies. Both flies were fed on food containing Bleomycin for 3 days before imaging. g,h. Traces of $\mathrm{Ca}^{2+}$ oscillations in $\mathrm{Dl}^{+}$stem cells from WT and Piezo mutant flies fed on Bleomycin for 4-5 days. Bleomycin treatment causes
some stem cells to maintain constant high $\mathrm{Ca}^{2+}$ levels, while others show reduced oscillation frequency but increased average GCaMP/RFP intensity ratio ( $\mathrm{G} / \mathrm{R}$ ratio). These data show that tissue damage by Bleomycin triggers stem cell proliferation, EE production, and an increase of cytosolic $\mathrm{Ca}^{2+}$, independent of Piezo. 30 cells from $\mathrm{n}=4$ (control), $\mathrm{n}=4$ ( $\mathrm{Bleo}^{+}$), and $\mathrm{n}=5\left(\right.$ Piezo $\left.^{K O}+\mathrm{Bleo}^{+}\right)$independent guts were plotted. i. Overexpression of Piezo ${ }^{G F P}$ in esg ${ }^{+}$cells $\left(32^{\circ} \mathrm{C}\right)$ increases the ratio of $\mathrm{Dl}^{+}$cells (labeled by Dl-lacZ) within the esg ${ }^{+}$ population. j. Piezo overexpression promotes $\mathrm{Dl}^{+}$stem cells ratio in $\mathrm{esg}^{+}$cells. Ratio between $\mathrm{Dl}^{+}$and esg ${ }^{+}$cells within $10,000 \mu \mathrm{~m}^{2}$ regions: $\mathrm{n}=21$ (control) and $\mathrm{n}=22$ (Piezo ${ }^{\mathrm{GFP}}$ ) from two independent replicates, are analyzed. k,l. Overexpressing Piezo or knocking down Serca in $\mathrm{Su}(\mathrm{H}) \mathrm{Gbe}^{+}$EB cells showed no significant phenotype, suggesting that their effect may be blocked by high Notch activity. Number of midgut areas quantified: $\mathrm{n}=18$ (control), $\mathrm{n}=20$ (Serca-i), $\mathrm{n}=16$ ( Piezo $^{G F P}$ ). Data are expressed as mean + s.e.m. values. P-values are calculated from two-tailed Student t-test with unequal variance. Scale bar: a,e,f, $50 \mu \mathrm{~m} ; \mathbf{i}, 20$ $\mu \mathrm{m} ; \mathbf{k}, 50 \mu \mathrm{~m}$.


Extended Data Figure 7. Cytosolic Ca $^{2+}$ triggers ISC proliferation and EP differentiation into EEs.
a. Image of chamber used for optogenetic activation of ChR . b,c. Flies expressing $G F P$ only in $\mathrm{Dl}^{+}$stem cells or Piezo ${ }^{+}$EP (EE precursor) cells were treated under either dark or light + ATR condition for two weeks like the flies expressing ChR. No significant phenotype was induced by the treatment alone. Number of midgut areas quantified: $\mathrm{n}=29$ (Dl, Dark), $\mathrm{n}=33$ (Dl, light+ATR), n=31 (Piezo, Dark), n=34 (Piezo, light+ATR). Representative results from two independent replicates are shown. d. Mitosis quantification of midgut from indicated genotype/condition. Activating ChR in $\mathrm{Dl}^{+}$cells significantly promotes stem cell proliferation. Only a mild increase of mitosis was detected in ChR active $\mathrm{Piezo}^{+} \mathrm{EP}$ cells, suggesting that the primary effect of $\mathrm{Ca}^{2+}$ in EP cells is to promote differentiation. Data are collected from 30 guts (DI>ChR); 30 guts (Piezo>ChR); 29 guts (DI); guts (Piezo) from two independent replicates. $\mathrm{pH}^{+}$cell number is quantified from the whole midgut. e,f. Activation of CsChrimson in $\mathrm{Dl}^{+}$stem cells with both Stim and InsP3R knocked-down shows reduced increase of stem cells and EEs compared to WT stem cells. Flies were raised at $18^{\circ} \mathrm{C}$ and shifted to $25^{\circ} \mathrm{C}$ during the experiment. Cell numbers are quantified within $10,000 \mu \mathrm{~m}^{2}$ area from 29 regions (dark) and 31 regions (light + ATR) from two independent replicates. g. Overexpression of Piezo in esg ${ }^{+}$cells increases MAPK pathway activity. Phosphorylation of extracellular signal-regulated kinase (dpErk) is significantly increased in Piezo-overexpressing cells. Representative images from two independent experiments are shown. h,i. Knocking down Ras significantly reduces stem cell proliferation caused by Piezo overexpression, but does not block Piezo triggered EE differentiation. Flies were kept at $32^{\circ} \mathrm{C}$ for $4-5$ days before analysis. esg ${ }^{+}$and EE cell number were quantified from $\mathrm{n}=29$ (control) and $\mathrm{n}=30\left(\right.$ Piezo $\left.{ }^{G F P}\right)$ midguts areas from two independent experiments. "Newborn" EEs, that are positive for both esg and Pros, are indicated by arrowheads. j,k. Knockingdown Yorkie using $Y k i^{R N A i}$ completely blocks stem cell proliferation but not the increase of EE cells induced by either Piezo overexpression or Serca knock-down. In addition, knocking-down Serca together with Yorkie also significantly reduced stem cell number, suggesting a depletion of stem cells caused by constant EE differentiation. Cell numbers were quantified within 30 midgut areas for each genotype. l. Midguts from flies fed on control ( $5 \%$ sucrose), Thap ( $5 \%$ sucrose $+0.5 \mu \mathrm{M}$ Thapsigargin), Thap+Tram ( $5 \%$ sucrose $+0.5 \mu \mathrm{M}$ Thapsigargin $+10 \mu \mathrm{M}$ Trametinib), and Tram ( $5 \%$ sucrose $+5 \mu \mathrm{M}$ Trametinib) for 4 days. Representative images from 3 independent experiments are shown. The increase of cytosolic $\mathrm{Ca}^{2+}$ by Thap promotes stem cell proliferation, EP (enteroendocrine precursor/ Piezo ${ }^{+}$cell) production, and EE differentiation. Newborn EEs, which are positive for esg, Piezo and Pros, are indicated by white arrowheads. m. Data are collected from Quantification of mitotic cells from $n=15$ (control), $n=16$ (Thap), $n=17$ (Thap + Tram), and $\mathrm{n}=16$ (Tram) midguts. Thap treatment triggers a significant increase in mitosis, which is largely reduced by the mitogen-activated protein kinase (MAP kinase) inhibitor Tram. n. Percentage of Piezo ${ }^{+}$cells within esg $^{+}$cell population. Number of areas quantified: $\mathrm{n}=29$ $(\mathrm{Ctl}), \mathrm{n}=31$ (Thap), $\mathrm{n}=32$ (Thap+Tram), $\mathrm{n}=29$ (Tram). o. Representative $\mathrm{Ca}^{2+}$ images of live midgut from control, Thap, and Thap+Tram treated flies. Similar results are collected from 4 independent guts for each condition. p,q. Thap treatment caused a reduction of oscillation frequency but an increase of average GCaMP/RFP ratio (G/R ratio). The increase of cytosolic $\mathrm{Ca}^{2+}$ by Thap is not affected by MAPK inhibition. Data are collected from 29 Cells from 3 independent guts for each condition. All data are expressed as mean + s.e.m.
values (shown in red). P-values are calculated from two-tailed Student t-test with unequal variance. Scale bar: $50 \mu \mathrm{~m}$.


Extended Data Figure 8. Over-feeding triggers stem cell proliferation and EE increase. a. Schematic illustration of fly midguts from control ( $5 \%$ sucrose) or MC ( $5 \%$ sucrose $+10 \%$ Methylcellulose) fed flies. b. "Smurf" assay of flies fed on both control and MC food shows no damage of gut integrity. Two independent replicates showed similar results. c,d. Image of a midgut feed on MC food. The cell proliferation phenotype is associated with midgut diameter increase but not food content. Data are collected from 23 midgut areas from two independent experiments for each condition. e,f. Midguts from flies fed on MC food with no increase of gut diameter shows no phenotype compared with control. Data are collected from 31 regions (control) and 28 regions (MC feed) from three independent experiments. g,h. Feeding-induced cell proliferation produces more Piezo ${ }^{+}$cells, which differentiate into EEs. All newborn EEs are indicated by white arrowheads. Data are collected from 27 areas from 2 independent experiments for each condition. i,j, Feeding-
induced midgut enlargement triggers a significant increase in EP/Piezo ${ }^{+}$cell number. Data are collected from $n=30$ (control) and $n=32$ (MC feed) midgut areas from two independent replicates. k,l. Feeding-trigged stem cell proliferation and EE increase are blocked in Piezo null mutant. Data are collected from $\mathrm{n}=27$ (control) and $\mathrm{n}=32$ ( $\mathrm{Piezo}^{\mathrm{KO}}$ ) midgut areas from two independent replicates. P-values for both esg and EE are smaller than 0.001. m. Linage tracing experiment (using Piezo-Gal4) under overfed condition shows a significant increase in cell number (2-3) in the same cluster compared to tracing result under control condition, suggesting that either more Piezo cells were created from ISCs or more Piezo ${ }^{+}$cells divide to create more progeny. Cells positive for both GFP and Pros are indicated by arrowheads. n, Images of live midguts from the following conditions/genotypes: control, MC fed without midgut diameter increase (normal size), MC fed with enlarged midgut diameter, MC fed with Piezo ${ }^{R N A i}$ and enlarged midgut diameter, and MC fed with $\operatorname{InsP3} R^{R N A i}+\operatorname{Stim}^{R N A i}$ and enlarged midgut diameter. o. Representative traces of $\mathrm{Ca}^{2+}$ oscillations in $\mathrm{Dl}^{+}$stem cells of flies from indicated treatment/genotypes. Data are collected from 3 independent experiments for each genotype/condition. p,q. $\mathrm{Ca}^{2+}$ oscillation frequency and GCaMP/RFP intensity ratio of 30 cells from 3 individual guts for each genotype are plotted. Mean $\pm$ s.e.m. is displayed in red. Enlarged midgut fed on MC food shows reduced $\mathrm{Ca}^{2+}$ oscillation frequency but increased average cytosolic $\mathrm{Ca}^{2+}$ level. MC food alone does not trigger any significant change of $\mathrm{Ca}^{2+}$ activity. Knocking-down either Piezo or both Stim and InsP3R blocks this feeding-induced increase of cytosolic $\mathrm{Ca}^{2+}$. Knocking-down InsP3R or Stim alone has no significant effect on cytosolic $\mathrm{Ca}^{2+}$ (Data not shown), which is probably due to the reduced expression level of Dl-Gal4 compared with esg-Gal4. The change of $\mathrm{Ca}^{2+}$ activity in MC-fed enlarged midguts is similar to some cells in the Bleomycin damaged midguts (Extended Data Fig. 6 f,g). However, the majority of cells from MC-fed enlarged midguts still oscillate, which is different from stem cells in Bleomycin-treated midguts in which a large portion of cells maintain a constant high level of $\mathrm{Ca}^{2+}$ (Extended Data Fig. 6 $\mathrm{f}, \mathrm{g})$. Data are indicated as mean + s.e.m. values. P-values are calculated from two-tailed Student t-test with unequal variance. Scale bar: e,i,k,n, $50 \mu \mathrm{~m} ; \mathbf{g}, 25 \mu \mathrm{~m} ; \mathbf{m}, 10 \mu \mathrm{~m}$.


Extended Data Figure 9. Direct mechanical activation of the Piezo channel triggers an increase of cytosolic calcium in stem cells.
a. Image of the microfluidic chip used for the ex vivo mechanical trigger experiment. b-c. Design of the channels on the microfluidic chip. Compressed air was delivered through left and right channels and controlled by a manual gauge. Dissected fly midguts were loaded into the main channel (center) from an inlet at the bottom. d. During each compression cycle, the midgut was squeezed to achieve $\sim 30-35 \%$ reduction in diameter from both sides. The switching time between compression and relaxation is $\sim 1 \mathrm{~s}$. e. Representative samples of ex vivo mechanical trigger experiment. Time 0 s and 40 s were taken immediately before and after compression. The total compression time is 40 s . Transmission light (up panel) and GCaMP6s signal (bottom panel) are shown. Compared to control, loss of Piezo significantly blocked activation of stem cells by mechanical compression. f. Plots of activated cells numbers during one triggering cycle ( 50 s ) for control ( $\mathrm{n}=12$ ) and Piezo $^{K O}(\mathrm{n}=15)$ fly midguts. Data were collected from 4-5 individual midguts. All GCaMP positive cells (brighter than the 5 folds of background signal) within the field were counted. Periods of
compression and relaxation are indicated by green and yellow colors, respectively. $\mathbf{g}$. Averaged response curves of multiple compression cycles ( $\mathrm{n}=12$ for control and $\mathrm{n}=10$ for PiezoKO) from control (blue) and PiezoKO (orange) midguts. h. Typical traces of $\mathrm{Ca}^{2+}$ activities in WT stem cells that respond to the mechanical stimulus. Data is represented in curve plot (first panel) and heatmap plot (second panel), respectively. Compression period is from 0 to 40 seconds (indicated by black box). Typical traces of $\mathrm{Ca}^{2+}$ activities with indicated genotypes. Stem cells with Piezo knockdown or mutant do not respond to the mechanical stimulus. Knocking-down Serca causes a constant high cytosolic $\mathrm{Ca}^{2+}$. Knocking-down both Stim and InsP3R significantly reduces random $\mathrm{Ca}^{2+}$ activities and largely blocks mechanically triggered a $\mathrm{Ca}^{2+}$ increase. Data are collected from 3 independent experiments for each genotype/condition. i. Images of cultured midguts from control, Piezo ${ }^{R N A i}$, Piezo ${ }^{K O}$, Serca ${ }^{R N A i}$, InsP3R $R^{R N A i}+$ Stim $^{R N A i}$ flies. j. Typical traces of $\mathrm{Ca}^{2+}$ activities in stem cells of indicated genotypes. Data are collected from 3 independent guts for each genotype/condition. k,l. $\mathrm{Ca}^{2+}$ oscillation frequency and GCaMP/RFP intensity ratio ( $\mathrm{G} / \mathrm{R}$ ratio) in cells from 35 cells (control), 35 cells ( Piezo $^{R N A i}$ ), 34 cells ( Piezo $^{K O}$ ), 36 cells (Serca $\left.{ }^{R N A i}\right), 33$ cells (InsP3R ${ }^{R N A i}+$ Stim $\left.^{R N A i}\right)$ from 3 independent experiment for each condition/genotype. Neither Piezo ${ }^{\text {RNAi }}$ nor Piezo ${ }^{K O}$ significantly affect $\mathrm{Ca}^{2+}$ activities. Knocking-down Serca induces a constant increase of cytosolic $\mathrm{Ca}^{2+}$ in most cells. Knocking-down both InsP3R and Stim stem cells significantly reduces their $\mathrm{Ca}^{2+}$ activities. Our data indicate that mechanical stresses generated during food digestion may activate Piezo and promote EE generation in vivo. However, we note that the time-scale between our ex vivo mechanical activation and in vivo cell proliferation and differentiation experiment is very different, especially as the in vivo property of Piezo-mediated $\mathrm{Ca}^{2+}$ activity in EP cells is unknown. According to our observations, only a small percentage ( $<5 \%$ ) of $\mathrm{Piezo}^{+}$cells become EEs every day under normal condition (interpreted from Piezo/Pros double positive cell number). Therefore, it is possible that either Piezo in vivo is difficult to activate through physiological level mechanical stimulus or that long-term cumulative Piezo activation is required to trigger EEs differentiation. Mean $\pm$ s.e.m. is displayed in red. P-values are calculated from two-tailed Student t-test with unequal variance. Scale bar: $50 \mu \mathrm{~m}$.


## Extended Data Figure 10. Model.

a. Under normal conditions, $\mathrm{Piezo}^{+}$cells, which we refer to as endocrine precursor (EP) cells, are unipotent stem cells that are mitotically quiescent and have a predetermined EE cell fate. In the presence of mechanical stimulation, the Piezo channel is activated and leads to an increase of cytosolic $\mathrm{Ca}^{2+}$ in Piezo ${ }^{+}$EP cells. $\mathrm{Ca}^{2+}$ increase in EP cells triggers strong
ell differentiation into EEs, which is probably mediated through inhibition of Notch activity and consequent increase of Sc/Ase transcription activity. b. The presence of food in the intestine triggers an elevated mechanical stress during food transport and visceral muscle contraction. Our results suggest that mechanical signaling activates the mechanosensitive channel Piezo in quiescent EP cells, leads to an increase in cytosolic $\mathrm{Ca}^{2+}$ level, which maintains the basal level EE cell production under-physiological condition and promotes fast EE generation under abnormal fed condition. We hypothesize that, as a key regulator of midgut function, EE cells might secrete hormones to enhance different long-term gastric functions including appetite, digestion, nutrient absorption, or gastric emptying.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Piezo $^{+}$cells are EE precursors in the fly midgut.
a. Piezo ${ }^{+}$cells are esg-GFP and DI-lacZ positive, but $S u(H) G b e-l a c Z$ negative. b. Percentage of Piezo ${ }^{+}$cells in esg ${ }^{+}$cells. For $\mathrm{Dl}^{+}: \mathrm{n}=238\left(\mathrm{Dl}^{+}\right)$, $\mathrm{n}=457\left(\mathrm{esg}^{+}\right)$. For Piezo ${ }^{+}$: $\mathrm{n}=151$ (Piezo ${ }^{+}$), $\mathrm{n}=682\left(\right.$ esg $\left.^{+}\right)$. c. "newborn" EEs (arrowheads) are Piezo ${ }^{+}$. d. Piezo ${ }^{+}$cells (RFP ${ }^{+}$) generate $\mathrm{GFP}^{+}$EEs (arrowhead). e. Statistics of $\mathrm{GFP}^{+}$ECs and EEs using Piezo-Gal4, $S u(H) G b e-G a l 4$, and DI-Gal4. Number of cells analyzed: $\mathrm{n}=561(\mathrm{DI}), \mathrm{n}=432(\mathrm{Su}(\mathrm{H})), \mathrm{n}=90$ (Piezo). f. Bleomycin and DAPT treatment increase $\mathrm{esg}^{+}$, EP and EE cell numbers. Areas quantified: $\mathrm{n}=23$ (Ctl), $\mathrm{n}=21$ (Bleo), $\mathrm{n}=32$ (DAPT). g,h. Elimination of Piezo ${ }^{+}$cells by conditional expression of Rpr. Areas quantified: $\mathrm{n}=27$ ( Ctl ), $\mathrm{n}=29$ (Rpr), $\mathrm{n}=27$ (Recover). i. pH3 staining of mitotic EPs (arrowhead). Data are expressed as mean + s.e.m. P-values are from two-tailed t-test. Scale bar: a, $20 \mu \mathrm{~m} ; \mathbf{c}, \mathbf{d , i}, 10 \mu \mathrm{~m} ; \mathbf{g}, 50 \mu \mathrm{~m}$.


Figure 2. Piezo regulates EE differentiation through cytosolic $\mathbf{C a}^{\mathbf{2 +}}$.
a,b. Midgut of flies homozygous for Piezo ${ }^{K O}$ shows reduced EE generation after 30 days after eclosion. Areas quantified: $\mathrm{n}=32$ (WT 5 days), $\mathrm{n}=32$ (WT 30 days), $\mathrm{n}=35$ (Piezo ${ }^{\text {KO }} 5$ days), $n=32$ (Piezo ${ }^{\text {KO }} 30$ days). c,d. MARCM clones of cells with indicated genotypes (arrowheads indicate the $\mathrm{GFP}^{+}$EEs). Ratio of EEs in the clone (normalized to control) is quantified. Number of clones quantified: $\mathrm{n}=32$ (FRT), $\mathrm{n}=35\left(\right.$ Piezo $\left.^{\text {KO }}\right), \mathrm{n}=26$ (Stim- 1$), \mathrm{n}=28$ ( Piezo $^{K O}$, Piezo ${ }^{O E}$ ), n=31 ( Piezo $^{\text {KO }}$, Pmca-1), n=35 (Piezo ${ }^{\text {KO }}$, Serca-1), n=28 (Piezo ${ }^{K O}$, O-fut-i). e. esg ${ }^{+}$and EE cell numbers were quantified in midgut expressing indicated genes using esg-Ga14. Number of areas quantified: $\mathrm{n}=22(\mathrm{Ctl}), \mathrm{n}=28$ ( Piezo $^{\text {OE }}$ ), $\mathrm{n}=23$ (Serca-1), $\mathrm{n}=21\left(\right.$ Piezo $^{\text {OE }}$, Stim-í), n=24 (Piezo ${ }^{\text {OE }}$, InsP3R-1), n=26 (Piezo ${ }^{\text {OE }}$, N-ICD). Data are expressed as mean + s.e.m. P-values are from two-tailed t-test. Scale bar: a, $50 \mu \mathrm{~m} ; \mathbf{c}, 25$ $\mu \mathrm{m}$.


Figure 3. Cytosolic $\mathbf{C a}^{2+}$ triggers cell proliferation and EE differentiation through different mechanisms.
 Piezo ${ }^{+}$, and EE cell numbers are quantified. Number of areas quantified: n=28 (Dark, DIGal4), n=30 (Light+ATR, Dl-Gal4), n=30 (Dark, Piezo-Gal4), n=31 (Light+ATR, PiezoGa14). c,d. Midguts of Thapsigargin (Thap) and Trametinib (Tram) treated flies. Number of areas quantified: $\mathrm{n}=29(\mathrm{Ctl}), \mathrm{n}=31$ (Thap), $\mathrm{n}=32$ (Thap+Tram), $\mathrm{n}=29$ (Tram). Data are expressed as mean + s.e.m. P-values are from two-tailed t-test. Scale bar, $50 \mu \mathrm{~m}$.


Figure 4. Mechanical stress increases cytosolic $\mathbf{C a}^{2+}$ through Piezo.
a. Flies fed on methylcellulose (MC) containing food. b,c. MC feeding increases esg ${ }^{+}$and EE cell numbers in the midguts, which is blocked by Piezo ${ }^{\text {RNAi }}$ and Stim ${ }^{\text {RNAi }}$. Number of areas quantified: $\mathrm{n}=25(\mathrm{Ctl}), \mathrm{n}=23(\mathrm{MC}), \mathrm{n}=20(\mathrm{MC}+$ Piezo- I$), \mathrm{n}=25(\mathrm{MC}+$ Stim- I$)$. d. An illustrated microfluidic channel that holds and compresses the midgut for ex vivo mechanical trigger experiment. e. Representative example of 3 cycles of consecutive mechanical activation. Number of calcium ${ }^{+}$cells is plotted over time. Green: compression period. Yellow: relaxation period. f. Average GCaMP activity during compression from control, Piezo ${ }^{K O}$, Piezo ${ }^{R N A i}$, Serca ${ }^{R N A i}$, and $\operatorname{Stim}{ }^{R N A i}+I n s P 3 R^{R N A i}$ flies. g. Model for mechanical regulation of EP differentiation in the fly midgut. $\mathrm{Ca}^{2+}$ plays different roles in ISCs (proliferation) and EPs (differentiation). Data are expressed as mean + s.e.m. P-values are from two-tailed t-test. Scale bar: a. 10 mm , b. $50 \mu \mathrm{~m}$.


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    J.H. and L.H. performed the initial Gal4 expression screen in fly gut. L. H. and N. P. designed the experiments. L.H. performed the Piezo-related experiments and analyzed the data. G.S. and A. S. designed and fabricated the microfluidic chip and together with L. H. optimized the experimental conditions. L. H. and N. P. wrote the manuscript with input from all of the authors.
    COMPETING FINANCIAL INTERESTS
    The authors declare no competing financial interests.

