Feedback regulation of steady-state epithelial turnover and organ size

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Epithelial organs undergo steady-state turnover throughout adult life, with old cells being continually replaced by the progeny of stem cell divisions1. To avoid hyperplasia or atrophy, organ turnover demands strict equilibration of cell production and loss2–4. However, the mechanistic basis of this equilibrium is unknown. Here we show that robustly precise turnover of the adult Drosophila intestine5 arises through a coupling mechanism in which enterocyte apoptosis breaks feedback inhibition of stem cell division. Healthy enterocytes inhibit stem cell division through E-cadherin, which prevents secretion of mitogenic epidermal growth factors (EGFs) by repressing transcription of the EGF maturation factor rhomboid. Individual apoptotic enterocytes promote divisions by loss of E-cadherin, which releases cadherin-associated β-catenin (Armadillo in Drosophila) and p120-catenin to induce rhomboid. Induction of rhomboid in the dying enterocyte triggers activation of the EGF receptor (Egfr) in stem cells within a discrete radius. When we blocked apoptosis, E-cadherin-controlled feedback suppressed divisions, and the organ retained the same number of cells. When we disrupted feedback, apoptosis and divisions were uncoupled, and the organ developed either hyperplasia or atrophy. Together, our results show that robust cellular balance hinges on the obligate coupling of divisions to apoptosis, which limits the proliferative potential of a stem cell to the precise time and place at which a replacement cell is needed. In this way, localized cell–cell communication gives rise to tissue-level homeostatic equilibrium and constant organ size.

Throughout an animal’s lifetime, mature organs undergo continuous cell turnover yet can maintain the same approximate size. This remarkable ability implies the existence of robust mechanisms to ensure that turnover is zero-sum, with cell production and loss held precisely equal1,2,4. In most organs, production of new cells ultimately depends on divisions of resident stem cells. Although much is understood about how equal rates of division and loss are sustained during the steady-state turnover of healthy tissues.

We investigated the regulation of turnover in the midgut epithelium of adult Drosophila6 (Extended Data Fig. 1a–e). To establish whether production of new cells equals loss of old cells, we measured the kinetics of cell addition and loss in the R4ab region using escargot>GFP flp-out labelling (esgF0>GFP)6,7 (Fig. 1a–e and Extended Data Fig. 1f, g). Newly added, GFP+ cells increased linearly over time and, after four days, comprised all cells in R4ab. Concomitantly, the total cell number remained near-constant. We conclude that the production of new cells quantitatively equals the loss of old cells.

To investigate the relationship between cell production and loss, we devised a system to manipulate mature enterocytes and simultaneously track divisions of stem cells by combining enterocyte-specific mexGAL4; GAL80H (mexH) with split-nlslacZ clonal labelling8,9 (Fig. 1f and Extended Data Fig. 2). Using this two-pronged system, we expressed the apoptotic inhibitor p35 in enterocytes and assessed the impact on stem cell divisions. Blocking enterocyte apoptosis resulted in fewer divisions, as indicated by smaller clones (Fig. 1g–i). Apoptotic inhibition also impeded S phase progression (Fig. 1j), consistent with a previous report9. Reduced divisions could be a compensatory means to keep a constant number of total cells. Indeed, the total cell number, as well as physical size and morphology, of apoptosis-inhibited midguts remained

Figure 1 | Enterocyte apoptosis regulates the rate of stem cell division for homeostatic maintenance of total cell number. a–e, Kinetics of midgut epithelial turnover: cartoon (a), images (b–d) and quantification (e) of esgF0>GFP labelling. Progenitors (a, small circles) express GFP upon induction; new, but not old, enterocytes (a, large hexagons) inherit GFP from progenitors after induction. Quantification of total and GFP+ cells over time shows complete replacement of unlabelled cells by GFP+ cells after four days (e). Mean ± s.d.; three midgut R4ab compartments per time point. See Extended Data Fig. 1. f, Genetic schema and experimental timeline for tracing stem cell divisions (split-lacZ clones) in a background of genetically manipulated enterocytes (mexH). See Extended Data Fig. 2. g–i, Sizes (g) and images (h, i) of stem cell clones following enterocyte inhibition of apoptosis (mexH>pt35). Clone sizes are reduced by apoptotic inhibition. Mean ± s.d.; P values by Mann–Whitney U-test; n = 5 midguts per genotype. j, EdU incorporation in diploid cells is reduced by apoptotic inhibition. k, Total cell counts of the R4ab compartment with control or apoptosis-inhibited (pt35 or diap1) enterocytes. j, K, For box-and-whisker plots, the boxes show median, 25th and 75th percentiles, and whiskers are minimum and maximum values; P values by unpaired t-test; NS, not significant; n = 4 midguts per genotype. e, g, j, k, One of three independent experiments is shown with n numbers as specified for each experiment. Scale bars, 25 μm.

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normal (Fig. 1k and Extended Data Figs 3a, 4a, b, d, e). These findings imply that enterocyte apoptosis and stem cell division are homeostatically coupled to maintain a constant cell number and organ size.

We sought to determine this coupling mechanism. The cell–cell adhesion protein E-cadherin (E-cad) drew our attention because in the mouse intestine, enterocyte E-cad represses stem cell divisions\(^{10}\), and in other epithelia, E-cad is degraded by caspases during apoptosis\(^{11}\). In the Drosophila midgut, we found that E-cad::mTomato was largely eliminated from the interfaces of dying, Sytox\(^*\) enterocytes (Fig. 2a), indicating that apoptotic enterocytes lose junctional E-cad.

To investigate whether E-cad couples divisions to apoptosis, we depleted E-cad (also known as shg) in apoptosis-inhibited enterocytes and assessed stem cell divisions by measuring clones. Depletion of enterocyte E-cad did not disrupt epithelial architecture or integrity (Extended Data Fig. 4a, c, d, f–j). It did, however, prevent the reduction in stem cell divisions that would otherwise have occurred following apoptotic inhibition (Fig. 2b–e). Consequently, the total cell number increased by 70%, and organs became markedly hyperplastic (Fig. 2h–j and Extended Data Fig. 3a). These effects were specific to E-cad, because the depletion of another cell–cell adhesion protein, echinoid, did not affect cell number (Fig. 2h). Without apoptotic inhibition, E-cad depletion caused excess divisions, but not hyperplasia (Fig. 2b), which was probably related to other, tissue-level effects (Extended Data Fig. 5). Conversely, E-cad overexpression suppressed divisions (Fig. 2f). These findings show that in apoptosis-inhibited midguts, homeostatic suppression of stem cell divisions requires enterocyte E-cad.

Because E-cad functions as an intercellular homodimer, we considered whether enterocyte E-cad acts by dimerizing with stem cell E-cad\(^{12,13}\). Notably, we found that manipulation of E-cad in stem and enteroblast cells did not alter the rate of stem cell divisions, at least as measured by four-day clones (Fig. 2k). Therefore, enterocyte E-cad does not act through stem cell E-cad, but through a distinct intermediary.

We sought to identify this intermediary. Notable candidates included four E-cad-associated pathways: Wingless/Wnt, Hippo, Jak–Stat and Egfr. To assess whether these pathways act downstream of enterocyte E-cad, we investigated whether E-cad knockdown induced pathway-specific target genes and reporters (Extended Data Fig. 6a–j). E-cad knockdown did not induce Wingless or Hippo targets, Jak–Stat pathway components or Stat signalling in stem cells, although Stat activity in enterocytes was mildly increased. By contrast, E-cad knockdown induced activation of Egfr targets in diploid cells, which probably included presumptive stem cells.

We therefore analysed the functional relationship between enterocyte E-cad, stem cell Egfr and organ size control. In the Drosophila midgut, a specific readout of Egfr activation is diphosphorylation of the effector kinase Erk (dpErk); endogenous diphosphorylation of Erk requires Egfr and occurs primarily in stem cells\(^{14,17}\) (Fig. 3d, e and Extended Data Figs 3b, 6k). We found that enterocyte E-cad knockdown caused the number of dpErk\(^*\) stem cells to increase, whereas overexpression caused dpErk\(^*\) stem cells to decrease (Fig. 3a–c and Extended Data Fig. 3b). Furthermore, Egfr was necessary for the excess divisions and organ hyperplasia that were induced by knockdown of E-cad in apoptosis-inhibited enterocytes (Fig. 3f–g and Extended Data Fig. 3a). Therefore, enterocyte E-cad inhibits stem cell Egfr to mediate homeostatic control of cell number and organ size.

We next investigated how enterocyte E-cad controls Egfr on stem cells. One possible mechanism involves a direct, E-cad–Egfr interaction\(^{18}\); another involves a dispersed signal. To examine these possibilities, we generated isolated, GFP-marked enterocytes that possessed for secretion\(^{19}\). We found that E-cad repressed the transcriptional

![Figure 2](image-url)
Enterocyte E-cad inhibits stem cell Egfr through a dispersed signal for homeostatic size control. a–e, Immunostaining of activated, diphosphorylated Erk (dpErk) following enterocyte manipulation of E-cad, without or with Egfr inhibition (AG1478, egfR fliu). Erk activation in stem cells (Extended Data Fig. 6c) is repressed by E-cad and requires Egfr. See Extended Data Fig. 3b. f, Sizes of stem cell clones after induction of enterocyte p35 and E-cad RNAi, without or with AG1478. Egfr inhibition suppresses stem cell divisions. Mean ± s.d.; P values by Mann–Whitney U-test; n = 4 midguts per condition. g, Total cell counts. Midgut hyperplasia (mex > p35, E-cad RNAi) requires Egfr and enterocyte spi and krn. Box-and-whisker plots as in Fig. 1j, k; P values by unpaired t-test.

The spatial distribution of dpErk cells around a single, E-cad–RNAi-expressing enterocyte distinguishes direct and dispersed activation mechanisms. i, dpErk + cells are both directly adjacent to and dispersed from GFP-marked, E-cad–RNAi-expressing enterocytes, consistent with a dispersed mechanism. j, dpErk – cells are infrequent near marked control enterocytes. k, Distances between dpErk + cells and E-cad–RNAi-expressing enterocytes (EC). n = 4 midguts (53 dpErk + cells total), five day induction. Green bar (k) represents dpErk + cells directly adjacent to enterocytes (0 μm), f, g, k. One of three independent experiments is shown with n numbers as specified for each experiment. Scale bars, 25 μm.

Figure 4 | Enterocyte apoptosis activates stem cell division by disrupting E-cad-controlled inhibition of rhomboid. a, qPCR of whole midgut mRNA following enterocyte-specific E-cad manipulation. E-cad represses rho but not Egfs (vn, spi, krn) or other Egf-related factors (egfr, aos, star). Control: dotted line. Mean ± s.d.; three biological replicates per condition. b-c, Enterocyte rho is required for Erk hyperactivation following depletion of enterocyte E-cad. See Extended Data Fig. 3b. d–f, rho-lacZ induction during physiological apoptosis. Immunostaining of β-gal and activated caspase-3 (Casp3) mark the same enterocytes. Images show different fields in planar (d) and vertical (e) sections; dotted line in e indicates basal. f, Percentage of Casp3 +, rho-lacZ + enterocytes compared to control; n = 4 midguts per condition. See Extended Data Fig. 3a.

We then investigated whether the E-cad–Rho–Egfr pathway couples stem cell divisions to enterocyte apoptosis. If this is the case, then: (1) apoptotic enterocytes, which lose E-cad, should upregulate rho; (2) loss of E-cad in apoptotic enterocytes should underlie stem cell Egfr activation; and (3) exogenous manipulation of rho should alter organ

stem cell Egfr by preventing Egf secretion through the repression of rho.

six days after eclosion. Mean ± s.d. of three independent experiments with n = 3 midguts per experiment; n = 188 enterocytes total. g–j, Erk activation is suppressed by apoptotic inhibition and depends on E-cad and rho. See Extended Data Fig. 3b. k, Total cell counts. Hyperplasia of mex > p35, E-cad RNAi midguts requires rho, arm and p120. Hyperplasia is induced by either rho or p120 alone. Box-and-whisker plots as in Fig. 1j, k; P values by unpaired t-test compared to control; n = 5 midguts (control) and 4 midguts (all other genotypes); one of three independent experiments is shown. See Extended Data Fig. 3a, i. Model for steady-state equilibrium. Scale bars, 25 μm.
size. We investigated each prediction. First, rho-lacZ predominantly marked apoptotic enterocytes during normal turnover (Fig. 4d–f). By comparison, the cardinal injury signal up3d (refs 20, 21) rarely marked apoptotic enterocytes; up3d was also dispensable for Egfr activation (Extended Data Figs 6l, 7i and Supplementary Discussion). Therefore, rho is silenced in healthy enterocytes but upregulated in enterocytes undergoing physiological apoptosis.

Second, we blocked enterocyte apoptosis and examined stem cell Egfr activation. Erk-activated stem cells were almost completely absent following apoptotic inhibition but were restored, in a rho-dependent manner, by additional deletion of enterocyte E-cad (Extended Data Figs 4g–j and Extended Data Fig. 3b). These results demonstrate that the loss of E-cad in apoptotic enterocytes underlies Egfr activation in stem cells.

Third, we manipulated enterocyte rho and measured cell number and organ size. Overexpression of rho in apoptosis-inhibited enterocytes led to organ hyperplasia (Fig. 4k and Extended Data Fig. 3a). Conversely, loss of rho in apoptosis-competent enterocytes resulted in organ atrophy (Extended Data Fig. 8). Moreover, combined loss of both rho and E-cad in apoptosis-inhibited enterocytes prevented the hyperplasia that would have resulted from the loss of E-cad alone (Fig. 4k and Extended Data Fig. 3a). These results show that downstream of E-cad, rho is the pivot point that balances division and apoptosis to sustain cellular equilibrium.

Finally, we investigated how E-cad controls rho expression by examining three transcription factors whose nuclear localization is precluded by binding to junctional E-cad: β-catenin (Armadillo (Arm) in Drosophila), p120-catenin (p120) and Yap (Yorkie (Yki) in Drosophila)22, arm and p120, but not yki, were required in E-cad knockdown enterocytes for induction of rho and hyperactivation of stem cell Egfr (Extended Data Figs 3b, 7a, g–l). In addition, loss of both arm and rho was sufficient for rho induction, Egfr hyperactivation and organ hyperplasia (Extended Data Figs 3b, 7b–f, p–r). Therefore, E-cad controls rho by inhibiting p120 and Arm, probably through physical sequestration at cell junctions.

Our results demonstrate that steady-state turnover is not driven by constitutive cycling of stem cells. Rather, healthy enterocytes enforce a default state of stem cell quiescence, while sporadic, apoptotic enterocytes trigger replacement divisions (Fig. 4l). Because divisions are coupled to apoptosis, turnover remains zero-sum over time.

The molecular mechanism of coupling suggests a simple model for how, during continuous turnover, total cell number is held constant with such robust precision. Apoptotic enterocytes trigger stem cell divisions through loss of E-cad, which induces rho to permit secretion of mitogenic Egfs. Crucially, a single enterocyte can efficiently activate Egfr on stem cells within an around 25-μm radius (Fig. 3k). We propose that this local zone of activation enables organ size homeostasis. If, by chance, stem cells produce excess enterocytes, the physical spacing of the stem cells would increase; consequently, fewer stem cells would reside in the activation zone of the next dying enterocyte, which would lead to fewer divisions. Similarly, insufficient production of enterocytes would place more stem cells in the activation zone, which would lead to more divisions. We propose that the radii of individual activation zones, when integrated over the entire epithelium, sets total cell number and organ size. In this way, localized cell–cell communication can give rise to tissue-level homeostatic equilibrium.

**Supplementary Information** is available in the online version of the paper.

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**Author Contributions** J.L. and L.E.O. designed all experiments and wrote the manuscript. J.L. and S.N. dissected and immunostained tissue samples. J.L., S.B. and S.N. performed confocal microscopy on tissue samples. J.L. performed qPCR assays, feeding assays and genetic crosses. J.L. also performed all quantitative analysis of microscopy data (for example, cell numbers, clone sizes, cell distances) and all statistical analysis.

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**METHODS**

*Drosophila* husbandry. Crosses using the GALA/GAL80 system were performed at 18 °C. Upon eclosion, adult flies were kept at 18 °C for four days, unless indicated otherwise. On adult day 4, flies were temperature shifted to 29 °C to inactivate GAL80 and induce GAL4-mediated expression. Midguts were collected for immunostaining four days after induction, unless specified otherwise in the figure caption. All other crosses were performed at 25 °C; refer to figure legends for individual time point information. Adult female flies were used in all experiments. See Supporting Table 1 for experimental genotypes in all figures.

**Fly stocks.** The following stocks were obtained from the Bloomington Stock Center: y,w;P{ey}1118 (ref. 23), UAS-shottgun RNAi (TRIP.HM500693, TRIP.JF02769 and TRIP.JF00646), UAS-chnidin RNAi (TRIP.JF06488), UAS-rhomboid RNAi (TRIP.JF01306), UAS-sptz RNAi (TRIP.HM501120), UAS-armadillo RNAi (TRIP.JF01251), UAS-p120ctn RNAi (TRIP.HM033276), UAS-yorkie RNAi (TRIP.JF03119), UAS-unpair3 RNAi (TRIP.HM50061), UAS-hisH2A.RFP, UAS-p35, UAS-diap1, UAS-rhomboid, UAS-armadillo, UAS-p120ctn, Egfr655/T2/3TSTL, Egfr647/T2/3TSTL, eye-lacZ and 10 × Stat-GFP. UAS-keren RNAi (KK104299) and UAS-armadillo RNAi (KK107344) were obtained from the Vienna Drosophila Resource Center. The following stocks were gifts: egl-fp-out line (from B. Edgar), mexGAL4 (from C. Thummel), UAS-E-cadherinGFP35 (from M. Fuller), rhoX411 (rho-lacZ) and Upd3.1-lacZ (from H. Jiang), UAS-groucho (from A. Orian). Other stocks used: egGAL4, y,w;hs-flp: X-15-29 w′ (‘split-lacZ’), y; y′ X-15-33 (‘split-lacZ’), w UAS-CDB-GFP hsph; tubGAL4; FRT82 tubGAL80; w; FRT82 (used in our previous study3). Detailed information on *Drosophila* genes and stocks is found on FlyBase (http://flybase.org/).

**Immunohistochemistry and microscopy.** Samples were fixed, immunostained and mounted as previously described3. Primary antibodies: mouse anti-β-galactosidase (1:400, Promega Z3781), mouse anti-Armadillo (1:100, DSHB N27A1), rabbit anti-cleaved caspase 3 (1:200, Cell Signaling), rabbit anti-dpErk (1:400, Cell Signaling 4370P), goat anti-HRP-Cy3 (1:100, Cappel), which anti-cleaved caspase-3 (1:200, Cell Signaling, gift from D. Bilder3), rabbit anti-lacZ (‘split-lacZ’8), and Upd3.1-lacZ (from A. Orian). Our split-lacZ resource center. The following stocks were gifts: Egfrtsla/T(2;3)TSTL (TRiP .JF03119), UAS-E-CadherinGFP35 (Extended Data Fig. 1b–e, g) and variable, incomplete turnover during the same time period, consistent with previous proximal-distal axis. Each compartment exhibits a characteristic digestive physiology.

**Immunostaining.** Animals were anaesthetized and fixed in 4% PFA solution in PBS, pH 7.4. Whole-mount midguts were dissected and mounted in ProLong (LifeTechnologies). Because Sytox is incompatible with fixation, live organs were imaged immediately after mounting. MARCM clone induction. MARCM clone inductions2 were performed by selecting flies to 20–30 min. 38.5 °C heat shocks separated by a 5-min chill on ice. For single-enterocyte MARCM clones, flies were dissected five days after induction and terminal clones consisting of one GFP + enterocyte (identified by its polyploid nucleus) were selected for analysis. GFP + enterocytes were excluded from analysis if another GFP + clone was present within an 80-μm radius. Fiji was used to measure the distance between the plasma membrane of the nearest GFP + enterocyte and the centre of dpErk + stem cells within a 60-μm radius. For mosaic analyses of multi-cell MARCM clones, flies were Sytox three days after induction and dissected. The proportion of labelled clone cells (GFP +) that were also Sytox + was quantified.

**Smurf assay.** Smurf assays were conducted by feeding adult flies yeast paste containing 2.5% Brilliant Blue FCF (Sigma) and scoring flies for leakage of dye into the abdomen. Flies were scored as ‘non-Smurf’ if the blue dye was confined to the gastrointestinal tract and ‘Smurf’ if the blue dye leaked outside the gastrointestinal tract. As a positive control, flies were fed dye in conjunction with 1% SDS. qRT–PCR. mRNA was extracted from midguts (five midguts per biological replicate) followed by cDNA synthesis with Invitrogen SuperScript III First Script Super Mix (Invitrogen). Real-time PCR was performed using the relative standard curve method with SYBR GreenER Supermix (Invitrogen) on a StepOnePlus ABI machine. Each biological replicate was assessed in three technical replicate experiments. Expression levels were normalized to mexGAL4 > CD4-GFP midguts; mef2 transcripts were used as a reference3. Primer sequences (Supplementary Table 2) were from refs 16 and 28 and from Fly Primer Bank (http://www.flyprimerbank.org/).

**Statistical analysis.** All statistical analyses were performed using Graphpad Prism 6. For comparisons of clone size distributions, unpaired two-tailed Mann–Whitney U-tests were used to assess statistical significance. (Clone size distributions are non-normal, independent and derived from a simple random sample.) For comparisons of cell numbers and gut length, unpaired two-tailed t-tests were used to assess statistical significance. (Organ cell number and size distributions are normal, independent and derived from a simple random sample.) For comparisons of rho gene expression, unpaired two-tailed t-tests were used to assess statistical significance. For all box-and-whisker plots: boxes are 25th percentile, median and 75th percentile. Whiskers are maximum and minimum values. Sample sizes were chosen based on our previous study3, which also characterized changes in organ cell number and clone sizes. In split-lacZ experiments, single enterocyte clones were excluded from analysis. No other exclusion criteria were applied. No sample randomization or blinding was performed, although automated, Imaris-based computer algorithms were used to analyse and quantify most of the data in this study.

**Data availability.** The authors declare that all quantitative data supporting the findings of this study are available as Source Data. Source Data for all graphs in Figs 1–4 and Extended Data Figs 3, 5–8 are provided.


Extended Data Figure 1 | Midgut lineage and morphology, esgF/O labelling system and workflow for semi-automated cell counting.

a, Lineage of the adult Drosophila midgut. Stem cells are, in general, the only cells capable of division. Asymmetric stem cell divisions typically produce absorptive enterocytes; less frequently, they produce secretory enteroendocrine cells. Enterocytes arise through direct maturation of transient, post-mitotic intermediates called enteroblasts. Stem and enteroblast cells express the Snail-family transcription factor escargot (esg).

b, Compartments of the female adult midgut are used for all experiments in this study. Midgut schematic based on ref. 7.

c,–e, Identification of R4ab using morphological landmarks. As defined in ref. 7, R4ab is bounded by the apex of the midgut tube’s most distal 180° turn (blue arrowheads in d) and the first prominent muscle constriction distal to this 180° turn (red arrowheads in d). The R4ab distal muscle constriction (red arrowheads) is particularly apparent in confocal optical sections. Visceral muscle stained with phalloidin. Midguts in c, d and in e are two different samples.

f, Genetic schema of esgF/O > GFP system. Stem and enteroblast cells are induced to express heritable GFP by temperature shift from 18 °C to 29 °C. The temperature shift inactivates GAL80 and allows the stem- and enteroblast-specific esgGAL4 to drive expression of both UAS-GFP and UAS-flp. The flp recombinase renders GFP expression permanent and heritable by excising a CD2‘flp-out’ cassette to generate a functional actGAL4. Once generated, actGAL4 drives expression of UAS-GFP (and UAS-flp) irrespective of cell type. Therefore, after the temperature shift, all mature cells that arise from undifferentiated cells will express GFP.

g, Pipeline for semi-automated, comprehensive cell counts of 3D-reconstructed midgut regions. (1) Confocal microscope z stacks capturing the entire depth of the organ are visualized in Fiji. (2) The R4ab region of the midgut (yellow outline) is digitally isolated and exported to Imaris. (Only the top half of the gut tube is shown.) Note that different midgut regions have different rates of turnover: R4ab undergoes complete turnover between adult days 4 and 8 (at 29 °C). However, other regions undergo slower turnover, as shown by large unlabelled regions outside of R4ab (see Methods). (3) To quantify total cells, nuclei (DAPI) are mapped to surface objects using Imaris. To quantify newly added cells in the esgF/O system, GFP+ cells are recognized in Imaris by co-localization of GFP and DAPI channels, and subsequently mapped to surface objects. Scale bars, 100 μm.
Extended Data Figure 2 | Genetic schema of system to simultaneously manipulate enterocyte expression and trace stem cell divisions.

a. Detailed explanation of the genetic system in Fig. 1f. Flies are raised at 18 °C; at this temperature, GAL80<sup>ts</sup> represses mex<sup>+</sup>-driven GAL4 in enterocytes (mex<sup>+</sup>) and lacZ labelling of stem cells is not induced. At four days after eclosion, flies are temperature-shifted to 29 °C; consequent inactivation of GAL80<sup>ts</sup> allows mexGAL4 to express genes of interest (UAS-gene X) specifically in enterocytes. After one day of UAS gene expression (five days after eclosion), flies are shifted to 38.5 °C for 1 h to induce ubiquitous expression of flp-recombinase, which is under control of a heat-shock promoter (hs-flp). Flp catalyses trans-recombination of the two FRTs to place the α-tubulin promoter upstream of the promoterless nls-lacZ cassette and, consequently, turn on permanent nls-lacZ expression. After heat shock, flies are returned to 29 °C to maintain UAS-transgene expression. Midguts are collected for clonal analysis four days after the 38.5 °C heat shock (nine days after eclosion). See Methods.

b. Validation of genetic system using mex<sup>+</sup>&gt;his2av::RFP. β-Galactosidase marks a stem cell clone (outlined) in a background of His2av::RFP<sup>+</sup> enterocytes. Within the 5-cell clone, only the enterocyte (yellow asterisk, polyploid) expresses his2av::RFP.
Extended Data Figure 3 | Quantification of organ size and Egfr activation in genetically manipulated midguts. 

a, Lengths of the R4ab compartment. $n = 15, 11, 12, 10, 11, 11, 9, 10, 12, 11, 9, 10, 13, 11, 13,$ and 11 midguts from left to right, analysed after four days of UAS-transgene expression. $n = 4, 4, 5, 4, 3, 4, 3, 4, 3, 4, 3, 4, 4, 4,$ and 4 midguts from left to right, analysed after two days of UAS-transgene expression. One of two replicate experiments was quantified.

b, dpErk $^+$ cells in the R4ab compartment. $P$ values by unpaired $t$-test compared to control.

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Extended Data Figure 4 | Analysis of epithelial architecture, polarity and barrier function. a–f, Apoptotic inhibition (b, e) or E-cad depletion (c, f) in enterocytes does not disrupt either epithelial architecture or apical–basal polarity. Images show vertical sections through the midgut epithelium after four days of either mexΔ>p35 or mexΔ>E-cad RNAi expression. Enterocytes remain as a coherent monolayer. Apical–basal polarity is intact, as revealed by immunolocalization of apical, actin-rich microvilli (a–f; SiR-Actin, red) and of two apico-lateral septate junction proteins, Coracle (a–c, green) and Discs-large (d–f, green). At the basal surface of the epithelium (white, dotted lines), midgut visceral muscle cells stain brightly for actin and Discs-large. Scale bars, 25 μm. g–j, Depletion of E-cad in enterocytes does not compromise the intestinal barrier. To test the intestinal barrier, flies were subjected to Smurf assays in which a blue, non-absorbable food dye is administered by feeding. The dye remains within the midgut (arrowhead) when the barrier is intact (g, non-Smurf), but leaks into the body cavity when the barrier is compromised, such as after the consumption of 1% SDS (h, Smurf). After 10 days of mexΔ>E-cad RNAi expression, midguts still retain the blue dye; no Smurf phenotypes were observed (i, j).
Extended Data Figure 5 | Depletion of E-cad has distinct cell-autonomous and tissue-level effects on cell death. In Fig. 2h, total cell counts show that midguts accumulate excess cells when E-cad is depleted from apoptosis-inhibited enterocytes, but not apoptosis-competent enterocytes. To shed light on this difference, we examined whether E-cad depletion itself promotes cell death. Two approaches, mosaic knockdown and pan-enterocyte knockdown, were used to distinguish direct, cell-autonomous effects from indirect, tissue-level effects. 

a–c, Mosaic knockdown of E-cad does not promote cell-autonomous death. To generate a mosaic epithelium, MARCM labelling27 was used to induce sparse, multicellular, GFP-marked clones in a background of unmarked, genetically unperturbed cells. a, b, Dotted outlines show representative control and E-cad RNAi clones (green). Sytox (red) identifies dying cells. c, Percentage of GFP⁺ cells that are also Sytox⁺. Dying cells occur with near-equal frequency within control and E-cad RNAi clones. Five midguts per genotype were analysed nine days after clone induction; n = 873 cells in control clones and 698 cells in E-cad RNAi clones. 

d–f, Pan-enterocyte knockdown of E-cad promotes cell death, probably through a non-autonomous effect. d, e, Representative images of mex⁵ control and mex⁵ > E-cad RNAi epithelia. Sytox (red) identifies dying cells.

f, Quantification of Sytox⁺ cells in the R4ab compartment. The number of dying cells increases approximately 2.5 × in E-cad RNAi midguts compared to control. n = 5 midguts per genotype, analysed after three days of transgene induction. c, f, Box-and-whisker plots as in Fig. 1j, k; P values by unpaired t-test. Scale bars, 25 μm. 
g, Summary. The unaltered frequency of dying cells in E-cad RNAi mosaic clones indicates that loss of E-cad does not cause cell-autonomous death. This result suggests that increased cell death in mex⁵ > E-cad RNAi guts is a non-autonomous, tissue-level effect, possibly due to excess divisions (Fig. 2b) and consequent crowding. These findings may explain why p35, E-cad RNAi guts accumulate excess cells, whereas E-cad RNAi guts retain a normal number of cells (Fig. 2h).
Extended Data Figure 6 | Loss of enterocyte E-cad activates Egfr, but not Wg, Hpo or Upd–Jak–Stat. a, Effect of enterocyte E-cad depletion on target mRNA expression of known midgut regulatory pathways. mRNA expression was measured by qPCR of mex control or mex > E-cad RNAi midguts. Relative to control (dotted line), mRNA expression levels do not increase for the Wg targets frizzled-3 (fz3) and senseless (sens), the Hpo–Yki targets expanded (ex) and diap1, the injury-associated cytokines upd and upd3, and the Jak–Stat target windpipe (wdp). The other Jak–Stat target, Socs36E, is increased, which probably reflects its occasional activation in enterocytes (f). By comparison, the Egfr target pointed (pnt) is slightly increased and the Egfr target cyclinE (cycE) is substantially increased. Values are mean ± s.d.; data points are individual biological replicates analysed four days after induction. b–d, The number of upd3-lacZ enterocytes in the R4ab compartment is unchanged by enterocyte E-cad depletion. Occasional activation of 10×Stat-GFP+ occurs in E-cad-depleted enterocytes (asterisk), consistent with increased Socs36E (a). h–j, The number of cycE+ diploid cells in R4ab increases after enterocyte E-cad depletion.

d, g, j, Box-and-whisker plots as in Fig. 1j, k; P values by unpaired t-test; NS, not significant. n = 4 midguts per condition, analysed two days after induction. One of two replicate experiments was quantified. k, dpErk immunostaining is limited to stem cells (Hrp+, Su(H)lacZ−; arrowheads) and does not mark enteroblasts (Hrp+, Su(H)lacZ+; asterisks), even in mex > E-cad RNAi midguts. l, Expression of upd3 is not associated with physiological apoptosis. Most enterocytes that express upd3-lacZ are non-apoptotic, as assessed by staining for cleaved caspase-3. Values are means ± s.d.; n = 4 midguts per condition, analysed six days after eclosion. Representative images are shown in all panels. Scale bars, 25 μm.
Extended Data Figure 7 | Two E-cad-associated transcription factors, Armadillo and p120-catenin, activate rho after loss of E-cad in enterocytes. a, rho mRNA levels were measured by qPCR of mex^{+}$E-cad RNAi midguts with additional manipulation of candidate rho regulators as indicated. Five candidates were examined: yki, a transcriptional co-activator in the Hpo pathway; groucho, a co-repressor known to target rho in some tissues; puckered (puc), an inhibitor of Basket/Jnk, the latter of which can enhance Egf signalling; and arm and p120, co-activators that are inhibited by sequestration at E-cad adherens junctions. Knockdown of either arm or p120 significantly reduces rho activation. P values by unpaired t-test compared to E-cad RNAi alone. b, Overexpression of p120, but not arm, in enterocytes is sufficient to increase rho mRNA levels.

a, b, Values are mean ± s.d.; three biological replicates per condition, analysed four days after induction. Dotted lines show rho mRNA levels in unmanipulated controls. c–f, Depletion of E-cad or overexpression of p120 induces rho-lacZ in enterocytes and not in diploid cells. f, Quantification.

g–l, Enterocyte arm and p120, but not yki or upd3, are necessary for activation of stem cell Egfr (dpErk immunostaining) following loss of E-cad. m–o, Enterocyte rho is necessary and sufficient for activation of stem cell Egfr. p–r, Enterocyte p120, but not arm, is sufficient to activate stem cell Egfr. See also Extended Data Fig. 3b, s, Overexpression of enterocyte rho increases the number of mitotic (phospho-histone H3^{+}) stem cells. f, s, Box-and-whisker plots as in Fig. 1j, k; assessed after two days of transgene expression; P values by unpaired t-test compared to control; n = 5, 5, 5, 4 and 3 midguts from left to right (f); and n = 5 midguts (control) and 4 midguts (rho) (s). Representative images are shown in all panels. Scale bars, 25 μm.
Extended Data Figure 8  |  Loss of rho, arm or p120 in enterocytes results in organ atrophy. a, Total cell counts. Depletion of rho, arm or p120 in enterocytes reduces total cells compared to control. b, Depletion of enterocyte rho, arm or p120 reduces the length of the R4ab compartment compared to control. a, b, Box-and-whisker plots as in Fig. 1j, k; n = 5 midguts per genotype, analysed after six days of induction; P values by unpaired t-test compared to control. One of three independent experiments is shown in each graph. c, d, Depletion of enterocyte rho leads to organ atrophy. Representative whole mount images are shown. A, anterior; P, posterior. Scale bar, 200 μm.