Supplementary Information

Regulation of *Drosophila* intestinal stem cell maintenance and differentiation by the transcription factor Escargot.

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Figure S1. Loss of Escargot induces ISC loss and a bias towards the enteroendocrine cell fate.

(A) Normal midgut esg mRNA expression in esg\textsuperscript{shof} mutants. FISH/IF for esg mRNA and Armadillo (Arm) protein in midguts from 2-do adults that are hetero- or homozygous for the esg shutoff (esg\textsuperscript{shof}) allele. The Arm staining of cell boundaries was used to identify ISC/EB pairs, based on their characteristic smaller size and bowtie shape. Scale bars = 20µm.

(C) Esg is required for maintenance of ISCs. Images of control and esg\textsuperscript{G66} MARCM clones (4 dphs, 25°C), as in Fig. 1E. Control clones had an expected distribution of cellular compositions: single GFP+ EE cells (i) or ECs (ii) that likely originated from the permanent GFP-labeling of an EB. In addition, there were clones with 2 or more cells that contain one or more Pros\textsuperscript{−} diploid cells (iii), originated from the permanent GFP-labeling of a functional ISC. By contrast, esg\textsuperscript{G66} mutant clones with more than one cell frequently deviated from this expected pattern: they lack a putative ISC and have only differentiated cells (shown are examples of clones with two ECs and one EE (iv) or two EE cells (v)). Scale bars = 20µm (upper panels) and 10µm (insets).

(B) Automated cell profiling within MARCM clones. CellProfiler was used to delineate GFP+ clones from images as in Fig. 1C (left panel) and identify all of the cells within based on DAPI and Pros staining (blue and red lines, respectively – middle panel). The histogram (right panel) shows the bimodal distribution of nuclear sizes in control clones (the red line denotes the cutoff used to classify cells as diploid or polyploid in downstream analyses). The combination of nuclear size and Pros staining allowed for the classification of cells within clones as EC (polyploid), EE (diploid and Pros+) or ISC/EB (diploid and Pros-). Scale bar = 20µm.

(D, E) Quantification of phenotypes induced by the loss of esg. (E) Proportion of multicellular clones that contain only differentiated cells for the indicated genotypes. *** and * denote significant differences in the proportion of ISC/EB-less clones for the indicated pairs (p<0.0001 and p=0.0118, respectively, in a two-sided Fisher’s exact test). (F) Further classification of the ISC/EB-less clones from (E) based on their cellular composition. Orange
bars correspond to clones that contain only ECs, grey bars correspond to clones containing a mix of ECs and EEs and green bars to clones with only EEs. * as in (E) (p=0.0211).

(F) Enrichment for EE cells following Esg knock-down with alternative RNAi lines. As in Fig. 1D, CellProfiler was used to quantify the relative proportion of EE cells following 6 days of Esg knockdown in ISC/EBs via the 5961-GAL4<sup>GS</sup>-driven expression of an alternative UAS-esgRNAi construct (Harvard TRiP collection). *** denotes a significant EE cell enrichment in RU486-fed flies relative to EtOH controls (p<0.001, two-tailed unpaired Student’s t-test).

Figure S2. Characterization of ISC and EB-specific drivers.

(A) ISC-specific transgene expression using an esg-Gal4/Su(H)-Gal80 driver. Midguts of the indicated genotypes maintained for 3 days at the indicated temperatures were stained as indicated following incubation for 3 days at the indicated temperatures. Bottom images are zoomed in images from the corresponding dashed boxes. Notice that YFP expression is highly restricted to only one diploid cell per ISC/EB pair in control flies, but YFP expression can be detected in more than a diploid cells when esgRNAi is expressed with this driver (and some larger cells as well, likely mature EBs or early ECs). The yellow arrowhead points to an example of an esg/Pros double positive cell. Scale bars = 20µm (main figure), 5µm (inset).

(B) EB-specific transgene expression using a Su(H)-Gal4 driver. To confirm the restricted activity of a temperature-sensitive and EB-specific GAL4 driver, midguts of the indicated genotype (Su(H)-GAL4<sup>ts</sup> > UAS-gfp<sup>CD8</sup> = Su(H)-GAL4,UAS-gfpCD8 ; tub-GAL80<sup>ts</sup> – derived from Zeng et al. (Zeng et al, 2010)) were stained as indicated following a 2-days incubation at 29°C. We noticed some variability in the intensity of GFP staining among EBs, which did not change upon longer incubations at 29°C. The insets show examples of GFP+ doublets that, upon closer inspection, were always adjacent to additional GFP+ diploid cells (arrowheads). The latter are likely ISCs, suggesting that both GFP+ cells in a doublet are true EBs (and not ISC/EB pairs). Scale bars = 20µm (both main figure and inset).
Figure S3. Rescue of *esg* loss of function phenotypes by ectopic Notch activation.

(A) Reduced Su(H) activity in EBs following Esg knockdown in ISC/EBs. Midguts of the indicated genotypes were stained with DAPI (all nuclei), GFP (ISC/EBs) and βGAL (EBs) following a 4 days incubation in 10µg RU486/mL food at 25°C. Scale bars = 20µm.

(B) Representative example of a midgut expressing NotchRNAi in ISC/EBs under control of an *esg*-GFP,5961-Gal4GS driver (6 days induction in 10µg/mL RU486).

(C,D) Rescue of the EE cell enrichment phenotype caused by Esg knockdown by expression of constitutively active Notch using the *esg*ts driver. (C) Midguts of the indicated genotypes were stained with for DAPI (all nuclei) and Pros (EE cells) following a 7-days incubation at 29°C (or 10 days at 18°C - not shown). Scale bars = 20µm. (D) Images as shown in (C) were quantified with CellProfiler to determine the relative proportion of EE cells. Midguts overexpressing the esgRNAi construct alone were the only sample that showed a significant EE enrichment over the baseline proportion in control midguts at 18°C (*** = p<0.001, 1-way ANOVA/Bonferroni test).

(E,F) Cell Profiler measurements of EC and EE/EC relative proportions following EsgRNAi expression ISC/EBs, alone or in combination with N\textsuperscript{intra}. (E) DAPI images as those shown in (Fig. 3C) were quantified with CellProfiler and used to identify polyploid ECs based on a distribution of nuclear sizes (as shown in Supplementary Fig. S1D). *** and * denote significant differences in the relative proportions of ECs from the sample pairs indicated by the corresponding bars (p<0.001 and p<0.05 respectively, based on a Kruskal-Wallis/Dunn test). (F) The proportion of EE cells relative to the corresponding ECs, as determined by CellProfiler quantifications as in Fig. 3D and (C). *** and * denote significant differences between the sample pairs indicated by the corresponding bars (p<0.001 and p<0.05 respectively, based on a Kruskal-Wallis/Dunn test). Some irrelevant pairwise comparisons (e.g. EtOH vs RU samples from different genotypes) are not shown for clarity.

Figure S4 Amun overexpression recapitulates *esg* loss of function phenotypes.
(A) **ISC/EB-specific expression of an Amun-HA transgene.** Midguts of the indicated genotype (also carrying the Su(H)-lacZ reporter – not shown) were stained with DAPI (all nuclei), GFP (ISC/EB) and HA (to detect expression of the UAS-AmunHA transgene) following a 6 days incubation in 25µg RU486/mL or ethanol-containing food (as indicated). Scale bars = 20µm.

(B) **Amun overexpression in ISC/EBs causes a partial loss of Notch phenotype.** Midguts of the indicated genotype were stained with DAPI (nuclei), GFP (ISC/EB) and βGAL [Su(H) activation]. Scale bars = 20µm.

(C) **CellProfiler quantification of the relative proportion of EE cells in midguts from (B).**

*** denotes a statistically significant difference between the samples (p<0.001, Mann-Whitney U-test).

**Figure S5. Amun upregulation following Esg knockdown in ISC/EBs.**

(A) **RT-qPCR measurements of relative transcript abundances for the indicated genes from whole guts dissected from esg-GFP, 5961^GS > UAS-esgRNAi flies incubated on ethanol or RU486 (10 mg/mL for 4 days).** Shown are representative means ± SEM of efficiency-corrected relative quantities for each primer set, normalized to the corresponding EtOH control. *** and * denote a significant reduction in Gfp and an increase in Amun transcript levels, respectively (p<0.001 and p=0.028, two-tailed unpaired Student’s t-test; n.s.: non-significant). RpL32 is a housekeeping gene used as a cDNA input control.

(B) **FACS gates used for sorting GFP+ and GFP- cells from flies treated with ethanol or RU486 (25µg/mL-3 days) as indicated.** Left panels: dissociated cells were stained with propidium iodide (PI), a DNA dye taken up by dead cells, and Draq5, a DNA dye taken up by living cells. The PI gate (Draq5-high/PI-low) captured mostly living cells. Middle panels: gates used for GFP+ and GFP- cells. Notice the presence of an intermediate population, which increased in abundance following esgRNAi expression (arrow), likely reflecting differentiating cells that express reduced levels of the esg-GFP reporter. Right panels: FSC/SSC plots for the gated GFP+ and GFP- cells.
(C) FISH/IF for Amun mRNA, GFP and Armadillo (Arm) in midguts of the indicated genotypes, after a 4-day incubation in RU486 (10µg/mL) or ethanol-containing food (“esgRNAi” and “control”, respectively). The dashed outlines in the right panels correspond to the ISC/EB cell membranes, as determined based on Arm staining (left panels). The strongly stained stripes in the FISH image correspond to non-specific labeling of the tracheae (“Tr”), a probe-independent phenomenon when performing FISH in midguts (Toledano et al, 2012). Scale bars = 20µm.

**Figure S6. An independent AmunRNAi line also rescues the EE cell bias caused by loss of Esg function.**

(A,B) Partial rescue of EE cell accumulation by co-inhibition of Amun and Esg. (A) These samples were obtained from the same experiment shown in Fig. 5A,B, but correspond to the cross using an independent insertion of the AmunRNAi (line2). The co-expression of AmunRNAi and esgRNAi significantly rescued the EE accumulation phenotype (blue ***), but did not fully restore baseline EE cell proportions, as indicated by a mild yet statistically significant enrichment of EE cells compared to the corresponding 18°C sample (red **). Scale bars = 20µm. *** and ** (p<0.001 and p<0.01, respectively, 1-way ANOVA/Bonferroni test).

**Figure S7. Depletion of Amun suppresses phenotypes caused by loss of Esg function.** Immunostaining of midguts following co-downregulation of Esg and Amun in ISC/EBs. The indicated genotypes were incubated for 5 days at 25°C on ethanol or 10µg/mL RU486-food, and stained for DAPI (nuclei), GFP (EBs) and βGAL (Notch activation). Co-expression of AmunRNAi rescued the expression of the Su(H)-lacZ reporter in EBs. Notice the remaining altered morphology of some EBs (as in Fig. 5A and Supplementary Fig. S6A). Scale bars = 20µm.
Table S1. MARCM clonal analysis of esg<sup>G66</sup> in midguts.

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Indicated are the number of clones analyzed with Cell Profiler (Supplementary Fig. S1D). An average of 4 images were analyzed per midgut scored. Clones were classified into uni- or multicellular (2 or more cells), and the latter were further subdivided into multicellular clones that contained diploid, Pros-negative cells (ISC/EB+, not shown), or “ISC/EB-less” clones that only contained fully differentiated polyploid ECs or Pros+ EEs. These were in turn sub-classified into clones that contained only ECs, only EEs or a mix of both cell types (as indicated).

Table S2. Putative Esg target genes based on <i>in vivo</i> DamID analysis (associated MS Excel file). List of genes that were identified as putative targets of Esg in the midgut based on the presence of an Esg binding region (EBR) within ± 5kb of its sequence in at least 2 of the 3 tiling array datasets (Array #1 - #3). For each gene, the corresponding EBR with the highest average intensity across probes is shown (Max avg ratio – the average of log2[Dam:Esg/Dam intensity ratio] values within the EBR) along with the chromosomal coordinate of their approximate midpoint. For example, anterior open (aop) had 3-4 associated EBRs in each of the three arrays,
but only the ones with the highest average ratio for each of the datasets are listed. Dashes indicate the absence of an EBR associated with a gene from a particular dataset.
SUPPLEMENTARY MATERIALS AND METHODS

*Fly stocks.* The *Su(H)-lacZ*, 5961- GAL4\textsuperscript{GS}, UAS-gfp and the *Su(H)-lacZ*: esg-GFP, 5961-GAL4\textsuperscript{GS} stocks were made from *Su(H)GBE-lacZ* (gift from H. Jasper (Bray & Furriols, 2001)), 5961-GAL4\textsuperscript{GS} (gift from B. Ohlstein) and esg-GFP/CyO (gift from L. Cooley). An alternative esg-GFP reporter used in this study is *esg-GAL4*, UAS-gfp (gift from A. Christiansen and B. Baker). The *esg-GAL4*, UAS-gfp, *tub-GAL80*\textsuperscript{s} stock was a gift from B. Edgar (Heidelberg University) (Jiang et al, 2009). The esgRNAi lines used are UAS-esgRNAi (Vienna *Drosophila* RNAi Center, VDRC, #9793) and UAS-esgRNAi (Harvard Transgenic RNAi Project, TRiP HMS00025). Two UAS-AmunRNAi lines from the VDRC were used in this study; #104808, referred to as "line 1", corresponds to an insertion on chromosome II, whereas #40622, or "line 2" is an insertion on chromosome X. The w; UAS-Amun:HA line is from the Bloomington Stock Center (#27600). The stocks used for MARCM clonal analysis (see below) were derived from *esg*\textsuperscript{G66}, FRT40A / CyO (Voog et al, 2008). The *esg shutoff* (shof) allele was recovered during a male sterility genetic screen (G.Hime, Stanford University - (Voog et al, 2014)). The *Su(H)-GAL4*, UAS-gfp\textsuperscript{CD8} used to derive *Su(H)-GAL4*, UAS-gfp\textsuperscript{CD8}; tub-GAL80*\textsuperscript{s} flies (Zeng et al, 2010), and the esg-Gal4,UAS-2xYFP; *Su(H)-Gal80*, tub-GAL80*\textsuperscript{s} (Wang et al, 2014) were generous gifts from S.Hou (NCI). The 10X-STAT-gfp reporter line was a gift from N. Perrimon and GH. Baeg (Harvard University)(Bach et al, 2007). The UAS-N\textsuperscript{intra} stock is from B. Glise (Centre de Biologie du Development, Toulouse, FR). See *in vivo* DamID section for generation of UAS-dam:esg flies. Oregon R flies were used for outcrossing drivers to a wild type control.

*GAL4/UAS systems.* GAL4 GeneSwitch (GAL4\textsuperscript{GS}) drivers expresses a fusion of the yeast GAL4 DNA binding domain, the human progesterone receptor and a transcriptional activation domain from human NFkB (Osterwalder et al, 2001). The activity of GAL4 is temporally controlled by feeding the flies the steroid hormone mifepristone (RU486, at 10 or 25\textmu g/mL in the food – Sigma, M8046), eliminating genetic background effects since flies differ only with respect to the presence (or not) of RU48 in their food. In the TARGET approach, a tissue-specific GAL4 is recombined with a constitutively expressed *tubulin (tub)-GAL80*\textsuperscript{s}. At the permissive temperature (18°C), GAL80*\textsuperscript{s} binds and inhibits the activity of the GAL4, whereas shifting the flies to the restrictive temperature (29°C) alleviates the GAL80 repression and allows the activation of UAS-transgenes.
The following is a list of the antibodies used, the host species, concentration and source: GFP (rabbit, 1:3,000, Molecular Probes A-11122); GFP (mouse, 1:200, Molecular Probes A-11120); GFP (chicken, 1:500, Aves Labs GFP-1010); DIG-HRP (Fab fragment, 1:500, Roche); β-GAL (rabbit, 1:2000, Molecular Probes 559761). The following antibodies were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. Pros (mouse, 1:20, MR1A); Arm (mouse, 1:20, N27A1); Delta (mouse, 1:100, C594.9B); DEcad (rat, 1:20, DCAD2).

**in vivo DamID.** The DamID technique was used to characterize the in vivo chromatin-binding profile of Esg in whole intestines (Choksi et al, 2006; van Steensel & Henikoff, 2000). Briefly, the expression of a Dam:Esg fusion construct permits Dam-mediated in vivo adenine methylation of GATC sequences nearby genomic sites bound by Esg. GA\textsuperscript{m}TC sites can be cut by the methylation sensitive restriction endonuclease DpnI, which allows for the isolation, PCR-based labeling and profiling of methylated fragments. The esg coding sequence was cloned into pUAST-NDam (Choksi et al, 2006) using NotI and XbaI sites. Transgenic flies were generated as previously described (Choksi et al, 2006), and midguts from 5-6 days old adult females were dissected and immediately frozen at -80°C for later processing. Genomic DNA was isolated from approximately 50 midguts per genotype. The frozen tissue was allowed to thaw on ice and immediately ground in 200µL of ice-cold PBS containing 100mM EDTA (pH=8.0), 1.25mg/mL RNaseA, 200µg/mL Proteinase K and incubated at 70°C for 10min. Debris was pelleted by centrifugation at maximum speed for 5min, and the supernatant was subjected to 3 rounds of phenol:chloroform:isoamyl alcohol (P:C:I) extraction. 2.5 μg of genomic DNA (gDNA) were processed following the protocol in ref. (Choksi et al, 2006) without any further modifications.

Perl scripts used for identification of putative Esg targets and data visualization with the USCS Genome Browser are available upon request and are slightly modified versions from those in Southall and Brand (Southall & Brand, 2009). A gene was identified as putative Esg target if an Esg binding region (EBR) was mapped within ± 5kb of its sequence. EBRs are strings of at least 8 consecutive probes with a Dam:Esg/Dam intensity ratio above a chosen threshold, which was adjusted for each dataset to generate similar distributions of EBR size. The search for EBRs was restricted to chromosomes X, 2 and 3. For the putative targets listed on Table E2, genes had an
associated EBR in at least 2 out of the 3 datasets, and only EBRs between 8-12 probes in length were considered.

RT-qPCR on whole guts. Whole guts from Su(H)-lacZ; esg-GFP, 5961-GAL4GS; UAS-esgRNAi females incubated on 10 µg/mL RU or EtOH food for 4 days were dissected and immediately frozen at -80°C, until approximately 250 guts per treatment group had been collected. Total RNA was extracted with Trizol® (Life Technologies, cat#15596026), following the manufacturer’s instructions. After confirming the integrity of the RNA sample by gel electrophoresis, 2 µg of RNA were treated with DNase Q1 (Promega, cat#M610A) in a 20 µL reaction volume. 10 µL of the DNaseQ1-treated RNA were reverse-transcribed using the iScript kit (Bio-Rad, cat#170-8841) - 2.5 µL were used to make a corresponding no-RT control sample. Standard qPCR reactions were carried out on a BioRad CFX96/C1000Touch system (Bio-Rad), using SsoAdvanced SYBR®Green (Bio-Rad, cat#1725-264). The following are the primer sequences used: RpL32 Fwd: ATCGTGAAGAAGCGCACCAA; RpL32 Rev: TGTCGATACCCTTGGGCTTG; GFP Fwd: TCCGCCCTGAGCAAAGAC; GFP Rev: GAACTCCAGCAGGACCATGTG; Amun Fwd: TAAACACCAGCCCGGTACTT; Amun Rev: GATGCGGATGTGTCGTTC.

Dissociation and FACS-sorting of intestinal cells. The following protocol was adapted with minor modifications from (Dutta et al, 2013), incorporating suggestions from Pedro Victor (H. Jasper lab, Buck Institute). Approximately 50-60 guts/genotype were dissected (5 at a time) in ice-cold DEPC/PBS, and immediately placed on a tube with DEPC-PBS/BSA1% on ice. Dissected guts were resuspended in 250uL of an enzyme cocktail containing elastase (1mg/mL) and trypsin (0.5% - in DEPC/PBS/1%BSA/0.5M EDTA, or “PBE buffer”). The guts were dissociated for 45min at 25°C, passing the tissue carefully but thoroughly through a 25 5/8 G-1mL syringe every 5-10min. 0.25uL of PI and 0.125uL of Draq5 (Thermo Scientific, cat# 62254) were added to each tube for the last 5 minutes of incubation with the enzyme cocktail. Cells were centrifuged for 20min at 1700rpm at 4C, and resuspended in 375 µL of ice-cold PBE buffer containing PI (2µg/mL) and Draq5 (1:1,000) and filtered through a 35-um mesh into FACS tube. Ten thousand cells were immediately sorted on a FACSARia Cell Sorter into 30uL of lysis buffer (ArrayPure™ Nano-scale RNA Purification Kit, Epicentre cat# MPS04050).
References:


