Enteroendocrine Cells Support Intestinal Stem-Cell-Mediated Homeostasis in *Drosophila*

**Highlights**
- The AS-C gene *scute* is necessary for the development of enteroendocrine cells.
- Enteroendocrine cells support nutrient-stimulated intestinal stem cell division.
- Tachykinin is a gut hormone mediating the enteroendocrine cell-regulated growth.
- Tachykinin regulates DILP3 expression in visceral muscle for intestinal growth.

**In Brief**
Amcheslavsky et al. show that enteroendocrine cells serve a niche function to regulate intestinal stem cell division. High-nutrient diet stimulates intestinal stem cell division and intestinal tissue growth in newly eclosed flies. Enteroendocrine cells act as an important link for this process by producing gut hormones such as Tachykinin to regulate the expression of an insulin-like peptide DILP3 in the visceral muscle. This *Drosophila* model helps to elucidate the function of enteroendocrine cells in complex whole-animal physiology.
Enteroendocrine Cells Support Intestinal Stem-Cell-Mediated Homeostasis in Drosophila

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SUMMARY

Intestinal stem cells in the adult Drosophila midgut are regulated by growth factors produced from the surrounding niche cells including enterocytes and visceral muscle. The role of the other major cell type, the secretory enteroendocrine cells, in regulating intestinal stem cells remains unclear. We show here that newly eclosed scute loss-of-function mutant flies are completely devoid of enteroendocrine cells. These enteroendocrine cell-less flies have normal ingestion and fecundity but shorter lifespan. Moreover, in these newly eclosed mutant flies, the diet-stimulated midgut growth that depends on the insulin-like peptide 3 expression in the surrounding muscle is defective. The depletion of Tachykinin-producing enteroendocrine cells or knockdown of Tachykinin leads to a similar although less severe phenotype. These results establish that enteroendocrine cells serve as an important link between diet and visceral muscle expression of an insulin-like growth factor to stimulate intestinal stem cell proliferation and tissue growth.

INTRODUCTION

The gastrointestinal (GI) tract is a complex organ essential for nutrient absorption and whole-body metabolism (Miguel-Aliaga, 2012). The Drosophila midgut is an equivalent of the mammalian stomach and small intestine. The midgut epithelium has no crypt-villus structure but instead is a monolayer of absorptive enterocytes (ECs), with interspersed intestinal stem cells (ISCs), enteroblasts (EBs), and enteroendocrine cells (EEs) located closer to the basement membrane (Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006).

All cells in the midgut likely constitute together the niche that regulates ISC proliferation and EB differentiation for tissue homeostasis. The visceral muscle secretes Wingless, insulin-like peptides, epidermal growth factor receptor (EGFR) ligands, and Decapentaplegic (Dpp)/bone morphogenetic protein (Guo et al., 2013; Jiang et al., 2011; Lin et al., 2008; O’Brien et al., 2011). The mature ECs are a major source of stress-induced Dpp, EGFR ligands, and the JAK-STAT pathway ligands Unpaired (Upd) 1–3 (Biteau and Jasper, 2011; Buchon et al., 2010; Guo et al., 2013; Jiang et al., 2009, 2011; Li et al., 2013a; Osman et al., 2012; Tian and Jiang, 2014; Xu et al., 2011). The differentiating EBs also produce Upds, Wingless, and EGFR ligands (Cordero et al., 2012; Jiang et al., 2011; Zhou et al., 2013). The surrounding trachea secretes Dpp, while the inwardly conducting neurons can also regulate intestinal physiology (Cognigni et al., 2011; Li et al., 2013b).

EEs constitute a major cell type in the Drosophila midgut epithelium. While the mammalian secretory lineage is differentiated into Paneth cells, goblet cells, enteroendocrine cells, and tuft cells (Gerbe et al., 2012), the entire population of secretory cells in the Drosophila midgut is collectively called EEs and marked by the homeodomain protein Prospero (Proe) (Michelli and Perrimon, 2006). Nonetheless, different subsets of hormones are produced from different subtypes of midgut EEs (Ohlstein and Spradling, 2006). In the mouse intestine, the Lgr5+ ISCs directly contact Paneth cells, and isolated ISC-Paneth cell doublets have higher efficiency to form organoids (Sato et al., 2011). However, mouse genetic knockout that has Paneth cells removed did not result in the loss of Lgr5+ ISCs (Durand et al., 2012). Only recently have Drosophila midgut EEs been shown to negatively regulate ISC proliferation via EGFR ligand production and to regulate ISC differentiation via the Slit/Robo pathway (Biteau and Jasper, 2014; Scopelliti et al., 2014). Therefore, the function of EEs in regulating stem cell activity largely remains to be investigated. Here, we show that Drosophila midgut EEs serve a niche function by producing hormones such as Tachykinin (Tk) to regulate insulin peptide expression in the surrounding muscle that in turn affects intestinal homeostasis.

RESULTS AND DISCUSSION

scute RNAi and Deletion Result in EE-less Flies

Previous evidence shows that adult midgut mutant clones that have all the AS-C genes deleted are defective in EE formation while overexpression of scute (sc) or asense (ase) is sufficient
Figure 1. EE-less Fly Guts after Loss of sc Function Have Growth Defects

(A) The number of Pros+ nuclei was counted within 0.08 mm² surface area of a microscopic image from a similar region of each posterior midgut. The scRNAi midguts were completely devoid of EEs.

(B) EE quantification in the midguts of flies with the genotypes indicated. Control was w- and the deficiency for sc was Df(1)sc10-1 and for ato was Df(3R)p13. Young flies were 7 days old, and aged flies were 21 days old. NS, nonsignificant (p > 0.05), and all p values are from the Student’s t test.

(C and D) Light microscope images of control and esg > scRNAi fly midguts. The arrow and hair line point to the posterior midgut region where images were taken to measure the diameter.

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to increase EE formation (Bardin et al., 2010). Moreover, the Notch pathway with a downstream requirement of ase also regulates EE differentiation (Michelli and Perrimon, 2008; Takashima et al., 2011; Zeng et al., 2013). To study the requirement of EEs in midgut homeostasis, we first attempted to delete all EEs by knocking down each of the AS-C transcripts using the ISC/EB driver esg-Gal4. The results show that sc RNAi was the only one that caused the loss of all EEs in the adult midgut (Figures 1A and S1A–S1F). The esg-Gal4 driver is expressed in both larval and adult midguts, but the esg > sc RNAi larvae were normal while the newly eclosed adults had no EEs. Therefore, sc is likely required for all EE formation during metamorphosis when the adult midgut epithelium is reformed from precursors/stem cells (Jiang and Edgar, 2009; Michelli et al., 2011).

The sc/10-1 hemizygous mutant adults were also completely devoid of midgut EEs (Figures 1B, S1G, and S1H), while other hemizygous combinations including sc/1, sc/38, and sc/5 were normal in terms of EE number. Df(1)sc10-1 is a small deficiency that has both ac and sc uncovered. sc/1 and sc/38 each contain a gypsy insertion in far-upstream regions of sc, while sc/5 and sc/6 are 1.3 and 17.4 kb deletions, respectively, in the sc 3′ regulatory region (Garcia-Bellido and de Celis, 2009). The sc/5/sc10-1 combination may affect sc expression during midgut metamorphosis and thus the formation of all adult EEs.

The atonal homolog 1 (Atoh1) is required for all secretory cell differentiation in mouse (Durand et al., 2012; VanDussen and Samuelson, 2010). However, esg-Gal4-driven atonal (ato) RNAi and the amorphic combination ato/Df(3R)ip13 showed normal EE formation (Figures 1A, 1B, S1G, S1I, and S1J). Nonetheless, we found that older ato/Df(3R)ip13 flies exhibited a significantly lower increase of EE number (Figure 1B), suggesting a role of ato in EE differentiation in adult flies.

Changing the Number of EEs Alters Lifespan
In sc RNAi guts, the mRNA expression of allatostatin (Ast), allatostatin C (AstC), Tachykinin (Tk), diuretic hormone (DH31), and neuropeptide F (NPF) was almost abolished (Figure S1K), to astatin C (AstC), Tachykinin (Tk), diuretic hormone (DH31), and neuropeptide F (NPF) was almost abolished (Figure S1K), the esg-Gal4 driver is expressed in both larval and adult midguts, but the esg > sc RNAi larvae were normal while the newly eclosed adults had no EEs. Therefore, sc is likely required for all EE formation during metamorphosis when the adult midgut epithelium is reformed from precursors/stem cells (Jiang and Edgar, 2009; Michelli et al., 2011).

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EE-less Flies Have Reduced Intestinal Growth as Observed under Starvation Conditions
One of the phenotypic changes we found for the sc RNAi/EE-less flies was that under normal feeding conditions, their midguts had a significantly narrower diameter than that of control midguts (Figures 1C and 1D). When reared in poor nutrition, WT flies had substantially bigger midgut diameter, while EE-less flies had grown significantly less (Figure 1E). The cross-section area of enterocytes in the EE-less midguts was smaller (Figure 1F), suggesting that there is also a growth defect at the individual cell level.

A series of experiments showed that ingestion of food dye by the sc RNAi/EE-less flies was not lower than control flies (Figure S2C). The measurement of food intake by optical density (OD) of gut dye contents also showed similar ingestion (Figure S2D). The measurement of excretion by counting colored deposits and visual examination of dye clearing from guts showed that there was no significant change in food passage (Figures S2E and S2F). The normal fecundity shown in Figure S1M also suggested that the mutant flies likely had absorbed sufficient nutrient for reproduction. Nonetheless, another phenotype we could detect was a substantial reduction of intestinal digestive enzyme activities including trypsin, chymotrypsin, aminopeptidase, and acetate esterase (Figures 1G, 1H, S2A, and S2B). These enzyme activities exhibit strong reduction after starvation of WT flies. The EE-less flies therefore have a physiological response as if they experience starvation although they are provided with a normal diet.

EE-less Midguts Have Reduced ISC Division and Dilp3 Expression
A previous report has established that newly eclosed flies respond to nutrient availability by increasing ISC division that leads to a jump start of intestinal growth (O’Brien et al., 2011). When we fed newly eclosed flies on the poor diet of 1% sucrose, both WT and sc RNAi/EE-less guts had a very low number of p-H3-positive cells (Figure 2A), which represent mitotic ISCs because ISCs are the only dividing cells in the adult midgut. When fed on normal diet, the WT guts had significantly higher p-H3 counts, but the sc RNAi/EE-less guts were consistently lower at all the time points. The sc/5/sc10-1 hemizygous mutant combination exhibited a similarly lower mitotic activity on the normal diet (Figure 2B).
Figure 2. EE-less Guts Have ISC Proliferation and Dilp3 Expression Defects

(A and B) Newly hatched flies (day 1) were collected and kept in normal food vials or plastic vials with filter paper soaked with 1% sucrose (starved). Each day after, midguts were dissected from flies of the indicated genotypes and stained for p-H3 to detect mitotic cells. Average number of p-H3+ cells is shown. The esg > GFP in (A) or sc/+ in (B) served as controls. The deficiency is Df(1)sc10-1.

(C) Dilp3 mRNA expression assayed by qPCR. Newly hatched esg > GFP (control) and esg > GFP, scRNAi flies were kept in normal food vials for 1 to 5 days as indicated. At each indicated day, ten flies from each sample were used for gut dissection, RNA isolation, and qPCR. Each qPCR cycle number of Dilp3 was

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When we investigated possible signaling defects in the EE-less flies, we found that in addition to other gut peptide mRNAs, the level of Dilp3 mRNA was also highly decreased in these guts while the head Dilp3 was normal (Figures 2C and S1L). This is somewhat surprising, because Dilp3 is expressed not in the epithelium or EEs but in the surrounding muscles (O’Brien et al., 2011; Veenstra et al., 2008). We used Dilp3 promoter-Gal4-driven upstream activating sequence (UAS)-GFP expression (Dilp3 > GFP) to visualize the expression in muscle (Figure 2D). Both control and sc RNAi under this driver showed normal muscle GFP expression (Figure 2E), demonstrating that sc does not function within the smooth muscle to regulate Dilp3 expression. We then combined the esg-Gal4 and Dilp3-Gal4, and the control UAS-GFP samples showed the expected expression in both midgut precursors and surrounding muscles (Figures 2F–2H). When these combined Gal4 drivers were used to drive sc RNAi, the smooth muscle GFP signal was clearly reduced (Figures 2I–2K). These guts also exhibited no Prospero staining and overall fewer cells with small sizes as expected from esg > sc RNAi (Figures 2I–2K).

The report by O’Brien et al. (2011) showed an increase of Dilp3 expression from the surrounding muscle in newly eclosed flies under a well-fed diet (see also Figure 2C). This muscle Dilp3 expression precedes brain expression and is essential for the initial nutrient stimulated intestinal growth. Our EE-less flies show similar growth and Dilp3 expression defects, suggesting that EE is a link between nutrient sensing and Dilp3 expression during this early growth phase.

Increasing the Number of EEs Promotes ISC Division Partly via Dilp3 Expression

WT and AS-C deletion (scB57) mutant clones in adult midguts did not exhibit a difference in their cell numbers (Bardin et al., 2010). Moreover, we performed esgts > sc RNAi in adult flies for 3 days but did not observe a decrease of mitotic count or EE number. Together, these results suggest that sc is not required directly in ISC for proliferation, and they imply that the ISC division defects observed in the sc mutant/EE-less flies is likely due to the loss of EEs. To investigate this idea further, we used the esgts > system to up- and downshift the expression of sc at various time points and measure the correlation of sc expression, EE number, and ISC mitotic activity. The overexpression of sc after shifting to 29°C for a few days correlated with increased EE number, expression of gut peptides, and increased ISC activity (Figure S3A–S3I). Then, we downshifted back to room temperature (23°C) to allow the Gal80ts repressor to function again. The sc mRNA expression was quickly reduced within 2 days and remained low for 4 days (Figure 3A). Although we did not have a working antibody to check the Sc protein stability, the expression of a probable downstream gene phyllodop (Reeves and Posakony, 2005) showed the same up- and downregulation (Figure 3B), revealing that Sc function returned to normal after the temperature downshift. Meanwhile, the number of Pros+ cells and p-H3 count remained higher after the downshift (Figures 3C and 3D). Therefore, the number of EEs, but not sc mRNA or function, correlates with ISC mitotic activity.

We performed another experiment that was independent of sc expression or expression in ISCs. The antiapoptotic protein p35 was driven by the pros-Gal4 driver, which is expressed in a subset of EEs in the middle and posterior midgut (Figures S4B–S4E). This resulted in a significant albeit smaller increase in EE number and a concomitant increase in mitotic activity (Figures S3J and S3K), which was counted only in the middle and posterior midgut due to some EC expression of this driver in the anterior region (Figure S4C). Therefore, the different approaches show consistent correlation between EE number and ISC division.

Dilp3 expression was significantly although modestly increased in flies that had increased EE number after sc overexpression (Figure 3E), similar to that observed in fed versus fasted flies (O’Brien et al., 2011). We tested whether Dilp3 was functionally important in this EE-driven mitotic activity. Due to the lethality, we could not obtain a fly strain that had esg-Gal4, Dilp3-Gal4, UAS-Dilp3RNAi, tub-Gal80ts, and UAS-sc to perform a comparable experiment as shown in Figure 2. So instead, we generated flies that contained a ubiquitous driver with temperature controlled expression, i.e., tub-Gal80ts/UAS-sc; tub-Gal4/UAS-Dilp3RNAi. These fly guts showed a significantly lower number of p-H3+ cells than that in the tub-Gal80ts/UAS-sc; tub-Gal4/+ control flies (Figure 3F). These results demonstrate that the EE-regulated ISC division is partly dependent on Dilp3. The expression of an activated insulin receptor by esg-Gal4 could highly increase midgut proliferation, and this effect was dominant over the loss of EEs after scRNAi (Figure S4A), which is consistent with an important function of insulin signaling in the midgut.

Tk-Secreting EEs Have a Role in Regulating Dilp3 and ISC Proliferation

As stated above, normally hatched flies did not lower their EE number after esgts > sc RNAi, perhaps due to redundant function with other basic-helix-loop-helix proteins in adults. The expression of proapoptotic proteins by the pros ts-Gal4 also could not reduce the EE number. We thus screened other drivers and identified a Tk promoter Gal4 (Tk-Gal4) that had expression recapitulating the Tk staining pattern representing a subset of EEs (Figures S4B and S4F–S4H). More importantly, when used to express the proapoptotic protein Reaper (Rpr), this driver caused a significant reduction in the EE number (Figure S4J), normalized with that of rp49 in a parallel reaction of the same RNA sample. The lowest Dilp3 expressing sample esg > scRNAi at day 1 was set as 1 (first black bar), and all other samples were calculated as relative level and plotted as shown. (D and E) Dilp3 promoter-Gal4 driven UAS-GFP expression (Dilp3 > GFP) illuminates the smooth muscle surrounding the adult midgut epithelium. This expression of muscle Dilp3 > GFP is not altered when the UAS-scRNAi construct is also driven by this Dilp3 promoter. (F–K) Confocal images of midgut at an anterior focal plane showing the visceral muscle staining, an inner focal plane showing the epithelium staining and 3D reconstruction of multiple focal planes. The control flies contained the combination of esg-Gal4 and Dilp3-Gal4 together driving UAS-GFP expression. The bottom panels (F–K) were from a fly strain that also contained the scRNAi construct. Data are presented as mean ± SEM (error bar).
Tk and Dilp3 mRNA (Figures 4A and 4B), and mitotic count (Figure 4C). The Tk-Gal4-driven expression of another proapoptotic protein, Hid, caused a less efficient killing of EEs (Figure S4J) and subsequently no reduction of p-H3 count (Figure 4C). The knockdown of Tk itself by Tk-Gal4 also caused significant reduction of p-H3 count (Figure 4D). A previous report revealed the expression by antibody staining of a Tk receptor (TkR86C) in visceral muscles (Poels et al., 2009), and our knockdown of TkR86C in smooth muscle by Dilp3-Gal4 or Mef2-Gal4 showed a modest but significant decrease in ISC proliferation (Figure 4E, F). There was a concomitant reduction of Dilp3 mRNA in guts of all these experiments (Figures S4K–S4M), while the head Dilp3 mRNA had no significant change in all these experiments. As a comparison, TkR99D or NPFR RNAi did not show the same consistent defect.

In conclusion, we show that among the AS-C genes, sc is the one essential for the formation of all adult midgut EEs and is probably required during metamorphosis when the midgut is less severe than that in the sc RNAi/EE-less guts. The results together suggest that Tk-expressing EEs are part of the EE population required for this regulatory circuit. The approach we report here has established the Drosophila midgut as a model to dissect the function of EEs in intestinal homeostasis and whole-animal physiology.

**EXPERIMENTAL PROCEDURES**

*Drosophila Stocks and Tissue Staining*

All *Drosophila* stocks were maintained at room temperature in yeast extract/cornmeal/molasses/agar food medium. UAS-mCD8-GFP and w1118 were used for crossing with Gal4 and mutant lines as control. The fly stocks sc10-1 (107663) were obtained from VDRC. esgRNAi (13392), lscRNAi (27237), scRNAi (206206), atoRNAi (26316), TkRNAi (25800), NPPRNAi (27237), sc1, sc2, sc2, sc1, ato1, Df(1)scRNAi, Df(2)scRNAi, and UAS-sc were obtained from Bloomington Stock Center. TkR86C RNAi (13392), TkR99D RNAi (43329), and NPFR RNAi (107863) were obtained from VDRC. ess-Gal4, Dilp3RNAi (33681), Dilp3-Gal4, Mef2-Gal4, and pros-Gal4 have been described previously (Micchelli and Perrimon, 2006; O’Brien et al., 2011;
Sen et al., 2004). The Tk-Gal4 line was among a set of Tk promoter Gal4 lines screened for expression in the adult midgut, and it contains an approximately 1 kb fragment 2.5 kb upstream of the

screened for expression in the adult midgut, and it contains an approximately 1 kb fragment 2.5 kb upstream of the transcription start (Song et al., 2014, in this issue of Cell Reports). Female flies were used for routine gut dissection and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.08.052.

Feeding, Fecundity, and Enzyme Assays

For feeding experiments, newly hatched or appropriately aged flies were kept in regular food vials or in plastic vials with a filter paper soaked with 1% sucrose in water and transferred to fresh vials every day. For dye-ingestion experiments, 20 flies were transferred to a plastic vial with a filter paper soaked with 5% sucrose and 0.5% bromophenol blue sodium salt (B5525, Sigma). At the indicated time, flies that showed visible blue abdomen were counted because of the bigger size. Immunofluorescence staining, antibodies used, microscope image acquisition, and processing were as described previously (Amcheslavsky et al., 2009, 2011).

Real-Time qPCR

Total RNA was isolated from ten dissected female guts and used to prepare cDNA for quantitative PCR (qPCR) using a Bio-Rad iQ System (Amcheslavsky et al., 2011). qPCR was performed in duplicate from each of at least three independent biological samples. The ribosomal protein 49 (rp49) gene expression was used as the internal control for normalization of cycle number. The primer sequences are listed in the Supplemental Experimental Procedures. Each genotype corresponded to five to six samples of ten midguts each.

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AUTHOR CONTRIBUTIONS

A.A., Q.L., Y.N., and Y.T.I. designed, carried out, and analyzed the experiments. W.S. and N.P. performed the experiments that identified the TK-Gal4 gut driver. Expression pattern, and cell-killing conditions. I.B. and D.F. designed and performed the gut digestive enzyme and feeding assays. A.A. and Y.T.I. wrote the manuscript. All authors amended the manuscript.

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Figure S1. Related to main text Figure 1A-B.

cRNAi

A B C

DAPI Pros Arm

D I

DAPI Pros Arm

G H

sc<sup>6/+</sup> sc<sup>6/Df</sup>

J

ato<sup>1</sup>/+ ato<sup>1/Df</sup>

K L

Gut expression

Relative mRNA levels

Control
esg>sc<sup>RNAi</sup>

M

Cumulative

N

% Survival

Days at 29°C

P<0.05

eggs/fly pupae/fly

Control
esg>sc<sup>RNAi</sup>

esg, ts>UAS-sc

Control
esg>sc<sup>RNAi</sup>

esg, ts>UAS-sc
Figure S1. EE number, hormone gene expression and life span after loss of sc function. (A-F) Confocal images of adult midgut surface views. esg-Gal4 flies were crossed with w- or the indicated UAS-RNAi constructs. 7 days old flies were used for dissection and staining. For all images in this figure, blue is DAPI for DNA, red membrane is Armadillo (β-catenin) and red nuclear is Prospero. The arrows point to examples of EEs. Scale bar is 20 μm. (G-J) Confocal images from the midguts of flies with the indicated genotypes. The deficiency for sc was Df(1)sc10-1 and for ato was Df(3R)p13. (K, L) Peptide hormone gene expression in midgut and head. 5 days old flies were used for dissection and RNA isolation from heads and midguts. The cycle number of each gene was normalized with that of rp49 in a parallel reaction of the same RNA sample. The level of expression relative to rp49 in control sample is shown at the top of each white bar, and this normalized expression was set as 1 for each gene. The expression of that gene in the scRNAi guts was calculated as the ratio to that in control. (M) For fecundity assay, males and females were put together and transferred to new vials every day. Eggs were counted in each vial for 10 days. Four independent experiments were performed and the average cumulative number of eggs laid per female fly was plotted. After flies were transferred out, the vials were kept and the embryos were let grown and the pupae were counted. Four independent experiments were performed and the average pupae number per female fly was plotted. (N) 100 flies of each genotype were kept at 29°C in normal food vials and transferred to fresh vials every other day. The percent of flies survived at each time was recorded. The experiment was performed 3 times, and the average at each time point was plotted. The P value was calculated by comparing the survival of control sample on the same day as 50% survival of the sc samples.
Figure S2. Related to main text Figure 1C-H.

A

% Activity

aminopeptidase N

acetate-esterase

B

C

Control
esg>scRNAi

D

esg>
esg>
esg>

E

Dye feeding (min)

P<0.01

P<0.05

P<0.05

P=0.112

P<0.05

P=0.117

Dye feeding (hrs)

P=0.156

P=0.226

P=0.226

P=0.226

P=0.117

Dye feeding (hrs)

% with ingested dye

% of cleared guts

P<0.01

P<0.05

P<0.05

P<0.01

P<0.01

P=0.05

P=0.05

P=0.05

P=0.05

P=0.05

P=0.05

P=0.05

P<0.05

P<0.05

P<0.05
Figure S2. Digestive enzyme activities, food intake and food passage assays in EE-less flies. (A-B) For digestive enzyme assay, midguts from 7 days old females were homogenized and mixed with the corresponding substrate for the indicated enzymatic assay. Each genotype analyzed had 5-6 samples of 10 midguts each. (C) 20 control and esg>scRNAi flies were transferred to plastic vials containing a filter paper soaked with 5% sucrose in water and 0.5% bromophenol blue (BPB) sodium salt. Every 30 minutes flies that had visible ingested dye in the abdomen were counted. Six independent experiments were performed and the average accumulative at each time point was plotted. (D) 10 midguts from 7 days old females of each genotype were pooled at 3 and 6 h after transferring the flies to a plastic vial containing a solution of sucrose 50 mM (1.71%) and 2.5% (w/v) blue dye (food colorant E131). Food intake was monitored by the increase in absorbance at 630 nm (Mithras LB 940, Berthold Technologies) of the supernatant after midgut homogenization and centrifugation (10.000x g 10 min). Average of 3 experiments was plotted. (E-F) The fly strains were kept in 5% sucrose and 0.5% BPB. At the indicated times, blue deposits on the plastic vials were counted. Similarly, 10 flies were well fed to have visible blue abdomens and then transferred to a new vial containing 5% sucrose solution. Flies that had clear abdomens were counted after the time as indicated. Three independent experiments were performed and the average was plotted.
Figure S3. Related to main text Figure 3.

A

![Graph showing mRNA levels](image)

**Control**  esg,ts>UAS-sc

B

![Immunostaining images](image)

esg,ts>GFP

C

D

E

F

G

H

![Graph showing mRNA levels](image)

**Control**  esg,ts>UAS-sc

I

![Graph showing pH3+ cells/gut](image)

**Control**  esg,ts>UAS-sc

J

![Graph showing pros+ cells/0.08 mm²](image)

**Control**  pros,ts>UAS-p35

K

![Graph showing pH+ cells/gut](image)

**Control**  pros,ts>UAS-p35
Figure S3. EE number correlates with ISC proliferation. (A) The flies approximately 7 days old were shifted to 29°C for 2 to 6 days to inactivate Gal80ts and allow Gal4 to activate UAS-sc expression. Quantification of Prospero+ nuclei were performed from midguts of esgts>GFP control and esgts>GFP, UAS-sc flies. (B-G) Representative confocal images of midgut surface views from control and sc over-expression flies. Blue is DAPI for DNA, red nuclear is Prospero and green is mCD8GFP. The scale bar is 20 µm. (H) The esgts>GFP control and the esgts>GFP, UAS-sc flies were shifted to 29°C for 4 days and used for gut dissection and RNA isolation. The primer sets for the indicated genes were used for qPCR. The cycle number of each gene was normalized with that of rp49 in a parallel reaction of the same RNA sample. The normalized expression of each gene in the control sample was set as 1 and the expression of that gene in the UAS-sc background was calculated as fold change. (I) Average number of p-H3+ cells in whole midguts of esgts>GFP control and esgts>GFP, UAS-sc flies. 7 days old flies were shifted to 29°C to initiate sc overexpression. At the days indicated, a portion of the flies was used for midgut dissection and p-H3 staining. (J-K) The pros promoter-Gal4 and tubulinGal80ts (pros-ts>)were crossed with UAS-GFP control and UAS-p35 transgenic flies. 5 days old flies were shifted to 29°C for 4 days and then used for gut dissection, Pros and p-H3 staining, and quantification.
Figure S4. Related to main text Figure 4.

A. Graph showing the relative Dilp3 mRNA levels compared to controls for different treatments.

B. Table showing the percentage of Gal4+ cells that are EEs and the percentage of EEs that are Gal4+ for different treatments.

C-H. Images showing the distribution of Pros+ cells in different regions.

I-I". Images showing the expression of TkGal4>GFP and Pros in different regions.

J-K. Graphs showing the count of pH3+ cells in the midgut for different treatments.

L-M. Graphs showing the relative Dilp3 mRNA levels for different treatments.

Additional notes:
- Pros+/prosGal4>GFP (%) vs. ProsGFP+/Pros (%)
- Control rpr hid
- Tk Tk + GFP
- % Gal4+ cells that are EEs % EEs that are Gal4+
- Control scRNAi InRAct esg> scRNAi InRAct
- TkRNAi NPFRNAi Control
- TkR86C RNAi TkR99D RNAi Mef2>
Figure S4. Midgut expression of pros-Gal4 and Tk-Gal4 and functional assays of TK signaling. (A) The esg-Gal4 was used to express an activated insulin receptor (InR<sup>act</sup>), together with the scRNAi. Flies 3 days after eclosion were used for gut dissection and p-H3 quantification. (B) Quantification of Pros+ staining overlapping with the Gal4 driven GFP+ cells. The ratios are calculated to show the percentage of GFP+ cells that should be EEs, or to show the percentage of EEs that have the Gal4>GFP expression. (C-I’’) UAS-GFP were crossed with the Gal4 drivers as indicated and midguts of 5-7 days old flies were dissected and stained using antibodies for Pros (C-H) or Tk (I-I’’) proteins. The arrows indicate examples of Pros+ nuclear staining, and the wide green arrows indicate overlapping GFP+ staining in some EEs. The GFP signal in panel C reveals pros-Gal4 expression in the ECs of a small anterior midgut region. Although we did not observe obvious increase of p-H3+ cells in this anterior region from the pros-Gal4/UAS-p35 experiments, we counted p-H3 staining from middle and posterior midguts only for these experiments. (J) The same experiments as in Fig. 4C, and the guts were stained and quantified for Pros+ EEs. (K) The same experiments as in Fig. 4D, and the guts were used for PCR quantification of Dilp3 mRNA. (L, M) The same experiments as in Fig. 4E and F, and the guts were used for PCR quantification of Dilp3 mRNA.
qPCR primer sequences. Related to Real-time qPCR in Experimental Procedures.

**scute**: 5'-CAATTCCGGCAACGAAGAT and 5'-CAGCGGTTGATTTTGAT

**allatostatin**: 5'-TCCGCAACCCCTTCAACTT and 5'-TGAATAAGTGCACCACCATCC

**allatostatin C**: 5'-AGTTACCGCGACCAAAGG and 5'-AGTTTTTCGGCCTTGATG

**neuropeptide F**: 5'-ACCATGGCAACCTTGAACTTA and 5'-AACAATTTGCCCCGAAAA

**diuretic hormone**: 5'-CAAACGAACCGTGGACCTT and 5'-CAGACCCATGCAGATGTTT

**tachykinin**: 5'-TACGCGAGCATTGGACA and 5'-GAAATCGATGCCTGAAG

**phyllodop**: 5'-CCCTCCGGGAATACCTGAAAC and 5'-GCCTGGATTAGCTGAACGATA

**Dilp2**: 5'-ATGGTGTGCAGTATAATCC and 5'-TCGGCACCAGGGCATG

**Dilp3**: 5'-AGAGAACTTTGGACCCGTGGA and 5'-TGAACCGAACTATCACTCAACAGTCT

**Dilp4**: 5'-CGGAGAGCTCGTCTAAGGA and 5'-TCATCCGGCTGCCTAGCTT

**rp49**: 5'-CGGATCGATATGCTAAGCTGT and 5'-GCGCTTGTTCGATCCGTA