Enteroendocrine cells support intestinal stem-cell-mediated homeostasis in Drosophila

Alla Amcheslavsky
University of Massachusetts Medical School

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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

**Authors**

Alla Amcheslavsky, Wei Song, ..., Norbert Perrimon, Y. Tony Ip

**Correspondence**

tony.ip@umassmed.edu

**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

Alla Amcheslavsky,1 Wei Song,2,3 Qi Li,1 Yingchao Nie,1 Ivan Bragatto,4 Dominique Ferrandon,4 Norbert Perrimon,2,3 and Y. Tony Ip1,1
1Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605, USA
2Howard Hughes Medical Institute, Harvard Medical School, Boston, MA 02115, USA
3Department of Genetics, Harvard Medical School, Boston, MA 02115, USA
4Unité Propre de Recherche 9022 du Centre National de la Recherche Scientifique, University of Strasbourg Institute for Advanced Study, Institut de Biologie Moléculaire et Cellulaire, 67084 Strasbourg Cedex, France
*Correspondence: tony.ip@umassmed.edu
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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-Aliga, 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 (Micchelli 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 innervating 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 (Pros) (Micchelli 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 (Micchelli 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; Micchelli et al., 2011).

The sc3/sc6 hemizygous mutant adults were also completely devoid of midgut EEs (Figures 1B, S1G, and S1H), while other hemizygous combinations including sc1, sc28, and sc5 were normal in terms of EE number. Df(1)sc10-1 is a small deficiency that has both ac and sc uncovered. sc1 and sc28 each contain a gypsy insertion in far-upstream regions of sc, while sc5 and sc6 are 1.3 and 17.4 kb deletions, respectively, in the sc3 regulatory region (García-Bellido and de Celis, 2009). The sc5/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)p13 showed normal EE formation (Figures 1A, 1B, S1G, S1I, and S1J). Nonetheless, we found that older ato+/Df(3R)p13 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), tostatin C (AstC), Tachykinin (Tk), diuretic hormone (DH31), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neuropeptide F (NPF) was almost abolished (Figure S1K), and neupe...
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 plotted as 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 esgAS > 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 esgAS > 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–S3l). 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/UAS-Dilp3RNAi. 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 esgAS > sc RNAi, perhaps due to redundant function with other basic-helix-loop-helix proteins in adults. The expression of proapoptotic proteins by the prosAS-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 outer 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 4C). A previous report revealed the expression by antibody staining of a Tk receptor (TkR86C) in visceral smooth muscle by Dilp3-Gal4 or Mef2-Gal4 showed a modest but significant decrease in ISC proliferation (Figures 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-mCD8GFP and $w^{1118}$ were used for crossing with Gal4 and mutant lines as control. The fly stocks scRNAi (29586), aseRNAi (31895), lscRNAi (27058), sc2RNAi (26206), atoRNAi (26316), TkRNAi (25800), NPFRRNAi (27237), sc1, sc5, sc6, ato1, Df(1)sc10-1, Df(3R)13, and UAS-sc were obtained from Bloomington Stock Center. TkR88C/RNAi (13392), TkR99D/RNAi (43329), and NPFRRNAi (107663) were obtained from VDRC. esg-Gal4, Dilp3-Gal4 (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 transcription start (Song et al., 2014, in this issue of Cell Reports). Female flies were used for routine gut dissection because of the bigger size. Immunofluorescence staining, antibodies used, microscope image acquisition, and processing were as described previously (Amcheslavsky et al., 2009, 2011).

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 (BS525, Sigma). At the indicated time, flies that showed visible blue abdomen were counted or used for gut-extract preparation and OD measurement. For defecation experiments, flies were placed in new vials with sucrose/bromophenol blue, and colored excreta on the vial wall were counted at 4 and 24 hr time points. For gut-clearance assays, flies were first fed with bromophenol blue, and ten flies that had blue abdomen were transferred to a new vial containing 5% sucrose only. At 2 and 24 hr after, flies were counted based on whether they still had blue abdomen or not. For fecundity assays, newly hatched male and virgin female flies were aged for 5 days on a normal diet. A group of ten females and five males were put together in a new food vial and transferred to a fresh food vial every day. The number of eggs was counted in each vial for 10 days. Vials were kept to allow larvae and pupae to develop, and the number of pupae was counted for every vial. For digestive enzyme assays, midguts from fertilized females (7–10 days old) were homogenized in 50 μl PBS at 5,000 rpm for 15 s (Precellys 24, Bertin Technologies) and centrifuged (10,000 × g for 10 min). Substrates for trypsin enzymatic assay (C8022) were purchased from Sigma-Aldrich, and the reaction was set up following the manufacturer’s instructions. Increase in absorbance (405 nm) or fluorescence (555 nm/460 nm) after substrate cleavage was monitored by a microplate reader (Mithras LB 940, Berthold Technologies). Each genotype corresponded to five to six samples of ten midguts each.

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 IQ5 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. All error bars represent SEM, and p values are from the Student’s t test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.08.052.
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