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STATE-OF-THE-ART REVIEW

# The specification and function of enteroendocrine cells in *Drosophila* and mammals: a comparative review

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Enteroendocrine cells (EECs) in both invertebrates and vertebrates derive from intestinal stem cells (ISCs) and are scattered along the digestive tract, where they function in sensing various environmental stimuli and subsequently secrete neurotransmitters or neuropeptides to regulate diverse biological and physiological processes. To fulfill these functions, EECs are specified into multiple subtypes that occupy specific gut regions. With advances in single-cell technology, organoid culture experimental systems, and CRISPR/Cas9-mediated genomic editing, rapid progress has been made toward characterization of EEC subtypes in mammals. Additionally, studies of genetic model organisms—especially *Drosophila melanogaster*—have also provided insights about the molecular processes underlying EEC specification from ISCs and about the establishment of diverse EEC subtypes. In this review, we compare the regulation of EEC specification and function in mammals and *Drosophila*, with a focus on EEC subtype characterization, on how internal and external regulators mediate EEC subtype specification, and on how EEC-mediated intra- and interorgan communications affect gastrointestinal physiology and pathology.

**Abbreviations**

5-HT, 5-hydroxytryptamine/serotonin; A/P, anterior/posterior; AstA, allatostatin A; AstC, allatostatin C; bHLH, basic helix-loop-helix; BMP, bone morphogenetic proteins; Burs, bursicon; Cas9, CRISPR-associated protein 9; CCAP, crustacean cardioactive peptide; CCHa1, CCHamide-1; CCHa2, CCHamide-2; CKK, cholecystokinin; CNS, central nervous system; CRISPR, clustered regularly interspaced short palindromic repeats; Dac, dachshund; DH31, diuretic hormone 31; Dl, Delta; DPP4, dipeptidyl peptidase 4; Drm, drumstick; E(spl), enhancer of split; EC cell, enterochromaffin cell; ECL cell, enterochromaffin-like cell; EEC, enteroendocrine cell; EEP, EE progenitor cell; Fer1, 48 related 1; FFAR, free fatty acid receptor; FOXA 1/2, forkhead box A1/2; Gfi1, growth factor independent protein 1; GI, gastrointestinal; GIP, gastric inhibitory peptide; GLP-1, glucagon-like peptide-1; GLUT, glucose transporter; Gpb5, glycoprotein hormone beta 5; GPCR, G protein-coupled receptor; HDAC, histone deacetylase; Hes1, hairy and enhancer of split 1; HHEX, hematopoietically expressed homeobox; HMG, high-mobility group; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome; INSL5, insulin-like peptide 5; ISC, intestinal stem cell; ITP, ion transport peptide; KLF4, Kruppel-like factor 4; Klu, Klumpfuss; LLPS, liquid–liquid phase separation; LRCs, label-retaining cells; MAPK, mitogen-activated protein kinase; Mip, myoinhibitory peptide; MNU, N-nitroso-methylurea; Myt1, myelin transcription factor 1; NeuroD1, neuronal differentiation 1; Ngn3, neurogenin 3; NP cell, neural precursor cell; NPF, neuropeptide F; Nplp2, neuropeptide-like precursor 2; NTS, neurotensin; OGT, O-GlcNAc transferase; OXM, oxyntomodulin; Pax4, paired box 4; PC, prohormone convertase; PEPT, proton-coupled oligopeptide transporter; Phyl, phyllpod; Pros, prospero; PYY, peptide YY; Rfx6, regulatory factor X6; Sc, scute; SCFA, short-chain fatty acid, scRNA-seq, single-cell RNA sequencing; SCT, secretin; SGLT, sodium-glucose transporter; sNPF, short neuropeptide F precursor; ssRNA, single-stranded ribonucleic acid; SST, somatostatin; T2DM, type II diabetes mellitus; TF, transcription factor; Tk, tachykinin; TPH1, tryptophan hydroxylase 1; Ttk, tramtrack.

## Introduction

The gastrointestinal (GI) tract serves as the main site for food digestion and nutrient absorption in all metazoans, with its luminal surface exposed to substances including nutrients and sometimes pathogens or toxic chemicals; the GI tract is also home to the gut microbiome and is therefore exposed to myriad products of microbial metabolism [1,2]. Enteroendocrine cells (EECs), which are scattered in the epithelium of the GI tract, are thought to function as major sensors of luminal content: EECs sense various substances and accordingly secret different types of neuropeptides or neurotransmitters that act either locally or remotely to regulate diverse biological and physiological processes, including appetite, food ingestion, food digestion, gut motility, metabolism, and immune responses [3–7].

Similar to the absorptive enterocytes, EECs are also derived from intestinal stem cells (ISCs) [8,9]. Despite comprising fewer than 1% of total epithelium cells in the mammalian intestine, the EEC population is highly heterogeneous: Previous studies have identified more than 10 subtypes from mammals and *Drosophila*. Dysfunction of EECs has been linked to various pathological disorders [10–12]. For instance, disrupted glucagon-like peptide-1 (GLP-1) secretion by EECs is associated with diabetes and obesity, while loss of gastrin-expressing enteroendocrine G cells leads to enhanced symmetric division of antral stem cells and increased risk of gastric tumorigenesis [13–15].

Historically, gut epithelial sensor cells were first documented by Rudolph Heidenhain in 1870, and secretin (SCT), the first gut hormone, was discovered in 1902 [16]. However, gut endocrinology developed slowly in subsequent decades until the emergence of new technologies including transmission electron microscopy, monoclonal antibodies for research, and solid-phase peptide synthesis technology in the 1960s [17]. In the following 20 years, more than 20 gut peptides were identified in the mammalian system [18], and the alphabet nomenclature for EECs—based on the ‘one cell-one hormone’ doctrine/hypothesis—was proposed at a scientific congress in 1977 and later widely implemented [17]. However, this classification is becoming inadequate as our understanding of EEC subtype diversification and specification increases. Specifically, single-cell RNA sequencing (scRNA-seq) studies have supported a high-resolution understanding of EEC heterogeneity, and it is now clear that each EEC subtype commonly secrets multiple peptide hormones [10–12,19,20]. Evolved EEC classification system has been proposed, which take account of species, anatomical location and genes or protein signatures [5]. For example, D cells in stomach are described as $\mathrm{G_M}^{\mathrm{SST+PYY+IAPP+CCK+GAST-}}$ cells with this new classification. However, such nomenclature is difficult to be widely used and needs further optimization.

In addition, CRISPR/Cas9-mediated genomic editing combined with *in vitro* culture of intestinal organoids has enabled sophisticated batch testing of the putative regulators of EEC specification and function, and robust multi-omics methods including proteomics and metabolomics continue to enable the identification of more and more bioactive neuropeptides and molecules that trigger EEC responses [10,21–24]. In this review, we summarize recent progress in our collective understanding of EEC cellular diversity, including characterization of EEC subtypes, transcriptional regulation of both cell fate specification and EEC sense-and-respond functions, and known links with EEC dysfunction-related pathological disorders.

## Specification of EEC lineage from ISCs

In the rapid-replenishing gastrointestinal epithelium, EECs are continually produced by multipotent ISCs [marked by Lgr5 in mammals, and Delta (Dl) in *Drosophila*] [9,25]. Similar to the differentiation processes leading to other mature cell types, specification of EECs is coordinately modulated by both internal transcription factors (TFs) and external niche signals.

### EEC lineage specification in mice

In mammals, the initial fate determination between absorptive enterocyte versus secretory lineages is controlled by Notch signaling (Fig. 1A). High Notch activity sustains strong expression of the bHLH type TF hairy and enhancer of split 1 (Hes1), which promotes an absorptive cell fate; both inhibiting Notch activity in ISCs and depleting Hes1 from ISCs can induce cell cycle arrest and secretory cell fate commitment [26–30].

Atoh1 is another bHLH TF that is believed to function as the pan-regulator of secretory cell lineage specification: Atoh1 depletion leads to loss of three secretory cell types from the epithelium: goblet cells, Paneth cells, and EECs [31]. Following the activation of Atoh1, distinct secretory cell lineage specifiers are subsequently activated, mediating further specification of the three secretory cell types (Fig. 1A). The HMG box TF Sox9, which functions downstream of Wnt signaling, is required for Paneth cell differentiation, and Paneth cell maturation also requires Wnt pathway activation [32,33]. Specification of goblet cell

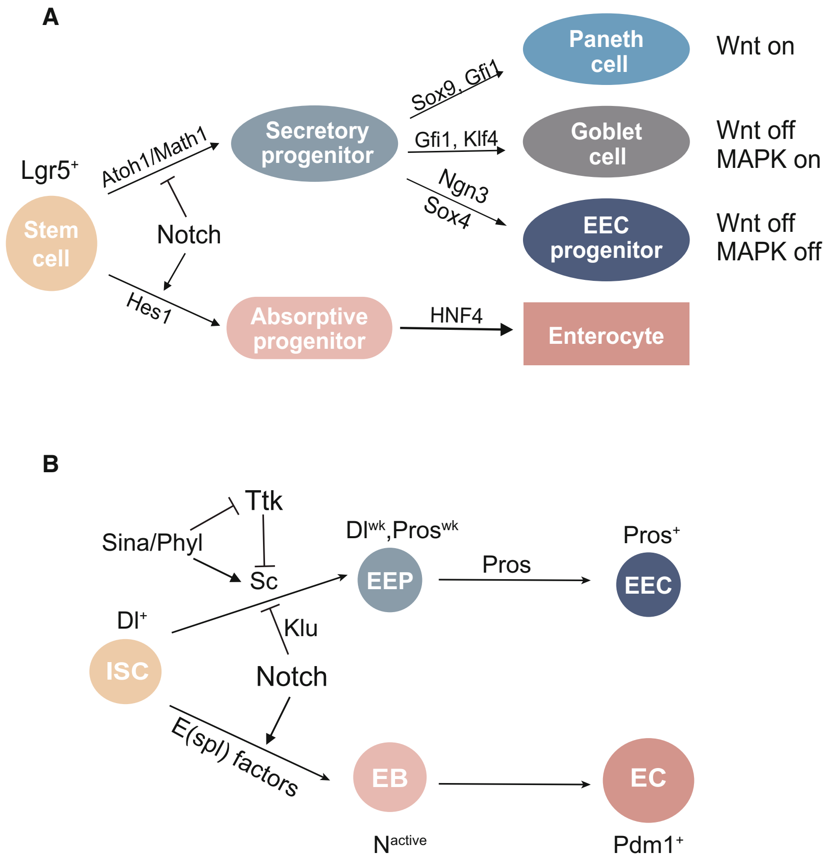

**Fig. 1.** Regulation of EEC lineage specification from ISCs in mammals and *Drosophila*. (A) Schematic overview of EEC lineage specification in the mammalian intestine. The binary fate choice between the secretory and absorptive lineages from Lgr5$^+$ cells is controlled by Notch signaling, and the transcription factor Atoh1/Math1 is required for differentiation of progenitor for the entire secretory lineages. Transient expression of Ngn3 specifically directs specification of EEC progenitor cells, while Sox9 and Klf4 direct Paneth and Goblet cell specification, respectively. Gfi1 is expressed in Paneth and Goblet cells, preventing their conversion into EEC lineage. (B) Schematic overview of EEC lineage specification in *Drosophila* midgut. Dl$^+$ ISCs give rise to two types of lineage-committed progenitor cells: the secretory EEP and absorptive EB cells. The transient expression of the bHLH family TF *sc* triggers the specification of EEPs, and Pros promotes EEC fate commitment and maturation. Notch activation-induced E(spl) factors promote EB differentiation, which is further differentiated into absorptive enterocytes. Other TFs including Ttk and Klu also carry out suppressive functions on EEC specification.

depends on the zinc finger TF growth factor independent protein 1 (Gfi1), which is induced by Atoh1, and high mitogen-activated protein kinase (MAPK) signaling activity [34,35]. Kruppel-like factor 4 (KLF4) is another zinc finger TF that has region-specific function in goblet cell specification: Its depletion leads to loss of more than 90% of this cell type in the colon, while the small intestine is not significantly affected [36]. As for EECs, the bHLH family TF neurogenin 3 (Ngn3) is specifically expressed in EEC progenitors and promotes EEC specification; the Ngn3 level is reduced gradually as these progenitor cells differentiate into mature, hormone-expressing EECs [12,37]. Gfi1, which is enriched in goblet and Paneth cells, suppresses the expression of Ngn3, thereby prevents fate conversion of the goblet and Paneth cells into EEC fate [34,38]. Interestingly, Sox4, a high-mobility group (HMG) protein that is expressed in cells close to the +4 position of intestinal crypts, functions in the early stages of EEC differentiation and contributes to the specification toward Tuft cell and EEC fates only [12,39].

In addition to *in vivo* genetic models, *in vitro* enteroid cultures and directed differentiation experiments have also highlighted the functional impacts of niche signals in modulating fate commitment of distinct secretory lineages (Fig. 1A). Simultaneous Notch suppression and Wnt activation lead to specification of

Paneth cells, whereas dual inhibition of both Notch signaling and Wnt signaling promotes robust specification of goblet cells. EEC lineage specification requires inactivation Notch and Wnt as well as repression of EGFR signaling [40,41]. Thus, extensive co-operation of internal TFs and niche signals determines proper specification of secretory lineages in the intestinal epithelium.

Using time-resolved lineage labeling combined with single-cell gene expression analysis, a recent study reveals that cell cycle exit of ISCs induced by a switch from canonical Wnt/β-catenin to noncanonical Wnt/PCP signaling represents the earliest step of Paneth cell and EEC lineage priming from ISCs, prior to Notch/Delta-mediated lateral inhibition [42]. Additionally, this study also argues against the existence of bi- or multipotent secretory progenitors and suggests that EECs and Paneth cells are directly allocated from ISCs via unipotent transition states [42–44]. Therefore, the exact cell lineage relationships based on which the four different types of secretory cells are generated from ISCs are still not fully clarified and require further investigation.

## EEC lineage specification in *Drosophila*

In *Drosophila*, Notch signaling also controls the binary fate choice between absorptive enterocyte versus secretory EECs (Fig. 1B). ISCs specifically express the Notch ligand Dl, which induces Notch activation of their immediate daughter progenitor cells to adopt an absorptive fate [8,45–47]. The generation of EEC progenitors from ISCs requires a transient activation of the bHLH family gene *scute* (*sc*), which induces asymmetric cell division to generate a new EE progenitor cell (EEP), and each EEP typically undergoes one round of cell division before the terminal differentiation which yields a pair of EECs [48]. This transient activation of *sc* in ISCs is achieved by a self-stimulatory transcription activation loop combined with a negative feedback loop between Sc and the Enhancer of split (E(spl)) factors (Fig. 1B). Therefore, it appears that *sc* is expressed in an oscillatory pattern in ISCs, possibly defining a clock-like mechanism through which ISCs periodically generate EECs; note that ISCs spend most of their time (> 70%) in a default mode that generates enterocytes [48].

A series of additional internal factors also participated in the regulation of *sc* expression (Fig. 1B). The C2H2 zinc finger family TF Klumpfuss (Klu) is induced in Notch-activated enteroblasts and guarantees enterocyte lineage commitment by suppressing *sc* expression [47]. In ISCs, a BTB domain-containing TF Tramtrack (Ttk, or Ttk69) maintains the intestinal epithelial identity of ISC and suppresses the proneural genes *sc* and *asense* (*ase*); *ttk* depletion leads to ectopic gain of neuroblast-specific transcription programs and production of excess EECs [49,50]. An adaptor protein Phyllpod (Phyl), which bridges Ttk to the E3 ligase Sina for proteolytic degradation, is transiently expressed in the EEC progenitor stage, which may function to support sustained Sc activation for EEC fate commitment, as loss of *phyl* causes failed EEC generation from ISCs [51,52].

Prospero (Pros), a homeobox family TF orthologous to mammalian Prox1, is induced by Sc and drives EEC fate commitment and maturation (Fig. 1B) [48]. Similar to its roles in the nervous system, Pros suppresses cell cycle and progenitor identity-related genes and activates EEC-specific genes, such as peptide hormones [49,53]. Immediately prior to the generation of a daughter EEP cell, Pros appears to be weakly expressed in ISCs, but it is sequestered in the cytoplasm and/or on the cell membrane. At the EEP stage, Pros starts to accumulate and initially appears in the form of punctate dots in the nucleus; it is soon richly present throughout the entire nucleus of differentiating and mature EECs [48]. In neural precursor cells (NP cell, also GMC), Pros also forms a similar punctate pattern in complex with HP1 via liquid–liquid phase separation (LLPS), and such structure is required for heterochromatin formation and terminal fate commitment [54]. Whether Pros mediates suppression of progenitor identity and/or activates the expression of EEC-identity-related genes and whether such functions are facilitated by these punctate structures remain unknown.

Specification of EECs from ISCs in the *Drosophila* midgut has also been reported to be regulated by external mechanical signals and Slit/Robo signaling. The stretch-activated Piezo channel, which is expressed in a subset of ISCs, is activated by mechanical stresses following gut filling to increase cytosolic $Ca^{2+}$ signaling and promote EEC specification [55]. The differentiated EECs are believed to exert a negative feedback mechanism on EEC generation from ISCs. EECs specifically express Slit, which then binds to its receptor Robo2 expressed in ISCs and suppresses ISC commitment toward the EEC lineage [56]. However, genetic manipulations that allow clonal depletion/accumulation of EECs or clonal depletion/overexpression of Slit in the intestinal epithelium fail to cause a significant effect on the distribution pattern and density of EECs in the epithelial regions surrounding the clones [57]. These observations indicate that a systematic rather than local feedback mechanism via Slit/Robo2 signaling might be involved in EEC generation from ISCs.

## Characterization of the heterogeneous EEC population

The particular functions of each EEC subtype are apparently determined by the specific complements of peptide hormones that each can secret in response to sensing of discrete messages. Numerous organelles belonging to the endolysosomal system (including early endosomes, late endosomes/multivesicular bodies, recycling endosomes, and lysosomes) and secretory system (including endoplasmic reticulum, Golgi, secretory granules, lipid droplets, and chylomicrons) exist in EECs to ensure that they are able to effectively synthesize and secrete peptide hormones with signaling capabilities [58]. Collectively, there are at least 20 peptide hormones that are expressed in all EECs of either mammals or *Drosophila*, and their expression patterns exhibit local and regional variations along the GI tract, highlighting substantial heterogeneity (Fig. 2A,B). Thus, a clear classification and characterization of EEC subtypes are important for further investigation of their biological functions. However, contrast to the characterization of EEC subtypes along the whole gut in *Drosophila* [11], studies of spatial patterning of EEC subtypes in mammals are far from adequate, hindered by the large size of the GI tract, and using organoids derived from different segments of the gut cannot completely simulate the situations of EECs *in vivo* [10,12,19,20]. Therefore, more studies and new technologies are needed to support more informative *in vivo* characterization of mammalian EEC subtypes and elucidate their sensing and hormone-secreting functions.

### EEC subtypes in the mammalian GI tract

As mentioned earlier, EECs in mice and humans have been historically classified by a letter code according to the specific hormone they secrete or based on ultrastructural features identified with electron microscopy. For instance, M cells were named based on their secretion of motilin, while L cells were named for containing large vesicles [59]. By using this nomenclature, at least 12 EEC subtypes have been identified, including D cells, enterochromaffin (EC) cells, enterochromaffin-like (ECL) cells, G cells, I cells, K cells, L cells, M cells (‘EC2’ or ‘MO’ cells), N cells, S cells, and X/A cells (P/D1 cells in human); these cells collectively secret more than 20 types of mature peptide hormones. We have summarized the expression patterns of peptide hormones and their chemosensors/receptors, as well as the spatial distribution and functions of distinct EEC subtypes, in Fig. 2A and Table 1. The spatial location of each EEC subtype is frequently reflective of regional adaptability to biological functions. For example, G and X/A cells are located in the stomach, where they secret gastrin and ghrelin, which regulate the secretion of gastric acid from enterocytes [60–62]. I, K, and L cells are enriched in the small intestine, the main region for food digestion and absorption, where they separately secret CCK, GIP, and PYY/GLP-1 to synergistically regulate gut motility, glucose homeostasis, and appetite [63,64]. In addition to the regional features along the length of the GI, EEC subtypes are also spatially segregated along the crypt–villus axis [12,65]. For example, L cells and *Tac1*-expressing enterochromaffin cells are found in the crypt, while I cells, N cells, and *Sct*-expressing enterochromaffin cells reside at various domains along the length of the villus.

It is noteworthy that the hormone production by EECs can be further diversified by prohormone organization and regulation, which can be achieved through alternative promoters, tissue-specific RNA splicing, cell-specific prohormone processing, and post-translational modifications [4,5,18,66]. One well-characterized example is the process of proglucagon-derived peptides. The glucagon (*GCG*) gene encodes a 180 amino acid preprohormone with a 20 amino acid N terminal signaling peptide. In pancreatic alpha cells, prohormone convertase 2 (PC2)-mediated posttranslational processing cleaves proglucagon into glucagon, glicentin-related pancreatic peptide, and major proglucagon fragment [67–70], while in intestinal L cells, the proglucagon is cleaved by PC1/3 into GLP-1, GLP-2, glicentin, and OXM [67,68,70]. Therefore, the expression of a hormone-related gene may not precisely represent the production of one specific hormone. The shared epitopes and sequence homology among different hormones, processed from the same proprotein, should be treated carefully when detecting these hormones at protein level.

Morphologically, most EECs in mammals have a teardrop shape, with a broad basolateral base and a narrow apical surface. Many ‘open type’ EECs have microvilli at their apical surface, and these structures increase their surface area exposed to luminal content. So-called ‘close type’ EECs, including some D, enterochromaffin, ECL, and X/A cells [3], are embedded in the epithelium and are regulated by receptors at their basolateral surface that are responsive to neuroendocrine or circulating factors. I and K cells can form a basal cytoplasmic protrusion termed a

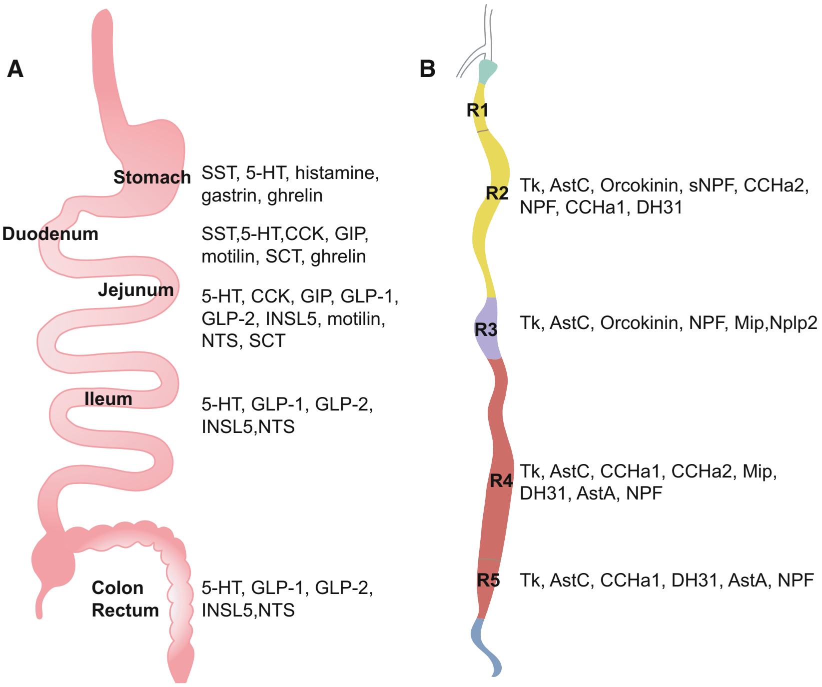

**Fig. 2.** Distribution of peptide hormones along the GI tract in mammals (mice or humans) (A) and *Drosophila* (B).

neuropod, which extends to the enteric nervous system [71,72]. Collectively, these structures and secreted peptide hormones enable EECs to carry out local or remote regulatory functions (Fig. 3).

## EEC subtypes in the *Drosophila* intestine

ScRNA-seq analysis of *Drosophila* intestinal cells revealed that Golgi-associated vesicle biogenesis genes were enriched in EECs, consistent with their secretory role [73]. The EECs in *Drosophil*a were previously divided into two major categories: the allatostatin C (AstC)-expressing class I cells and tachykinin (Tk)-expressing class II cells [74]. Guo *et al.* recently reported high-resolution mapping of EEC heterogeneity and classified EECs in the *Drosophila* midgut into 10 subtypes based on single-cell RNA sequencing analysis. These EECs collectively express genes encoding at least 14 different peptide hormones, including allatostatin A (AstA), AstC, Tk, neuropeptide F (NPF), short neuropeptide F (sNPF), diuretic hormone 31 (DH31), CCHamide-1 (CCHa1), CCHamide-2 (CCHa2), myoinhibitory peptide (Mip), myoinhibitory peptide (Nplp2), glycoprotein hormone beta 5 (Gpb5), ion transport peptide (ITP), crustacean cardioactive peptide (CCAP), and orcokinin [11]. Bursicon (Burs) is also reported to be expressed in some EEs in the posterior midgut after starvation [75,76], although it is not detected by *in situ* hybridization and scRNA-seq under normal conditions [11,75]. Table 2 summarizes the spatial distribution of *Drosophila* EEC subtypes and presents related information for both hormone expression and receptor expression.

Similar to mammals, each EEC subtype in *Drosophila* expresses 2–5 peptide hormone genes, yielding at least 2–5 peptide hormones. Most of these 10 midgut EEC subtypes are AstC$^{+}$ class I or Tk$^{+}$ class II cells [11]. Subtypes expressing NPF, DH31, and Nplp2 belong to Tk$^{+}$ class II EECs, while the AstC$^{+}$ class I subtype EECs express AstA, CCHa2, or orcokinin. There is a third ‘TK$^{-}$, AstC$^{-}$’ EEC class located in the anterior region of the intestine; these cells express CCHa2 and orcokinin [11]. The adult *Drosophila* midgut can be subdivided into six major anatomical regions along the anterior/posterior (A/P) axis: R0–R2 (anterior midgut), R3 (middle midgut, copper cell region), and R4–R5 (posterior midgut) [77]. Based on the expression profiles and experiments with *in vivo* genetic reporter lines, the spatial distribution of the EEC subtypes along the midgut has been characterized (Fig. 2B). The distribution of many EEC subtypes is well associated with their predicted biological functions: For example, the peptide hormone ITP that regulates ion transport is mainly expressed in the II-p EEC subtype, which is distributed at the most posterior region of the midgut, proximal to the hindgut region where ions and water are reabsorbed [78]. In addition to hormone peptides, different EEC subtypes also express many G protein-coupled receptors (GPCRs) that can respond to these hormones.

**Table 1.** Mammalian EEC subtypes, expressed receptors, peptide hormones, and biological functions.

| Subtype name | Regional distribution | Receptors expressed [1,112] | Peptide hormones expressed | Function |
|---|---|---|---|---|
| D cell | Stomach and duodenum | CaSR, GPRC6A, LPA$_5$R; FFAR4 | Somatostatin (SST) | Inhibit gastrin release [163,164] |
| Enterochromaffin cell | Entire GI tract | FFAR2–3; TRPA1; toxin receptors; TLRs | Serotonin (5-HT) | Gut motility [165], nausea, visceral hypersensitivity [7], platelet aggregation [166], bone density [167], liver regeneration [168], inflammation [169], permeability of the blood–brain barrier (BBB) [160], lipolysis [102], hematopoietic stem/progenitor cell proliferation [170] |
| | | | Tachykinin 1 (Tac1, substance P) | Unknown in EECs |
| Enterochromaffin-like cell (ECL cell) | Stomach | Closed cells | Histamine | Increase gastric acid [163] |
| G cell | Stomach | CaSR, GPRC6A, LPA$_5$R; FFAR3; TRPA1 | Gastrin | Stimulate acid secretion [62] |
| I cell | Upper small intestine | CaSR, T1R1-T1R3; FFAR1, FFAR3, FFAR4; TRPA1, TLRs | Cholecystokinin (CCK) | Promote digestion, promote nutrient absorption, cease food intake, delay gastric emptying and motility [63,64], increase satiety [112] |
| K cell | Duodenum and upper Jejunum | GPR119, FFAR1 | Gastric inhibitory peptide (GIP) | Stimulate insulin secretion [116,117], promote lipid uptake and storage [113] |
| L cell | Jejunum, ileum, and colon | T1R2-T1R3, SGLT1, K$_{ATP}$ channels; GPRC6A, T1R1-T1R3; FFAR1–4, GPR119; TRPA1, TLRs, GBAR1 | Peptide YY (PYY) | Digestion, nutrient absorption, food intake [63,64], increase satiety [111], maintain fluid homeostasis [171] |
| | | | Glucagon-like peptide-1 (GLP-1) | Digestion, nutrient absorption, food intake [63,64], bile acids metabolism [114], satiety, gut motility [111,112], insulin, and glucagon secretion [172] |
| | | | Glucagon-like peptide-2 (GLP-2) | Adaptation and recovery of intestinal mucosa in response to injury [4], growth of small intestine [173,174] |
| | | | Oxyntomodulin (OXM) | Body weight homeostasis [4] |
| | | | Insulin-like peptide 5 (INSL5) | Promote food intake, glucose production [91] |
| M cell (EC2 or MO cell) | Duodenum and jejunum | Bile receptors | Motilin | Increase gastrointestinal motility, increase appetite [175,176] |
| N cell | Jejunum, ileum, and colon | FFARs | Neurotensin (NTS) | Slow gastrointestinal motility [177], regulate insulin secretion [178] |
| S cell | Duodenum and jejunum | Acid receptor | Secretin (SCT) | Slow gastrointestinal motility, reduction of gastric acid [179], decrease appetite [115] |
| X/A cell (P/D1 cell in human) | Stomach and duodenum | SGLT1, K$_{ATP}$ channels<br>CaSR, GPRC6A, T1R1-T1R3<br>FFAR2, FFAR4 | Ghrelin | Increase appetite [180], fat storage [112], inhibit insulin release, increase gastrointestinal motility, increase gastric acid [60,61] |

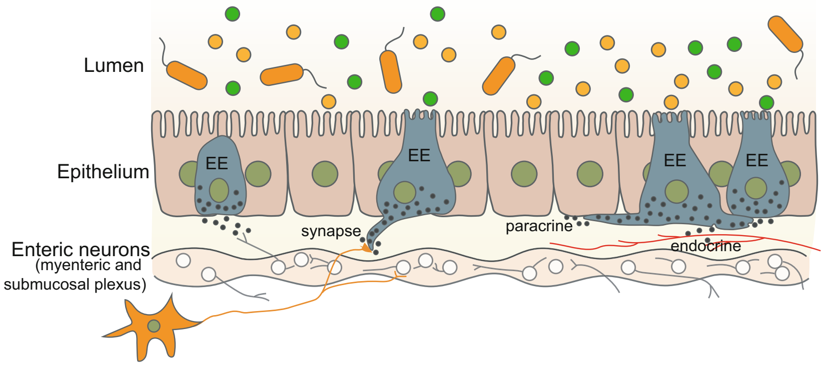

**Fig. 3.** Enteroendocrine cell morphology and modes of hormone action in the mammalian intestine. 'Open type' EECs with microvilli locate at the apical surface and 'close type' EECs reside in villi of the gut epithelium. 'Open type' EECs sense contents in the gut lumen and secret hormones to the circulatory system (red) or adjacent cells through an endocrine or a paracrine manner. Some EECs have basal cytoplasmic process termed as neuropod, which can form synaptic connection with vagus nerves (orange) to directly connect to the nervous system.

**Table 2.** EEC subtype characterization in *Drosophila*.

| Subtype name | Peptide hormones | Peptide receptors | Regional distribution |
|---|---|---|---|
| I-a | AstC, Orcokinin | CG30340, CCHa1-R, CG32547 | R2a, R2b |
| III | sNPF, CCHa2, Orcokinin (low) | AstC-R2 | R2a, R2b |
| I-ap-a | AstC, Orcokinin, CCHa1 | CG13575, CG32547, TrissinR | R2 |
| II-a | Tk, NPF, DH31 (part of), CCHa1 (part of) | Pdfr | R2 |
| I-m | AstC, Orcokinin | NPFR, CCHa1-R, CCKLR-17D3, CG12290, CG30340 | R3 |
| II-m1 | Tk, NPF, Mip (part of), Nplp2 (part of) | AstA-R2, AstC-R2, DH44-R2, Pdfr | R3 |
| II-m2 | Tk, NPF, Mip (part of), Nplp2 (part of) | AstA-R2, DH44-R2 | R3 |
| I-ap-p | AstC, CCHa1, CCHa2, Mip | CG13575, CG32547, TrissinR | R4 |
| I-p$^{CCHa1}$ | AstC, CCHa1 | AstC-R2, NPFR, CG13229, CG13575 | R4, R5 |
| I-p$^{AstA}$ | AstC, AstA, CCHa1 | CG13229, CG13575, CG32547, ETHR, TrissinR | R4c, R5 |
| II-p | Tk, CCHa1, DH31, NPF (part of) | AstA-R2 | R4c, R5 |

However, the expression of many peptide hormones and their corresponding receptors are (in general) mutually exclusive in each EEC subtype [11]. For example, NPF is expressed in Tk$^+$ class II EECs, while its receptor NPFR is expressed in AstC$^+$ class I EECs [11]. This suggests the potential for cell-to-cell communications among different EEC subtypes.

## Internal and external regulations of EEC heterogeneity

Apart from the general EEC lineage differentiation program from ISCs, a set of TFs have been identified to regulate the specification of EEC subtypes in both *Drosophila* and mammals. Notably, studies in *Drosophila* have revealed that in addition to its early roles during cell fate specification of ISCs, Notch signaling also continuously participates in specifying a subset of EEC subtypes, and the regional definition of many EEC subtypes can be attributed to the A/P body patterning that occurs during early embryonic development [11,74]. Studies in mammals have revealed a role for a 'morphogen gradient' along the crypt–villus axis in orchestrating different hormone production and secretion programs from EECs [65]. Generally, it is becoming clear that external factors including niche signals, nutrients, as well as gut microbiota and the metabolites they produce can impact EEC generation and EEC subtype specification. However, how these external factors and internal transcriptional network are connected to regulate EEC specification and function is still not well-understood.

### Transcriptional regulation of EEC diversity in mammals

After their specification, Ngn3$^+$ EEC progenitors are further specified into two main branches: the 5-HT-producing enterochromaffin cells and other peptide hormone-producing 'non-enterochromaffin cell trajectory' cells including D, I, K, L, and X/A cells (Fig. 4A) [12]. Specification of the enterochromaffin cell lineage requires the paired homeobox TF paired box 4 (Pax4), and a recent study has also revealed the involvement of additional TFs including Tox3 and myelin transcription factor 1 (Myt1) during this process by combining scRNA-seq analysis and organoid-based genetic validation [12,79]. These factors are transiently expressed during enterochromaffin cell specification and maturation (Fig. 4A), and their depletion leads to significantly compromised enterochromaffin cell specification and compromised 5-HT production. Additionally, the LIM homeobox TF Lmx1a is expressed in both early and mature enterochromaffin cells (Fig. 4A), and work with intestinal organoids has shown that it is required for 5-HT biosynthesis and enterochromaffin cell specification both in mouse and human GI tracts [10,12,80].

As for specification of most EEC subtypes within the non-enterochromaffin cell secretory trajectory, the paired-like homeobox TF Arx has been shown as a critical regulator, as its loss leads to depletion of CCK, SCT, GIP, GLP-1, PYY, and NTS producing cells [79]. However, the SST-expressing D cells are apparently a unique subtype in the non-enterochromaffin cell secretory trajectory, as the proportion of these cells is increased in *arx* mutant mice, whereas *pax4* depletion impairs its differentiation and upregulates L cell generation, exhibiting an antagonistic regulatory impact on these two distinct subtypes. The D cell-specific TF hematopoietically expressed homeobox (HHEX) may also contribute to the allocation of EEC progenitors into this lineage [12,79,81].

Among other subtypes within the non-enterochromaffin cell secretory trajectory, neuronal differentiation 1 (NeuroD1) regulates specification of SCT-producing S cells and CCK-producing I cells, while the winged helix factors forkhead box A1/2 (FOXA 1/2) function in specifying GLP-$1^+$ L cells [82,83]. In mature X/A cells, Zcchc12 is highly expressed, and its depletion also leads to suppression of ghrelin expression and X/A cell loss [12]. Possibly due to the different research systems used, there are apparently conflicting reports about the function of the transcriptional regulatory factor regulatory factor X6 (Rfx6): Using an *in vitro* organoid system, Gehart *et al.* [12] showed the dependence of Rfx6 for the specification of both enterochromaffin cells and non-enterochromaffin cell lineages, whereas Piccand *et al.* [84] showed that Rfx6 promotes *Arx*, *Pax6*, and *Isl1* expression to trigger the differentiation of peptidergic non-enterochromaffin cell lineages and blocks 5-HT biosynthesis in enterochromaffin cells by repressing *Lmx1a* and *Tryptophan hydroxylase 1* (*Tph1*) expression.

## Transcriptional regulation of EEC diversity in *Drosophila*

Based on scRNA-seq analysis combined with *in vivo* functional analysis, Guo *et al.* [11] have identified a number of TFs involved in EEC subtype specification in adult *Drosophila* midgut (Fig. 4B). Among them, the Irx family mirror (Mirr) and the paired-like homeobox factor Ptx1 control specification of $\mathrm{Tk}^+$ class II and $\mathrm{AstC}^+$ class I EECs, respectively [11]. In addition, Mirr is also required for the expression of all other class II EEC-specific peptide hormones tested (DH31 and NPF), indicating that Mirr is essential for the specification of class II EECs [11]. The C2H2 zinc finger TF Klu also carried out a suppressive role on class I/II EEC specification: Its depletion leads to a biased specification of EECs toward $\mathrm{AstA}^+$ class I fate [73].

Transcription factors whose expression is highly corelated with the expression patterns of peptide hormones in the EEC scRNA-seq analysis commonly show regulatory roles on transcription of related peptide hormones. Specifically, NK7.1, 48 related 1 (Fer1), and dachshund (Dac) are co-expressed with the expression of Tk, CCHa1/CCHa2, and orcokinin, respectively, and depletion of these TFs individually leads to specific downregulation of the corresponding peptide hormone; the expression of *nlp* is inversely corelated AstA, and its knock-down indeed causes upregulation of AstA expression in the gut [11]. Thus, examining TFs enriched in certain EEC subtypes or highly corelated to certain peptide hormone would be a promising approach for identification of novel regulators on EEC subtype specification.

Transcription factors with region-restricted expression commonly participate in regulating the regional identity of EEC subtypes along the *Drosophila* midgut. For example, Esg and Ptx1 are enriched in EECs in the R3 region and are required for transcription of *NPF* and *Orcokinin* in R3. Similarly, the odd-skipped family TF drumstick (Drm) is required for the expression of the peptide hormone ITP, and both Drm and ITP are enriched in the posterior end of the midgut [11,77,85]. Given that Hox genes are known to control segmentation during early embryonic development, it is tempting to speculate that these regionally expressed TFs are established during A/P body patterning during

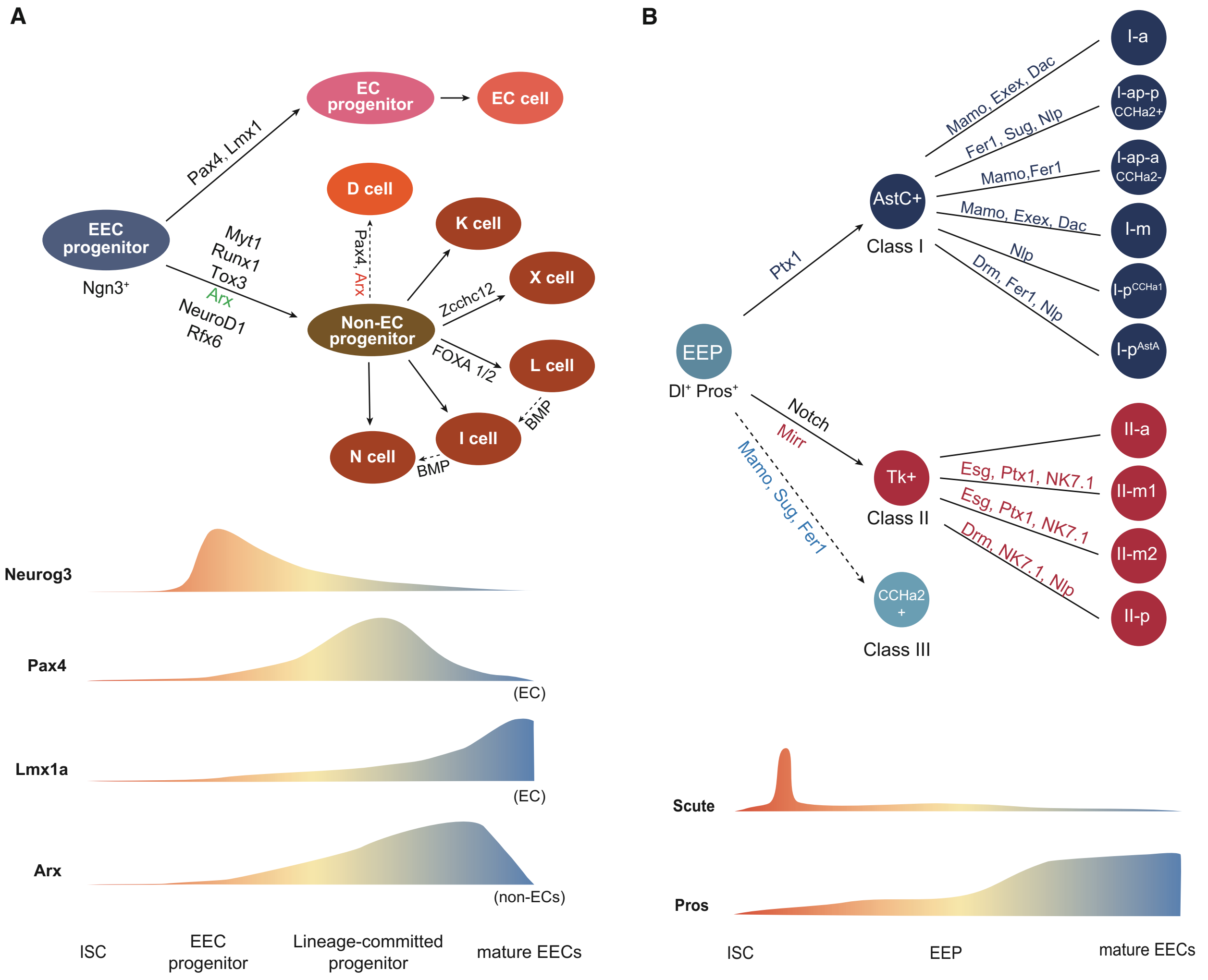

**Fig. 4.** The TF expression dynamics that lead to EEC subtype specification. (A) Schematic overview of TFs involved in EEC subtype specification in the mammalian intestine. EC progenitor: enterochromaffin cell progenitor; EC cell: enterochromaffin cell; Non-EC progenitor: non-enterochromaffin cell progenitor. Upper panel: Ngn3$^+$ EEC progenitor cells give rise to two major branches: via Pax4 and Lmx1 to promote differentiation and maturation of 5-HT-producing enterochromaffin cell; via Arx together with other TFs including NeuroD1, Rfx6, Runx1, and Tox3 to direct differentiation of non-enterochromaffin cell lineages. Note that D cell specification is suppressed by Arx and requires Pax4. For specific EEC subtypes, Zcchc12 is required for X/A cell specification, while FOXA1/2 is required for L cell specification. The BMP signaling gradient along the crypt–villus axis is able to reprogram GLP-1-producing L cell into I cell and finally N cell as the cell migrating from crypt to the villus tip. Bottom panel: expression dynamics for a few selected TFs: Neurog3 is enriched in EEC progenitors and decreased during further differentiation and maturation; Pax4 and Lmx1a are enriched in enterochromaffin cell committed lineages, Pax4 transiently expressed in enterochromaffin cell biased progenitors, and Lmx1a is gradually increased during EEC maturation. Arx is specifically enriched in non-enterochromaffin cell biased progenitors and downregulated during maturation. (B) Schematic overview of transcription factors that regulate EEC subtype specification in *Drosophila*. Upper panel: a hierarchy of TFs specifies EEP into distinct mature EEC subtypes. Among them, Mirr is required for specification of class II Tk$^+$ EECs, while Ptx1 is required for AstC$^+$ Class I EECs. Bottom panel: expression dynamics for Sc and Pros: Sc is transiently expressed in ISCs to induce EEP generation and is rapidly diminished as EEP undergoes further differentiation and maturation; Pros, which is initially induced by Sc, is gradually accumulated as EEC differentiates and matures.

early development; this could help explain their capacity in later regulating the regional identities of EEC subtypes. The EEC subtypes found in adult *Drosophila* midgut have been summarized in Table 2.

## External signals modulate EEC plasticity and adaptability

In addition to internal regulators, the continually changing environment of the GI tract can also exert profound effects on the generation of EEC diversity. The different EEC subtypes that reside at different regions along the crypt–villus axis of the mammalian intestine are not necessarily derived from distinct lineage progenitors. Lineage tracing experiments in mice revealed reprogramming of hormone repertoires in distinct EEC lineages during EEC maturation and migration along the crypt–villus axis, including decreased GLP-1 production and upregulation of PYY level in L cells, as well as increased SCT production in K cells [65]. This hormonal switch leads to alternation of the single-letter characterization of EEC subtypes, for example, a single GLP-1 producing L cell in the crypt can change into I cell and finally N cell during its migration to the villus (Fig. 4A) [12,65]. Such reprogramming appears to be controlled by a crypt-to-villus BMP signaling gradient, as *in vitro* BMP treatment of human intestinal organoids produces a similar reprogramming of hormone peptide profile [65]. It remains unclear, however, whether all I cells or N cells are originated from the reprogrammed L cells, and whether all L cells can undergo such crypt–villus reprogramming.

In *Drosophila*, Notch signaling controls binary fate choice between absorptive and secretory lineages and also exerts a role in specifying EEC subtypes: Notch depletion leads to formation of only AstC$^+$ class I EECs, while specification of Tk$^+$ class II EEC is abrogated [74]. As the two daughter EECs produced by each EEP generally express different peptide hormones (one is Tk$^+$ and another is AstC$^+$), it is likely that Notch-mediated lateral inhibition may function at the EEP stage to mediate specification of the two daughter cells, leading to adoption of either class I or class II fate. Apart from the Notch pathway, whether hormone repertoire switching in *Drosophila* also occurs during EEC maturation—and whether morphogens such as BMP or Wnt also modulate regional diversity of EEC subtypes—remains to be examined.

Enteroendocrine cells carry out chemosensory functions in the GI tract, responding to dietary nutrients and triggering the production and secretion of gut peptide hormones [1]. And in turn, dietary nutrients can also modulate specification of certain EEC subtypes [86]. Several studies have reported that high-fat diets lead to decreased total EEC numbers in mouse intestine, with especially evident reductions in the number of L cells; these reductions are accompanied by reduced levels of circulating gut hormones, which is caused by downregulation of bHLH TFs known to function in EEC specification such as Math1/Atoh1, NEUROD1, and PAX6/PAX4 [87,88]. However, another study reported increases in the number of L cells and in the plasma GLP-1 concentration after 8 weeks of high-fat feeding and linked these observations to Ngn3 activation [89]. In *Drosophila*, increased dietary cholesterol leads to reduced Delta expression and consequently reduced Notch activity, which causes increased EEC specification from ISCs [90].

Bacteria-derived metabolites have also been reported to effect EEC diversity [91]. For example, short-chain fatty acids (SCFAs), bacterial products from the fermentation of dietary fiber, have been shown to increase the PYY$^+$ cell number and circulating PYY levels in mouse models, possibly due to histone deacetylase (HDAC) inhibition [92,93]. In addition, there is an increase in the number of colonic L cells and in the plasma GLP-1 level in germ-free mice, and bacterial colonization rapidly reduced L cell numbers [94]. It is found that microbiota-derived SCFAs drive O-GlcNAc transferase (OGT)-mediated FOXO1 O-GlcNAcylation and inhibited Ngn3 expression, thus suppressing EEC progenitor specification toward L cell fate [95]. The gut microbiota also modulates colonic 5-HT production without altering enterochromaffin cell specification. This may be triggered by the spore-forming (Sp) bacteria-associated metabolites including deoxycholate, α-tocopherol, PABA, and tyramine, or via fecal microbial single-stranded RNA (ssRNA)-mediated activation of the Piezo1 receptor [96,97]. The gut microbiota can also regulate the morphology and function of EECs. *Acinetobacter* sp. *ZOR0008* in the GI tract of zebrafish is able to induce changes in EEC morphology, which cause EECs to become nutrition-insensitive, a state termed as ‘EEC silencing’ [98].

Collectively, these studies underscore the highly susceptible nature of EEC identity and hormone repertoires to both internal and external regulators; such flexibility enables adaption to the environment.

## Investigation of EEC diversification using mammal and *Drosophila* models: conservation and variance

Model organisms have been extensively used to investigate EEC biology, including mice, *Drosophila*, as well as enteroids derived from human gut tissues. Based on expression profiles and regulatory patterns of EEC subtypes in different organisms, we can evaluate evolutionary conservation of EEC-expressed peptide hormones and chemosensors, as well as TFs that modulate EEC subtype specification.

Among them, peptide hormones show less sequence conservation between *Drosophila* and mammals, although they might be functionally similar. Several sequence-conserved peptide hormones also carry out similar biological functions, such as sulfakinins (DSK) and CCK, both exist in *Drosophila* and mammals, respectively, and regulate both satiety and gut emptying [99–101] (Table 3). In contrast, transcriptional regulators that are involved in EEC subtype specification generally show more sequence conservation during evolution. As is shown in Table 4, most TFs that have been identified to exert regulatory roles on EEC subtype specification and/or peptide hormone expression in mammals have orthologues in *Drosophila*, and *vice versa*. Moreover, among these conserved TFs, there is evidence for conserved regulatory functions in mammals and *Drosophila*. For example, Arx is required for specification of non-enterochromaffin cell secretory lineages in mammals, and its orthologue in *Drosophila*, Hbn, also carries out a suppressive role on DH31$^{+}$ class II EEC specification in the posterior midgut [11,12]. Additionally, the class II specifier TF Mirr in *Drosophila* is homologous to the mammalian IRX family TFs, and IRX3 has been reported as a marker of TPH1$^{+}$ enterochromaffin cells, implying a potential regulatory role for IRX3 in enterochromaffin cell specification [10,11]. Moreover, EEC subtypes exhibit regional specificity in both mammalian and the *Drosophila* GI tracts, and the small size of *Drosophila* midgut enables the investigation of regional identities in a more discrete manner. Therefore, identification of regional and class-specific regulators in *Drosophila* may provide insights that could deepen understanding of the local and regional diversity of EECs in the mammalian GI tract.

## EECs mediate intra- and interorgan communication to modulate multiple biological processes

The gastrointestinal epithelium represents the largest surface among different organs to the external environment. It must acutely sense and broadcast nutritional status to other organs, recognize, and defend against pathogenic bacteria, as well as respond to and protect against injuries to epithelial cells of the GI tract. EECs are believed to be an important sensor and information transmitter in the gut. With recent progress, more detailed illustrations on signal sensing, peptide secretion, and physiological regulatory cascades and intra-/interorgan crosstalk mediated by these peptides have been achieved. In this section of the review, we summarize known functions of EECs in the regulation of metabolism, gut homeostasis, and crosstalk with nervous system and microbiota, specially focused on the chemoreceptors present on EEC cell membranes and on EEC secretion of peptide hormones.

**Table 3.** Evolutionary conservation of EEC produced hormones between *Drosophila* and mammals.

| EEC produced hormone in *Drosophila* | Homologue in mammals | EEC produced hormone in mammal | Homologue in *Drosophila* |
|---|---|---|---|
| Tk | Tac1 [119,181] | SST | AstC [182] |
| NPF | Neuropeptide Y [183,184] | Tac1 | Tk [119,181] |
| DH31 | Calcitonin gene-related peptide (CGRP) [185] | Serotonin (5-HT) | Serotonin (5-HT) |
| AstC | SST [182] | Histamine | Histamine |
| AstA | Galanin [101] | Gastrin | N.D. |
| CCHa1 | N.D. | CCK | Dsk [99,100] |
| CCHa2 | N.D. | GIP | N.D. |
| Orcokinin | N.D. | PYY | N.D. |
| MIP | N.D. | GLP-1 | N.D. |
| Nplp2 | N.D. | GLP-2 | N.D. |
| Gpb5 | Gphb5 [186] | Motilin | N.D. |
| ITP | N.D. | Neurotensin (NTS) | N.D. |
| sNPF | N.D. | Secretin (SCT) | N.D. |
| CCAP | N.D. | Ghrelin | N.D. |
| | | Oxyntomodulin (OXM) | N.D. |
| | | Insulin-like peptide 5 (INSL5) | N.D. |

### Regulation of metabolic homeostasis

Abundant chemoreceptors on EEC membrane enable EECs to be stimulated by metabolites and other compounds in the gut lumen [102] (Table 1). In mammalian GI tracts, carbohydrates such as simple sugars are sensed by sodium-glucose transporters (SGLTs), glucose transporters (GLUTs), and sweet taste receptors (T1R2-T1R3) in enterochromaffin, K, L, and X/A cells [103–105]. Lipids can be broken down by lipases and be sensed by free fatty acid receptors (FFARs) [106,107]; lipid amides are sensed by G protein receptor 119 (GPR119) in enterochromaffin, I, K, and L cells [108]. Oligopeptides and amino acids produced from proteins by proteases are sensed and translocated by proton-coupled oligopeptide transporter (PEPT1), umami taste receptor (T1R1-T1R3), and different GPCRs (such as CaSR, GPR142, and GPRC6a, etc.) in I, K, and L cells [109,110]. Ingestion and sensing of these dietary metabolites and other compounds (such as bile acids and bitter compounds) is known to stimulate the secretion of a range of gut hormones in the corresponding EEC subtypes (Table 1); these hormones act on multiple tissues including the GI tract, pancreas, liver, adipose tissue, and the central nervous

**Table 4.** Evolutionary conservation of EEC-regulating TFs between *Drosophila* and mammals. N.D., not determined.

| TF classes | Functionally tested TFs in mammals | Orthologs in *Drosophila* | TF classes | Functionally tested TFs in *Drosophila* | Orthologs in mammals |
|---|---|---|---|---|---|
| bHLH family | Atoh1 | Amos | bHLH family | Sc | Ascl1 |
| Zinc finger C2H2-type | Gfi1 | Sens-2 | Homeodomain family | Pros | Prox1 |
| bHLH family | Ngn3 | Tap | Iroquois homeobox | Mirr | Irx4/6 |
| HMG domain family | Sox4 | Sox14 | Paired-like homeobox | Ptx1 | Pitx2 |
| Paired homeobox | PAX4 | Eyeless | bHLH family | Fer1 | PTF1A |
| LIM homeobox | LMX1a | Lmx1a | Snail C2H2-type | Esg | Snai2 |
| Zinc finger C2H2-type | Myt1 | N.D. | NK-like homeobox | NK7.1 | N.D. |
| Runt domain family | Runx1 | Runt | Odd-skipped family | Drm | N.D. |
| HMG domain family | Tox3 | N.D. | Nucleoplasmin family | Nlp | Npm3 |
| Paired-like homeobox | Arx | Hbn | Zinc finger C2H2-type | Mamo | N.D. |
| bHLH family | neuroD1 | N.D. | NK-like homeobox | Exex | Mnx1 |
| RFX family | Rfx6 | Rfx | SKI/SNO/DAC domain | Dac | Dach1/2 |
| Forkhead family | FOXA1/2 | Fkh | Zinc finger C2H2-type | Sug | Glis2 |
| Zinc finger | Zcchc12 | N.D. | Paired homeobox | Poxn | PAX family |

system (CNS), modulating metabolic homeostasis. For example, free fatty acids can be sensed by I, K, and L cells via FFARs expressed at the apical membrane and stimulated the release of CCK, GIP and GLP-1, respectively [106,107]. All these three hormones have the ability to promote digestion, nutrient absorption [63,64], and satiety [111,112], while GIP can promote lipid uptake and storage [113], and GLP-1 is able to regulate bile acid metabolism [114].

Regulation of gut motility and metabolism during feeding requires crosstalk among gut, liver, and pancreas. In general, the participated hormones can be divided into two types, appetite promoting orexigenic and appetite suppressing anorexigenic hormones. During a fasting period, the orexigenic hormones ghrelin and motilin are increased to inhibit insulin release and to increase gastric acid secretion, gastrointestinal motility, and appetite [115]. 5-HT increases hepatic gluconeogenesis and glycogenolysis to maintain glucose homeostasis in the circulatory system [63,64,102]. INSL5 also helps to induce hunger and drive feeding behavior [91]. Postprandially, anorexigenic hormones including CCK, GLP-1, PYY, SCT, and OXM decrease appetite [111,112,115]; it is also known that released CCK, GLP-1, GIP, SST, and PYY slow down gastrointestinal motility to facilitate meal digestion and absorption [63,64]. To promote nutrient absorption, CCK triggers gallbladder contraction and secretion of pancreatic enzymes [63,64]. PYY, CCK, SST, and SCT reduce the secretion of gastric acid [112,115]. GLP-1 and GIP promote thermogenesis and uptake of triacylglycerides in adipose tissue, and PYY inhibits lipolysis [59,113]. Increased circulatory levels of GLP-1, GIP, CCK, and 5-HT stimulate insulin secretion from pancreatic β cells, while GLP-1 and CCK suppress hepatic glucose output, and both of these two processes contribute to the maintenance of glucose homeostasis [63,64,115–117]. Thus, modulation of gut EEC activities and peptide hormone secretion with dietary or pharmacological interventions may point to a potential treatment for human metabolic disorders, such as diabetes and obesity.

In *Drosophila*, gustatory receptors expressed in EECs to sense luminal contents have been characterized and examined for the colocalization with specific peptide hormones [118]. For example, A transporter GLUT1, which is the closest *Drosophila* homolog of SLC2A2 in mammals, is found to mediate the perception of dietary sugars in Bursα$^+$ EECs [76]. However, most of these gustatory receptors lack the information of what their ligands are. As for the function of peptide hormones, recent studies have revealed physiological roles of EEC-derived Tk and Burs in the regulation of intestinal lipid metabolism, and CCHa2 in the regulation of appetite. Tk suppresses lipid production in enterocytes by targeting the TKR99D receptor on enterocytes and thereby blocking PKA/SREBP signaling [119]. The secreted Burs, in response to nutrients, can bind to the neuronal receptor DLgr2 and restrict energy utilization through the repression of adipokinetic hormone (AKH)/AKHR signaling in fat bodies [76]. CCHa2 has been reported to bind its receptor in the brain [120], while loss of its expression in EECs and neurons reduced food intake, as well as leading to delayed larval development [121].

## The gut–brain axis

Enteroendocrine cells play an important role in intestinal chemotaxis, coordinating appropriate functional responses to various stimuli. Some progress has been made toward understanding the bidirectional communication between EECs and the brain. For example, Beutler *et al.* [122] found that nutrients in the gut lumen inhibited feeding behavior by stimulating the secretion of three satiety signals: 5-HT, PYY, and CCK. Buchanan *et al.* (Preprint) [123] and Tan *et al.* [124] found that SGLT1 expressed in EECs transduced gut–brain sugar signals and mediated sugar preference. Some EECs were also reported to extend basal cytoplasmic protrusion called neuropods, which might form synaptic connections with neurons to transmit information from the gut lumen to the brain [72,125,126]. Although this structure is supported by anatomical evidence, the study of functional signaling through these connections is proceeding slowly. By combining high-throughput quantitative PCR, RNA sequencing, and *in situ* hybridization, Egerod *et al.* [127] described the expression profiles of all GPCRs in the afferent vagal neurons of mice and gave particular focus to neurons innervating the GI tract. The study provided a basis for the investigation of the communication between intestinal afferent nerves and EECs and intestinal lumen.

Also, microbiome in the gut lumen can modulate physiological status of host through gut–brain axis. A study combining biophysics, genetics, pharmacology, and cell coculture methods in organoids showed that enterochromaffin cells can sense microbial metabolites in the intestinal lumen and regulate the release of 5-HT to directly communicate with neurons through synaptic interactions [7]. Microbial metabolites and other derivatives produced by food fermentation (e.g., SCFAs and bile acids) can impact intestinal peptide hormone secretion from EECs and through the microbiota–gut–brain axis, affect the nervous system, and regulate the host metabolism [96,97,128,129]. There is also evidence that microbiota composition is closely associated with many psychiatric disorders such as anxiety and depression, autism spectrum and neurodegenerative diseases including Parkinson's disease and Alzheimer's disease [130–132].

Communication through the gut–brain axis—based on EECs—has also been demonstrated in adult *Drosophila*. Chen *et al.* [133] found that specific activation of AstA expressing PLP peptidergic neurons and EECs reduces food intake as well as promotes sleep. Ren *et al.* [121] found that *ccha2* null mutant flies exhibit reduced food intake. Release of peptide hormones from EECs also contributes to postmating remodeling of food intake and reproductive capacity through gut–brain axis. Release of NPF and increase of Burs$^+$ EECs are observed in female flies after mating in response to the seminal-fluid protein sex peptide (SP). NPF promotes germline stem cell proliferation via ovarian NPFR activity, while Burs promotes food intake via Burs receptor Rickets (Rk, also called dLgr2) expressed in gut innervating myosuppressin (Ms) neurons via regulating crop enlargement [134,135].

It is worth noting that, since almost all peptide hormones secreted by EECs in *Drosophila* intestine also function as neuropeptide transmitters in the brain, distinguishing between these two sources of hormones when studying the mechanism of gut–brain communication should be carefully considered.

## Regulation of intestinal epithelial homeostasis

Homeostasis in the intestinal epithelium is maintained by ISCs in both *Drosophila* and mammals. In mammals, niche signals from epithelium cells (such as Paneth cells) and nonepithelium cells (such as mesenchymal cells) have been intensively investigated for their roles in regulating ISC self-renewal and intestinal homeostasis [136], while EECs also play a regulatory role on ISCs. In mice, GLP-2 can promote regeneration of Paneth cells and ISCs in crypts, which facilitates the preservation and regeneration of the damaged intestinal epithelium [137]. A subset of postmitotic, chromogranin A$^+$ (ChgA) EECs appears to have stem cell potential as these cells are located at the crypt of the mouse small intestine and express stem cell markers Lgr5 [138]. Indeed, ChgA$^+$ EECs with high levels of Sox9 expression exhibit regenerative capacity following irradiation [139,140]. Consistent with these observations, another study demonstrates that a subset of EECs is able to dedifferentiate into 'reserve ISCs' in response to radiation-induced injury [141].

A relationship between EECs and gastrointestinal cancers has also been implicated. EECs are observed in some primary colorectal cancers, and a worse prognosis is correlated with increased enterochromaffin cell differentiation [142]. These EECs are ChgA$^+$, 5-HT$^+$, or synaptophysin$^+$, and a subset of the ChgA$^+$ cell in normal human small intestinal and colonic crypts has been found to specifically express *Brachyury*, a putative oncogene, raising a possibility that they could act as reserve stem cells [143]. In N-nitroso-methylurea (MNU)-treated stomach, a decrease in the number of gastrin-expressing G cells helps switch of Cck2r$^+$ antral stem cells from predominant asymmetric division to symmetric division, thereby promoting tumorigenesis [15].

In *Drosophila*, EECs can also regulate ISCs by secreting peptide hormones as regulatory signals. Burs, secreted from EECs as a paracrine signal, binds to its receptor DLGR2 on visceral muscles to suppress Vn/EGF expression through cAMP signaling and finally maintains the quiescence of ISCs [144]. Another gut hormone Tk, secreted by EECs upon reception of high-nutrient diet, can induce the expression of an insulin-like peptide DILP3 in the visceral muscle, which functions as a paracrine signal for the adaptive ISC proliferation and intestinal growth [145].

## Disorders related to EEC dysfunction and pharmacological implications

Although EECs in the GI tract can regulate various biological processes, few clinical or basic studies have clearly defined their specific etiological impacts in gastrointestinal and other diseases, perhaps due to their very low number among the total gastrointestinal epithelial cell population [13]. It could be argued that the most relevant pathological disorders related to EEC dysfunction may be obesity and diabetes. Levels of fasting PYY and postglucose GLP-1 are downregulated in obese individuals, while incretins (e.g., GLP-1 and GIP) are decreased in type II diabetes mellitus (T2DM), leading to an abolished incretin effect that is now understood as a very early and specific feature of T2DM [146,147]. However, to date, no causal relationships have been demonstrated between T2DM and incretin defects. The hormone GLP-1 is necessary for normal glucose-regulation, and it is produced by both intestinal L cells and α-cells in pancreases islet, despite a relatively low level in the latter [148]. GLP-1 from both of these two sources has been demonstrated to be essential for regulating glucose homeostasis in mice models [149,150]. In new-onset type 2 diabetes patients, an increase in GLP-1-producing L-cell number could be observed, while GIP-producing K cell is not significantly altered [151]. An increased dose of this hormone can restore glucose-induced insulin secretion, a response that has been harnessed as the basis for the therapeutic use of GLP-1 receptor agonists to treat T2DM [152]. Combinational utilization of engineered stable GLP-1 agonists and inhibitors of dipeptidyl peptidase 4 (DPP4), a component which rapidly inactivates circulating GLP-1, has been licensed for clinical treatment of T2DM; the addition of basal insulin treatment or GIP agonists may further enhance the therapeutic activities of GLP-1 agonists [153–155]. Meanwhile, additional benefits such as weight loss and cardio protection can also be achieved with GLP-1 agonist treatment. Further pharmacological investigations targeting chemosensory receptors including FFAR1, GPR119, and GPR120 are currently underway, aiming to achieve a more systematic modulation of hormone production for the cure of metabolic disorders [156].

Another pathological disorder which may be impacted by EEC dysfunction is inflammatory bowel disease (IBD) [13]. GLP-2 produced by L cells enhances tight-junction integrity and decreases intestinal permeability; it also protects against oxidative stress, apoptosis, and cell cycle arrest induced by TNFα, a pro-inflammatory cytokine involved in IBD pathogenesis [157,158]. Based on the *ob*/*ob* mouse model, treatment of prebiotic like *Bifidobacterium* spp increases endogenous GLP-2 production, alleviating immunological disorders, suggesting the potential utility of prebiotics for the treatment of inflammation [159]. Enterochromaffin cell hyperplasia and increased 5-HT bioavailability in the gut are also associated with symptom generation in IBD and irritable bowel syndrome (IBS) [96,160,161]. Thus, targeting enterochromaffin cell specification and 5-HT production might be a promising therapeutic strategy for these disorders, possibly via microbiota transplantation, given that gut microbiome-derived ssRNA or metabolites produced by Sp bacteria species have been reported to regulate 5-HT production [96,97].

## Concluding remarks and perspectives

Extensive studies with multiple model organisms and recent technological advances have helped in clearly describing EEC diversity. As techniques for establishing and maintaining the biological functions of specific EEC lineages become increasingly available, it will be possible to conduct characterization and regulatory network studies at a subtype-specific level. This will almost certainly open a series of exciting research directions in EEC biology and beyond. Manipulating gene expression in specific EEC subtypes could be achieved using specific drivers, thus promoting our capacity to experimentally characterize their specific functions. Another important point is likely to be the definition of the full spectrum of the regulatory networks controlling EEC subtype specification and peptide hormone expression. Large numbers of TFs have been identified to exhibit subtype-specific expression patterns, potentially functioning in regulating specification of these EEC subtypes. Functionally, EECs carry out important roles in signal transduction between the host and the intestinal lumen contents, including the gut microbiome and their derivatives. How distinct EEC subtypes sense and respond to these variable stimuli and mediate the microbiota–gut–brain axis? Revealing nodes including ligand-GPCR receptor interactions, as well as crosstalk between EECs and surrounding vagus nerves, would bring insight to these questions. Based on the accumulation of scRNA-seq datasets of different organs, systematic illustration of interorgan crosstalk via ligand-receptor signaling between EECs with pancreases, liver, muscle, and adipose tissues could be achieved, pushing forward understanding of EEC-mediated endocrine modulation of systematic metabolism. Moreover, under stress conditions such as chronic inflammation or tumorigenesis, how specification of distinct EEC subtypes and their hormone production activities are affected? And, conversely, how do these altered EECs and peptide hormones affect the progression of disease states?

Model systems could be powerful tools to answer these questions. For example, given that zebrafish are amenable for performing live image and real-time monitoring of EEC activity or hormone secretion, this species may serve as an informative model to clarify how particular EEC subtypes respond to particular stimuli [98,162]. Organoid cultures combined with large-scale CRISPA/Cas9 screening enable systematic investigations of how TFs control EEC subtypes specification. And in addition to these model organisms, more clinical sample-based investigations with distinct disease also needs to be carried out, in order to reveal inner associations between human diseases and EEC dysfunctions.

Regarding the potential therapeutic exploitation of these basic discoveries and mechanistic insights, presently active applied research areas for potential treatment of immunological or metabolic disorders include modulation of EEC subtype specification, targeting of various chemosensors, and using endocrine hormone agonists/antagonists such as GLP-1 agonist to treat T2DM. Further studies, including basic investigations with model organisms and patient cohort-based clinical studies, focusing on elucidating the causal relationships between EECs and the etiology of several highly prevalent human disorders seem very likely to greatly expand our understanding of EEC biology and promote development of effective precision treatments.

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## Conflict of interest

The authors declare no conflict of interest.

## Author contributions

XG, JL, and RX conceptualized the idea, drafted, and revised the manuscript.

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