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Identification and characterization of GAL4 drivers that mark distinct cell types and regions in the *Drosophila* adult gut

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ABSTRACT

The gastrointestinal tract in the adult *Drosophila* serves as a model system for exploring the mechanisms underlying digestion, absorption and excretion, stem cell plasticity, and inter-organ communication, particularly through the gut–brain axis. It is also useful for studying the cellular and adaptive responses to dietary changes, alterations in microbiota and immunity, and systematic and endocrine signals. Despite the various cell types and distinct regions in the gastrointestinal tract, few tools are available to target and manipulate the activity of each cell type and region, and their gene expression. Here, we report 353 GAL4 lines and several split-GAL4 lines that are expressed in enteric neurons (ENs), progenitors (ISCs and EBs), enterocytes (ECs), enteroendocrine cells (EEs), or/and other cell types that are yet to be identified in distinct regions of the gut. We had initially collected approximately 600 GAL4 lines that may be expressed in the gut based on RNA sequencing data, and then crossed them to *UAS-GFP* to perform immunohistochemistry to identify those that are expressed selectively in the gut. The cell types and regional expression patterns that are associated with the entire set of GAL4 drivers and split-GAL4 combinations are annotated online at http://kdrc.kr/index.php (K-Gut Project). This GAL4 resource can be used to target specific populations of distinct cell types in the fly gut, and therefore, should permit a more precise investigation of gut cells that regulate important biological processes.

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Drosophila; gut; GAL4; regional; cell-type specificity; resource

Introduction

The gastrointestinal tract is not only the site where food digestion and nutrients absorption take place, but it also regulates key biological processes, including immune defenses, interactions with the microbiome, nutrient sensing, and inter-organ communications. For example, since the discovery of somatic stem cells in the adult *Drosophila* midgut more than a decade ago (Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006), the fly gut has been used to study the biology of stem cells, homeostasis and organ size control (Colombani & Andersen, 2020). Because of the ease of genetic manipulation, the fly gut has been used to investigate intestinal physiology and diseases (Miguel-Aliaga, Jasper, & Lemaitre, 2018).

The gastrointestinal tract in the adult *Drosophila* can be divided into three regions – the foregut, midgut, and hindgut – based on morphology, function, and developmental origin (Guo, Lucchetta, Rafel, & Ohlstein, 2016; Miguel-Aliaga *et al.*, 2018). The foregut, which is originated from ectodermal progenitors, consists of the esophagus, crop and proventriculus (Pr; also known as the cardia). This region of the gut regulates the passage of food and the accumulation of ingested food in the crop. The hindgut, which is also ectodermal in origin, carries out functions similar to the mammalian large intestine, including water/ion exchange. This region can be divided into four distinct regions: the hindgut proliferation zone (HPZ), the pylorus, the ileum, and the rectum (Guo *et al.*, 2016). In contrast to the foregut and hindgut, the midgut originates from the endoderm.

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Supplemental data for this article can be accessed here.

Functionally equivalent to the mammalian small intestine, the midgut can be divided roughly into three regions – the anterior, middle, and posterior midgut – and subdivided further into several distinct subregions based on distinct morphological, molecular, physiological and genetic characterizations (Buchon & Osman, 2015; Buchon *et al.*, 2013; Marianes & Spradling, 2013; Miguel-Aliaga *et al.*, 2018). The adult *Drosophila* midgut is lined with a single epithelium layer consisting of four cell types: pluripotent intestinal stem cells (ISCs), postmitotic and differentiating enteroblasts (EBs), absorptive enterocytes (ECs), and secretory enteroendocrine cells (EEs) (Micchelli & Perrimon, 2006; Miguel-Aliaga *et al.*, 2018; Ohlstein & Spradling, 2006).

GAL4 transgenesis technique that permits targeting and manipulating the activity of specific groups of cells and their gene expression has been extremely useful for carrying out *in vivo* experiments in *Drosophila*. Thousands of GAL4 lines that are expressed in distinct subsets of cells in the adult fly brain have already been generated (Pfeiffer *et al.*, 2008). Although these GAL4 drivers were initially developed and generated to study the nervous system in the fly, these tools could be used to study other tissue types, including the gastrointestinal tract. Documenting GAL4 expression in specific spatial and functional regions of the *Drosophila* gastrointestinal tract would be useful for investigating the functions of distinct cell types in this region of the fly (Jenett *et al.*, 2012; Manning *et al.*, 2012).

The goal of this study was to develop a comprehensive collection of GAL4 lines and split GAL4 lines that can be used to easily manipulate the cells along the gastrointestinal tract of the fly. Such a tool would ideally direct the expression of GAL4 in specific cell types (e.g. ECs) in a specific region of the gut or perhaps in a single cell. We examined the expression of 585 GAL4 drivers in the gastrointestinal tract and built a database of images for 353 lines that demonstrate GFP reporter expression. This database will be useful for researchers who are interested in manipulating distinct populations of cells along the gastrointestinal tract.

Methods

Drosophila stocks and transgenic flies

Flies were cultured on standard cornmeal agar medium at an average temperature of 25 °C. 5–7 day-old female flies were dissected and analyzed for all experiments. *UAS-mCD8-GFP* or *UAS-myr-GFP* were used as the GFP reporter.

Dissection and immunostaining

The process of dissecting the fly gastrointestinal tract to immunolabeling and mounting was based on a previous protocol (Park & Kwon, 2011) with minor modifications. Only female flies were dissected. Guts of males and females, in general, are anatomically and functionally very similar except their overall size. Based on the pilot test, the expression patterns of Gal4 drivers in males are overall comparable to those in females except few lines. Reproductive organs and other debris were removed from the fly abdomen. The remaining gastrointestinal tracts were fixed for 30 min at RT in 4% paraformaldehyde dissolved in phosphate-buffered saline containing 0.2% Triton X-100 (PBS-T, pH7.2). After washing three times for 10 min in PBS-T, samples were blocked for at least 1 h in PBS-T containing 3% normal goat serum. Samples were then stored overnight at 4 °C with the primary antibody diluted in blocking solution. Three washes for 10 min in PBS-T were followed by incubating samples overnight at 4 °C with the secondary antibody diluted in blocking solution. Samples were then washed three times for 10 min in PBS-T followed by DAPI (1:1000) staining for the confocal microscopy or DAPI (1:1000) and Alexa 555-Phalloidin (1:1000) (Thermo Fisher, Seoul, Republic of Korea) staining for the light-sheet microscopy. After three additional washes for 10 min in PBS-T, samples were mounted on a slide glass with 50% glycerol in PBS-T for the confocal microscopy or imbedded in 0.8 or 1.0% agarose PBS solution in the light-sheet capillary with inner diameter of 0.68 mm (BRAND, Catalogue number 701902) for the light-sheet microscopy. Samples in the capillary were kept in PBS and imaged within 24 h after sample preparation. The primary antibodies used were rabbit anti-GFP (1:1000), and mouse anti-Prospero (1:100). The secondary antibodies used were goat anti-mouse and goat anti-rabbit IgG conjugated to Alexa 568 and Alexa 488 (1 : 1000) (Molecular Probes, Seoul, Republic of Korea), respectively. At least, five gut specimens per a Gal4 line were dissected and examined for their GFP expression.

Variations in EN labeling among samples from the same cross were observed. The variations were observed mostly in EN cell bodies (i.e. SG neurons) and processes loosely attached to the gut, but not in EN processes deeply penetrating into the gut tissues (i.e. those in the crop and rectum).

Microscopy and image analysis

Zeiss LSM 700 laser-scanning confocal microscopy was used to image fluorescent samples, and Leica DM2500 microscopy with a digital camera (Canon EOS 700 D) through epi-fluorescence was used to image the whole gastrointestinal tract. For high resolution anatomical analysis of the enteric neurons, we used the light-sheet microcopy, Light-sheet Z.1 (Zeiss, Seoul, Republic of Korea).

Results

Annotation of GAL4 expression in the gut

We began our search for a tool that targets intestinal cells in the fly by the region-specific RNA seq and single-cell RNA seq data (Hung *et al.*, 2020) to identify genes that are expressed in the intestine. We then selected FlyLight (Janelia) GAL4 lines (Pfeiffer *et al.*, 2008) that had been constructed using enhancer sequences for the identified genes. A total of 585 GAL4 drivers were selected and crossed to *UAS-GFP* reporter to determine and view possible GAL4 expression in the gut. The gut specimens were dissected

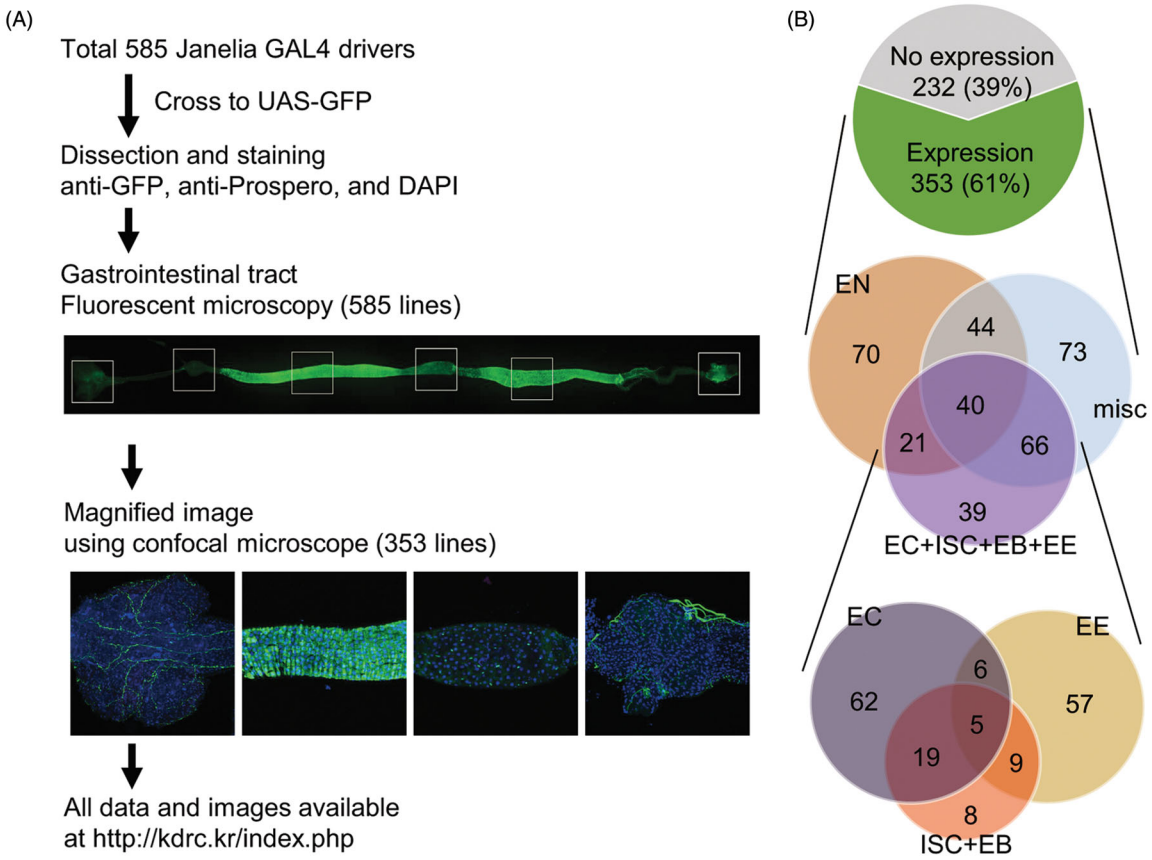

Figure 1. Overview of experimental strategy and outcome of a search for GAL4 drivers expressed in the gastrointestinal tract. (A) The flowchart to identify GAL4 lines that are expressed in the gut. Fluorescent images of GAL4 expression in the gut are annotated and available at the Korea Drosophila Resource Center (KDRC) website (http://kdrc.kr/index.php..). (B) The number of GAL4 drivers that are expressed in each cell type in the gastrointestinal tract are indicated. The size of the circle in the diagram reflects the relative number of GAL4 lines. EN, enteric neuron; EC, enterocyte; ISC, intestinal stem cell; EB, enteroblast; EE, enteroendocrine cell; misc, miscellaneous cell.

from 5- to 7-day-old female flies and co-stained with DAPI and anti-GFP and anti-Prospero antibodies. DAPI staining allowed us to distinguish ECs (which are relatively larger cells) from progenitor cells (ISCs and EBs) and EEs based on the overall size of the cell. Prospero-positive cells were scored as EEs. GAL4-driven GFP expression was observed along the entire gastrointestinal tract, encompassing a part of the foregut (including the crop and Pr) through the midgut and extending to the hindgut and rectum. Enteric neurons (ENs), muscle, and uncharacterized cells were included in our database (Figure 1(A)). Among the 585 GAL4 drivers tested, 353 exhibited GFP expression in at least some of the cells in the gastrointestinal tract between the crop and the anus. We divided the gastrointestinal tract into 10 regions to organize the GAL4 expression patterns: the crop (including the link between the crop and Pr), Pr, R1 to R5, midgut-hindgut junction (consisting of the HPZ and pylorus), ileum, and rectum. All data and images are available at the Korea Drosophila Resource Center (KDRC) website, http://kdrc.kr/index.php (Figure 1(A)).

Among the 353 GFP-expressing drivers, 175 GAL4 lines were expressed in the ENs; this included 70 lines that exhibited EN-specific expression. 166 GAL4 lines were expressed in midgut ISC, EB, EC, and EE cells. Because of the difficulty in distinguishing ISCs from EBs, these cells were initially grouped together as progenitor cells. The remaining 223 GAL4 lines were expressed in miscellaneous (misc) cells in the gastrointestinal tract, which include gastric stem cells (GaSCs) and injury-responsive stem cells in the HPZ (Figure 1(B)).

GAL4 drivers label distinct types of adult enteric neurons in the adult fly

ENs mediate interactions between the intestine and other organs (e.g. the brain) and between different regions of the intestine (Miguel-Aliaga *et al.*, 2018). In *Drosophila*, EN processes mainly innervate the foregut and hindgut regions, along the midgut region associated with each (i.e. the R1 and R5 regions) (Figure 2(A)) (Cognigni, Bailey, & Miguel-Aliaga, 2011). A limited number of GAL4 lines has been reported to target ENs innervating the intestine in adult *Drosophila* (Kuraishi, Kenmoku, & Kurata, 2015). In this study, we identified 175 GAL4 lines that label EN processes and analyzed 27 GAL4 lines using high resolution light-sheet microscopy (Supplementary Table 1).

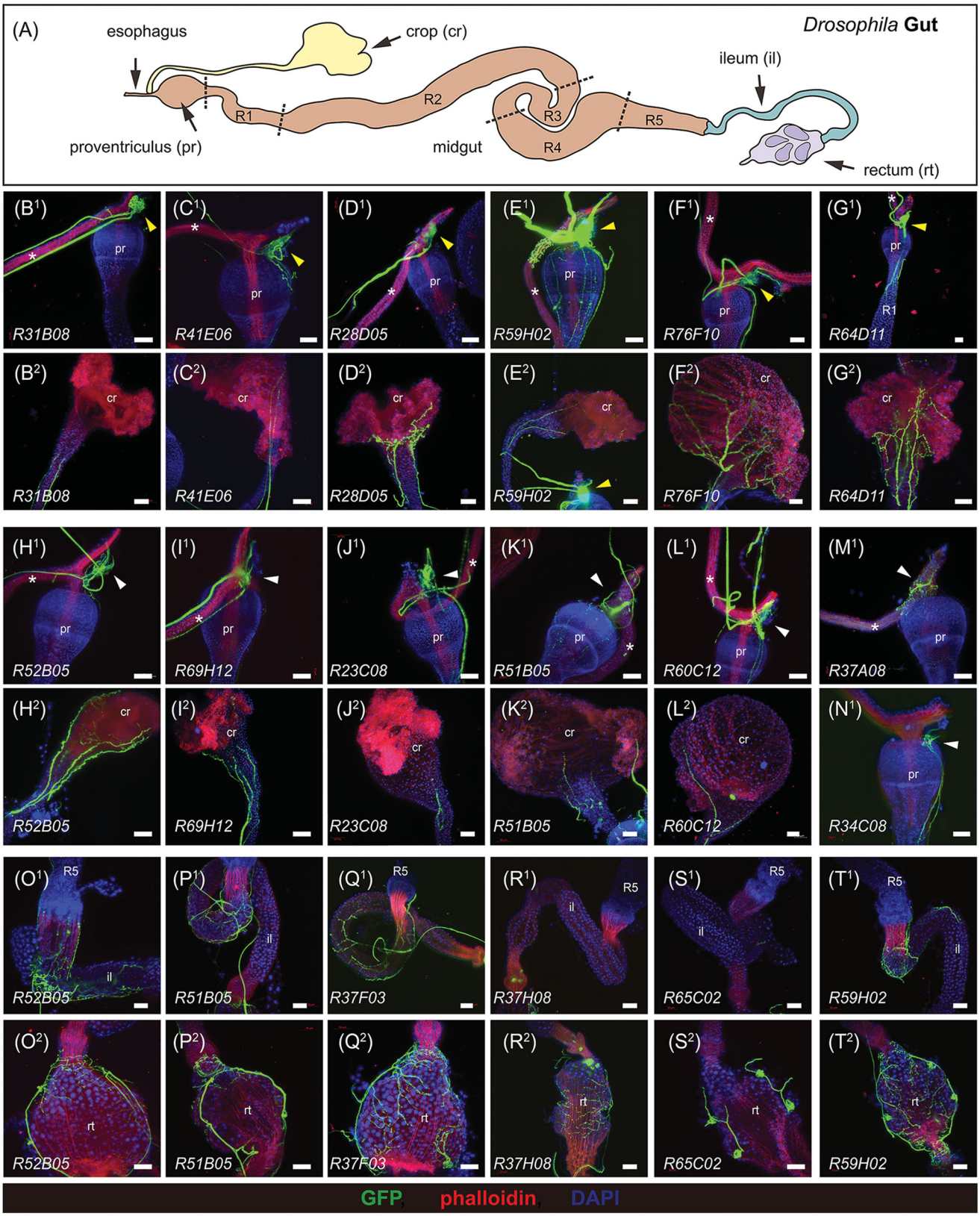

Figure 2. GAL4 drivers expressed in ENs. (A) Cartoon image illustrating the foregut, midgut, and hindgut regions of the *Drosophila* gastrointestinal tract. The midgut is divided into R1–R5 (dotted lines). (B–T) Light-sheet images of ENs expressing myrEGFP under the control of denoted GAL4 drivers in the foregut (B–N) and in the hindgut (O–T) regions, and associated midgut regions. (B^1–N^1) Light-sheet images of ENs in the proventriculus and the stomatogastric ganglion (arrowheads). The color of arrowhead defines the number of the sg neuronal cell bodies positive for GFP; yellow – more than 10 cell bodies; white – less than 5 cell bodies. White asterisk indicates the crop duct. (B^2–L^2) Light-sheet images of ENs in the crop. Varying degrees of complexity in their innervation pattern are observed. (O^1–T^1) Light-sheet images of ENs in the midgut-hindgut junction. R^1 and S^1 exhibit no enteric innervation. (O^2–T^2) Light-sheet images of ENs in the rectum. Distinct innervation patterns in the rectum are identified by different GAL4 drivers. Proventriculus (pr); crop (cr); ileum (il); rectum (rt). Scale bars, 50 μm.

In adult *Drosophila*, the hypocerebral ganglion and the endocrine corpora cardiacum fuse to form the stomatogastric ganglion (sg), which is located at the junction between the esophagus and Pr (yellow or white arrowheads in Figure 2(B^1–N^1)) (Lee & Park, 2004; Perea *et al*., 2017). All of the GAL4 lines that targeted the foregut region in this study labeled the sg neuronal cell body and/or nerve fiber. We examined GAL4 lines that labeled a cluster of more than 10 sg cell bodies (Figure 2(B^1–G^1)). These GAL4 lines labeled a pair of neuronal processes projecting along the crop duct (white asterisk) that innervated the crop (Figure 2(B^2–G^2)). We observed distinctive innervation patterns in the crop. The *R31B08* and *R41E06* lines, for example, labeled a pair of neuronal processes that terminated at the crop without branching further (Figure 2(B^2,C^2)), whereas the *R28D05, R76F10, and R64D11* lines labeled a pair of processes that branched multiple times to produce complex innervation patterns that covered the entire crop (Figure 2(D^2,F^2,G^2)). *R59H02* labeled an innervation pattern that was intermediate in complexity, consisting of a pair of neuronal processes that bifurcated once to produce four neurites that branched along the crop (Figure 2(E^2)). Additionally, neuronal processes emanating from the sg arborized along the crop duct close to the Pr (Figure 2(E^1)), the surface of the Pr (Figure 2(E^1)), and the R1 region in the midgut (Figure 2(F^1,G^1)).

We also identified several GAL4 lines that labeled a small number (3–4 cells) of the sg neuronal cell bodies (Figure 2(H^1–N^1)), some of which projected a pair of neuronal processes along the crop duct to innervate the crop after forming an arbor that was intermediate (*R52B05, R69H12, R23C08*, and *R51B05*) or simple (*R60C12*) in complexity. This group of GAL4 lines was rarely associated with complex crop innervation patterns (Figure 2(F^2, G^2)). Two GAL4 lines did not label processes innervating the crop: *R37A08* labeled 1 or 2 neurons that innervated a three-way junction connecting the esophagus, crop, and Pr (Figure 2(M^1)); *R34C08* labeled neuronal processes that arborize along the surface of the Pr and innervated the anterior R1 region of the midgut (Figure 2(N^1)).

The hindgut receives extensive neuronal innervations. Many of the GAL4 lines we have identified labeled neuronal processes that innervated the midgut–hindgut junction, ileum, and rectum (Figure 2(O–T)). All of the GAL4 lines expressed in ENs innervating the midgut–hindgut junction in this study were also found expressed in ENs innervating the rectum. Because the innervation patterns in the midgut–hindgut junction were similar, we used another criterion to classify ENs in the hindgut: the pattern of innervating the rectum. For example, the *R52B05* and *R51B05* lines labeled a pair of neuronal processes arborizing along the lateral edge of the rectum and innervating the junction between the ileum and rectum extensively (Figure 2(O^2,P^2)); *R37F03* labeled ENs that initially project laterally and then arborize medially in the rectum (Figure 2(Q^2)); *R37H08* labeled neuronal processes that innervate more medially than *R37F03* (Figure 2(R^2)); and *R65C02* labeled neuronal processes arborizing over a specific position on the rectal papilla (Figure 2(S^2)). Some GAL4 lines labeled all or part of these innervation patterns (i.e. Figure 2(T^2)). We found GAL4 lines that were expressed in neuronal processes projecting to the rectum, but were not expressed in processes innervating the midgut–hind junction. The *R37H08* and *R65C02* lines, for example, drove GAL4 expression in neuronal processes in the rectum but not in the midgut-hindgut junction (Figure 2(R, S)).

GAL4 drivers label different types of adult midgut epithelial cells

The *Drosophila* midgut contains progenitor cells (ISCs and EBs) that replenish gut epithelial cells (Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006). ISCs are the only proliferating cells in the gut epithelium; EBs are intermediate progenitor cells that undergo further differentiation into ECs. Because new progenitor drivers may be useful for investigating and understanding gut biology, we attempted to characterize GAL4 drivers that are expressed in progenitors. As with other epithelial cells in the midgut, the progenitors can be identified by their relatively small nuclei compared with the polyploid nuclei of ECs when stained with DAPI. Progenitor cells can also be distinguished from Prospero-positive EEs. Of the 166 GAL4 lines expressed in the midgut epithelium, 41 were expressed in progenitors (ISCs and EBs). These progenitor GAL4 drivers were subcategorized into three groups: a group of 8 GAL4 lines that were expressed specifically in progenitors, a group of 19 GAL4 lines that were expressed in progenitors and in ECs, and a group of 9 GAL4 lines in progenitors and in EEs (Figure 1(B), Supplementary Table 2). Among the progenitor-specific drivers (Figure 3(A',B')), *R59F09* was expressed throughout the midgut epithelium, whereas *R28A12* was expressed mainly in regions R2, R4, and R5 (Figure 3(A,B)). Among the progenitor GAL4 lines that were also expressed in ECs or EEs (Figure 3(C,D)), *R77G01* was selectively expressed in progenitors throughout the midgut (Figure 3(C')) except in the R4 region where GAL4 was expressed in both progenitors and ECs (Figure 3(C")); and *R29F11* was specifically expressed in progenitors throughout the midgut (Figure 3(D')) except in the R3 region where GAL4 was expressed in both progenitors and EEs (Figure 3(D")).

To identify GAL4 lines that are expressed in ECs, we selected GAL4 drivers that expressed the GFP reporter in relatively large gut cells that were positive for DAPI and negative for anti-Prospero antibody staining. The large polyploidy nuclei of ECs were clearly distinguishable with small diploid nuclei of progenitors when stained with DAPI, so the task of distinguishing ECs from other cell types was relatively straightforward. Of the 585 GAL4 lines, we identified 92 lines that were expressed in ECs in the gut; 57 were expressed only in the midgut; 25 were expressed only in the hindgut; 10 were expressed in both the midgut and hindgut (Supplementary Figure 1). Of 92 EC-expressing GAL4 lines, we found that only 10 that expressed GAL4 exclusively in ECs and not in any other cell types (Supplementary Table 3). Among those, 7 expressed GAL4 in a specific region of the gut (Figure 4(A,B)), 2 expressed GAL4 in multiple regions of the midgut (Figure 4(C)), and 1 was broadly expressed throughout the midgut (Figure 4(D)). Of the 7 lines, 2 expressed GAL4 in the R4 region, 3 expressed GAL4

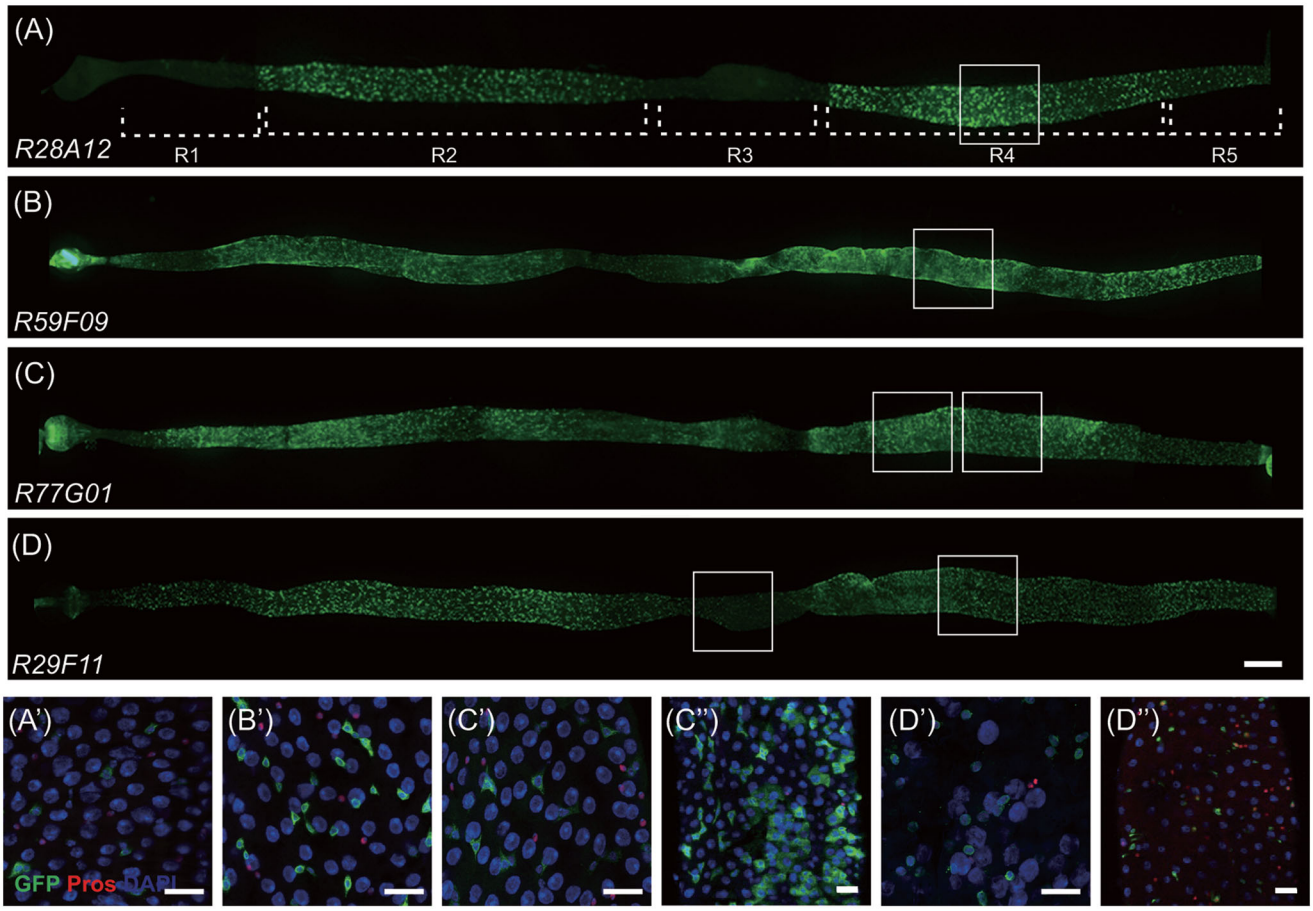

Figure 3. GAL4 drivers expressed in the progenitor cells. Epi-fluorescent images of the whole gut expressing native GFP under the control of denoted GAL4 drivers (A–D) and confocal immunofluorescent images labeled with DAPI and anti-GFP and anti-Prospero antibodies corresponding to white insets (A′–D′). (A, A′) *R28A12-GAL4 > UAS-mCD8-GFP* labels progenitors in the R2, R4 and R5 regions. (B, B′) *R59F09-GAL4 > UAS-mCD8-GFP* labels progenitors in all midgut regions. (C, C′, C″) *R77G01-GAL4 > UAS-mCD8-GFP* labels progenitors in all midgut regions (C′) and both progenitors and a portion of ECs in the R4 region (C″). (D, D′, D″) *R29F11-GAL4 > UAS-mCD8-GFP* labels progenitors in all midgut regions (D′) and both progenitors and a portion of EEs in the R3 region (D″). Scale bars: 300 μm (A–D), 20 μm (A′–D″).

in the R5 region of the midgut, and 2 expressed GAL4 in the hindgut.

EEs were identified based on co-labeling of the GFP reporter and anti-Prospero antibody. A total of 77 GAL4 drivers directed GAL4 expression in EEs (Figure 1(B)). 57 GAL4 drivers exhibited EE-specific expression in the midgut (Figure 1(B)). Among these, 19 lines were expressed exclusively in EEs without any other expression throughout the entire gastrointestinal tract (Supplementary Table 4). Among the 77 lines expressed in EEs, 10 lines were broadly expressed throughout the anterior, middle, and posterior midgut. Interestingly, 42 lines were expressed in the middle midgut only, whereas 5 were expressed in the anterior-middle midgut, 8 were expressed in the middle-posterior midgut, 2 were expressed in the anterior-posterior midgut, 2 were expressed in the anterior midgut expression, and 8 were expressed in the posterior midgut (Supplementary Figure 2). As shown in Figure 5, several GAL4 drivers represent broadly expressed and regionally specific GAL4 lines. Magnified images showed co-localization of GAL4-driven GFP with anti-Prospero (Figure 5(A'–D'), Supplementary Figure 3).

In whole mount preparations of dissected gut tissue, GAL4 drivers that directed GFP reporter expression in cells other than the ENs or midgut epithelial cells were classified as miscellaneous. These miscellaneous cells included GaSCs, muscle tissue, as well as unidentifiable cell types. The *Drosophila* Pr, which is located at the intersection of the esophagus, crop, and midgut (Figure 6(A)), functions as a valve that regulates the passage of food into the anterior midgut and crop (Singh, Zeng, Zheng, & Hou, 2011). Based on the location and morphology of GFP reporter-expressing cells, we determined that some GAL4 drivers were expressed in GaSC (Figure 6(A')), whereas others were expressed in the anterior outer layer of the Pr and GaSCs (Figure 6(A'')). Some drivers were expressed in the inner layer of circular muscles enveloping the midgut epithelium (Figure 6(B')), whereas others were expressed in the outer layer of longitudinal muscles (Figure 6(C')). Some drivers were expressed in the midgut–hindgut boundary and HPZ where stem cells exist (Figure 6(D')), while others in the region where subsequent proliferation and differentiation of ISC descendants occur (Figure 6(D'')). GAL4-driven GFP expressions were observed in the rectum, where GAL4 lines were expressed in structures such as rectal papilla caps (Figure 6(E')), or structures that appear to be muscles surrounding the rectum (Figure 6(E'')).

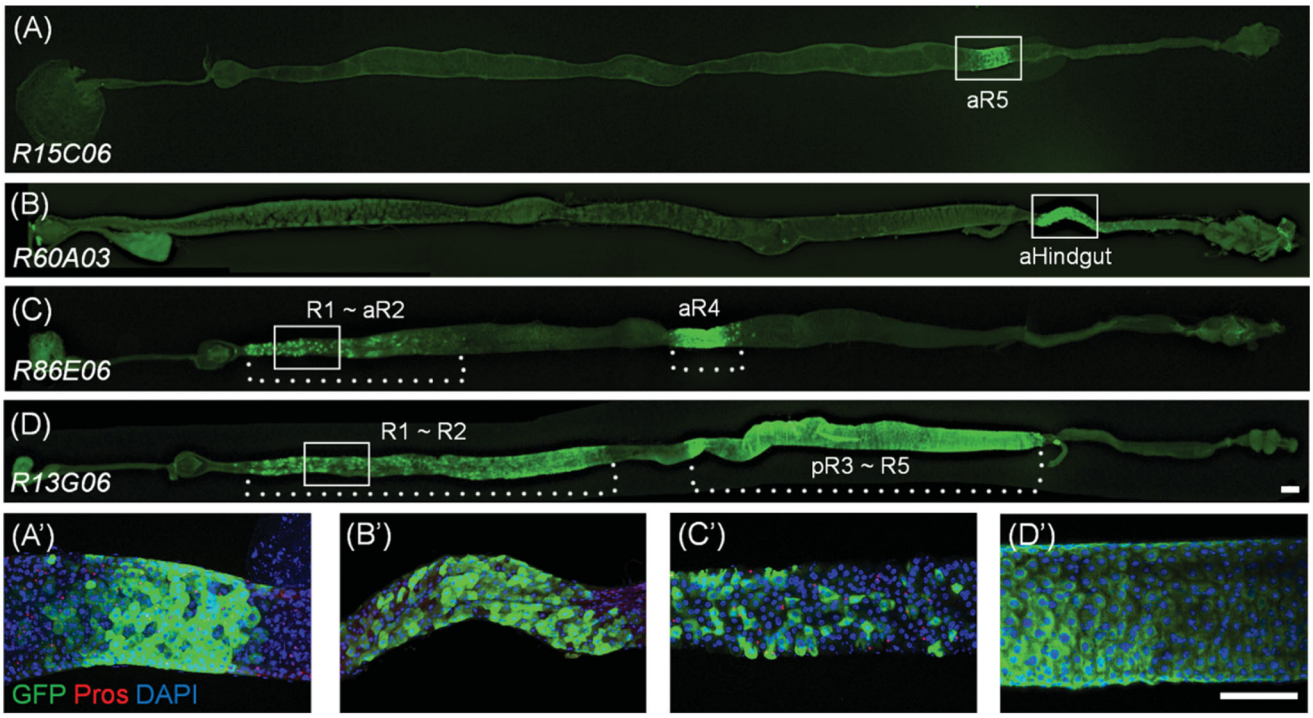

Figure 4. GAL4 drivers expressed in ECs. Epi-fluorescent images of the whole gut expressing native GFP under the control of denoted ECs-GAL4 drivers (A–D) and confocal immunofluorescent images labeled with DAPI and anti-GFP and anti-Prospero antibodies corresponding to white insets (A'-D'). (A) *R15C06-GAL4 > UAS-mCD8-GFP* specifically labels ECs in the anterior part of the R5 region. (B) *R60A03-GAL4 > UAS-mCD8-GFP* selectively labels ECs in the anterior hindgut region. (C) *R86E06-GAL4 > UAS-mCD8-GFP* labels ECs in the R1, anterior R2, and anterior R4 regions. (D) *R13G06-GAL4 > UAS-mCD8-GFP* labels ECs throughout the midgut except the posterior R2 and anterior R3 regions. Dotted lines indicate the regions of GFP expression. Scale bars: 300 μm (A–D), 100 μm (A'–D').

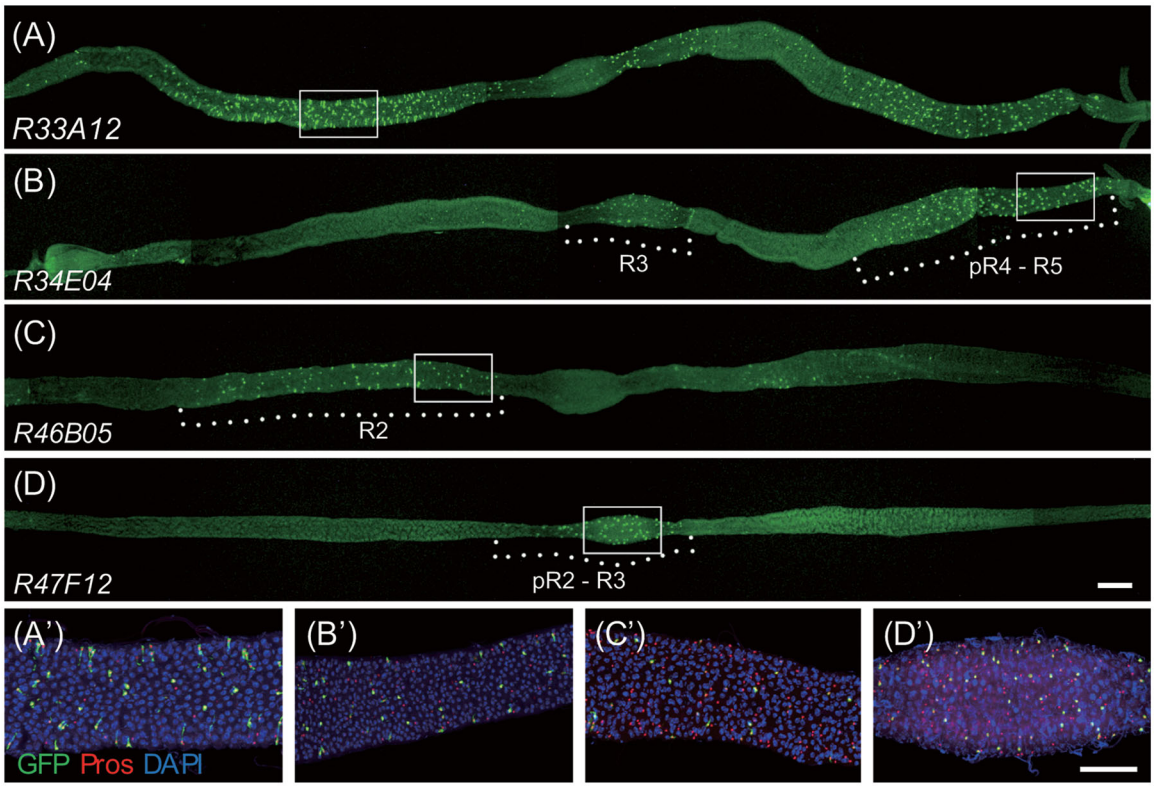

Figure 5. GAL4 drivers expressed in EEs. Epi-fluorescent images of the whole gut expressing native GFP under the control of denoted EEs-GAL4 drivers (A–D) and confocal immunofluorescent images labeled with DAPI and anti-GFP and anti-Prospero antibodies corresponding to white insets (A'–D'). (A) *R33A12-GAL4 > UAS-mCD8-GFP* labels EEs throughout the midgut. (B) *R34E04-GAL4 > UAS-mCD8-GFP* labels EEs in the R3, posterior R4, and R5 regions. (C) *R46B05-GAL4 > UAS-mCD8-GFP* selectively labels EEs in the R2 region. (D) *R47F12-GAL4 > UAS-mCD8-GFP* labels EEs in the posterior R2 and R3 regions. Dotted lines indicate the regions of GFP expression. Scale bars: 300 μm (A–D), 100 μm (A'–D').

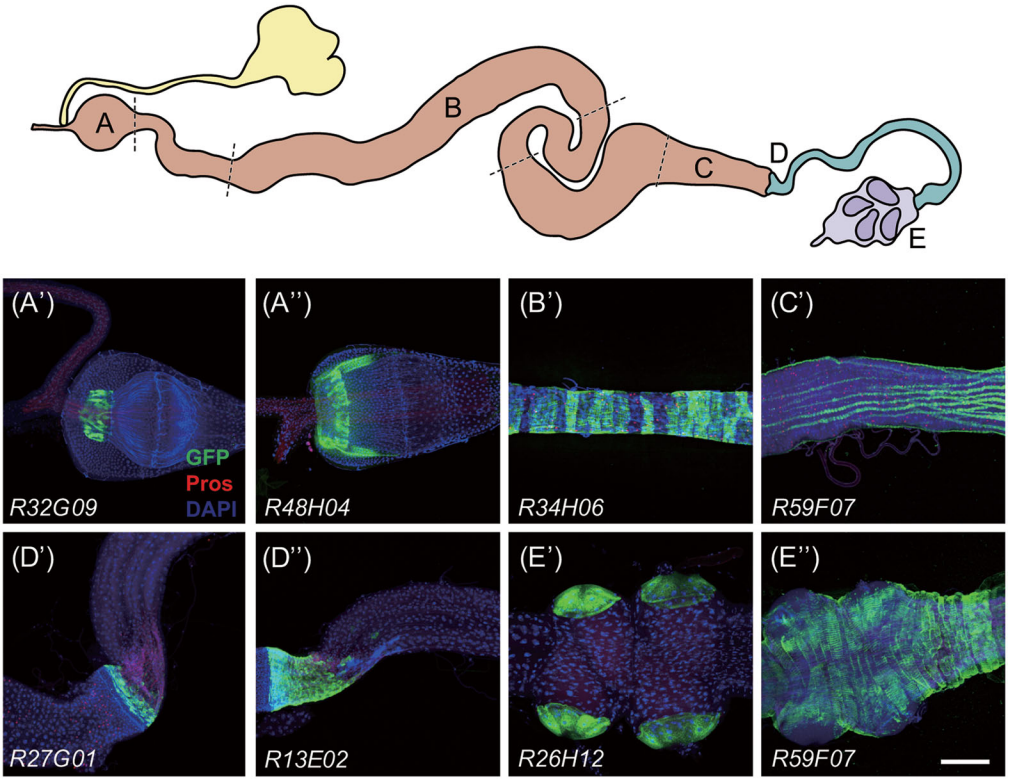

Figure 6. GAL4 drivers expressed in miscellaneous cell types of the gastrointestinal tract. Cartoon schematic of the gastrointestinal tract (top): Proventriculus – A; midgut – B, C; midgut–hindgut junction – D; rectum – E. (A′–E″) Confocal immunofluorescent images of various structures corresponding to the abovementioned gastrointestinal regions. Denoted GAL4 drivers crossed to *UAS-mCD8GFP* are stained with DAPI and anti-GFP and anti-Prospero antibodies. Scale bar: 100 μm (A′–E″).

split-GAL4 combinations label specific subsets of enteroendocrine cells

Many of the GAL4 drivers expressed in the gastrointestinal tract show cell type or subregion specificity. Split-GAL4 combinations would allow us to target even specific populations of cells, making this resource even more useful for genetic manipulation. We tested this Split-GAL4 approach in EEs. We found that *R61H08* is expressed in EEs over the entire midgut (Figure 7(A), Supplementary Table 5). We crossed the split *R61H08-DBD* (GAL4 DNA-binding domain) line into 8 EE-expressing p65-activation domain (AD) split lines. GAL4-induced GFP expression was observed in 3 of these combinations, as anticipated, but not in five other combinations (Figure 7(B–D), Supplementary Table 6). For example, *R65D05* was expressed in the EEs of the posterior midgut and in ENs wrapped around the posterior midgut and hindgut (Figure 7(B), Supplementary Table 5). By contrast, the combination of *R61H08-DBD* and *R65D05-AD* specifically labeled posterior midgut EEs only, revealing no expression in ENs (Figure 7(C), Supplementary Table 6). This result suggests that *R61H08-GAL4* may be expressed broadly over the entire midgut, but not in all of the EEs. Additionally, five transgenes that did not show GAL4 expression when combined with *R61H08-GAL4* were probably expressed in populations of EEs that were excluded from the *R61H08*-expressing EEs.

Discussion

We identified 353 GAL4 drivers that are expressed in the gastrointestinal tract of the adult fly and built an open-access, web-based image database based on the results of our expression analyses. We plan to provide continuous updates to this site. The GAL4 lines identified in this work are useful for studying the function of each type of cells in the gut. One potentially informative application of this database would involve searching for images obtained from two GAL4 lines for cells that express both GAL4 lines. We were successfully using this approach to target more specific populations of cells in the gut using split-GAL4 approach. Even more restricted subsets of a cell type could be manipulated using constructs that express the transcriptional activator LexA and the repressor GAL80, combined with the split-GAL4 system (Dionne, Hibbard, Cavallaro, Kao, & Rubin, 2018; Luan, Peabody, Vinson, & White, 2006).

In adult flies, enteric neuronal processes innervating the foregut originate from the sg and brain (Cao & Brown, 2001; Cognigni *et al.*, 2011; Lee & Park, 2004; Perea *et al.*, 2017). Some brain neurons, especially those in the pars intercerebralis (PI) (i.e. dilp2-GAL4 neurons and Dh44-GAL4 neurons), projected to the sg (Cao & Brown, 2001; Dus *et al.*, 2015). Nearly all of foregut-innervating GAL4 lines that were analyzed by the high-resolution imaging in this study showed strong labeling in neuronal processes that

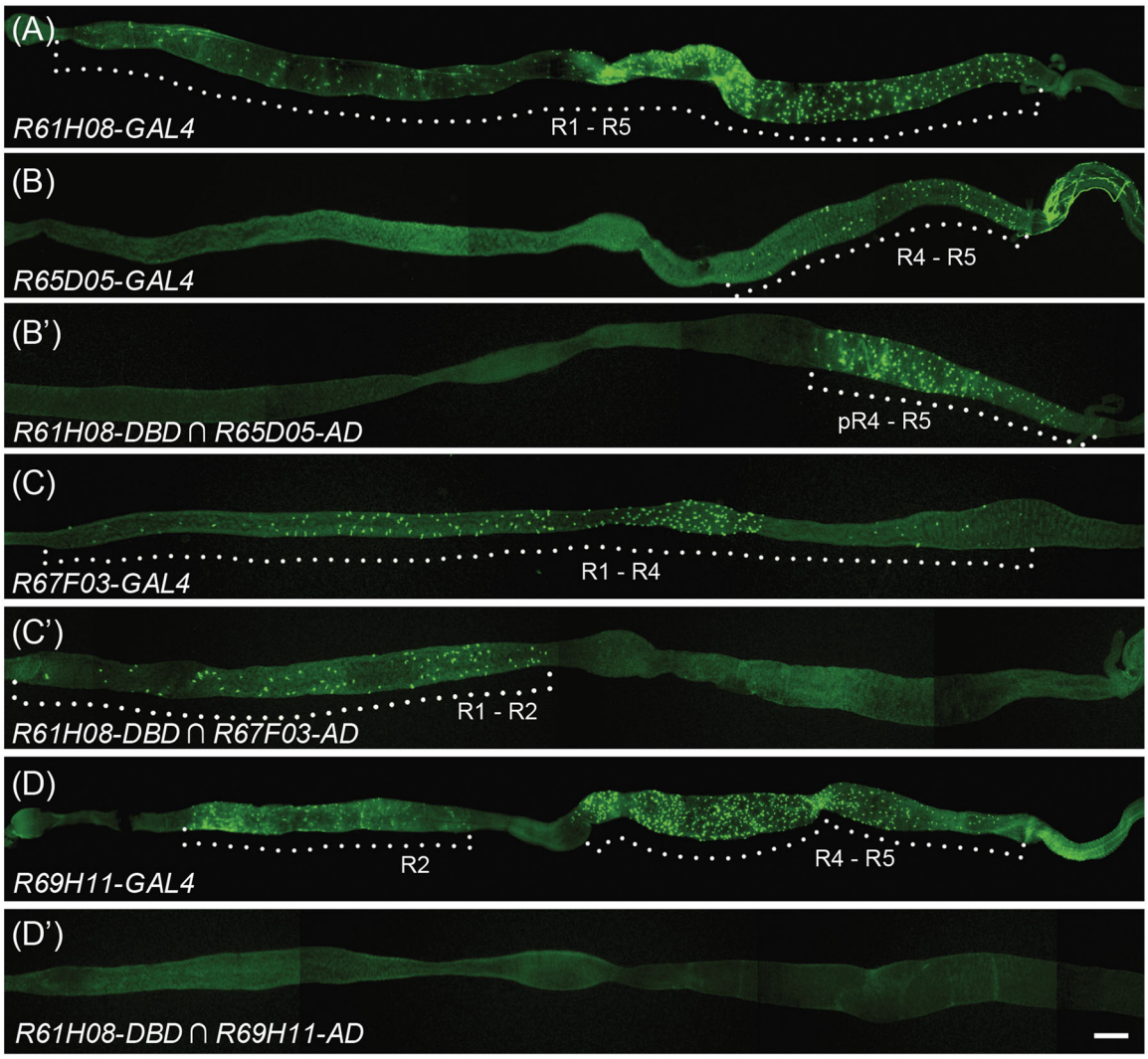

Figure 7. Split-GAL4 drivers label intersecting EEs. (A–D) Epi-fluorescent images of the whole midgut expressing native GFP under the control of denoted GAL4 drivers. (B′-D′) Illustrations of GFP reporter expression by *R61H08-DBD* when combined with *R65D05-AD* (B′), *R67F03-AD* (C′), or *R69H11-AD* (D′), respectively. Dotted lines indicate the regions of GFP expression. Scale bar: 300 μm (A–D′).

infiltrate the sg region and then project to the other foregut regions (i.e. crop), but many of them had only a small number of the sg neuronal cell bodies labeled. Hence, we speculate that a part of neuronal processes seemingly generated by the sg-derived ENs were indeed originated from the brain. We found a contrasting case, however; *R31B08* was expressed in the crop-innervating neuronal processes that were emanated exclusively from the sg-derived ENs.

Unlike the foregut that is innervated by the sg-derived ENs, the source of enteric innervation to the hindgut is the central nervous system. For example, *dilp7-GAL4-*, *pdf-GAL4-*, *HGN-GAL4-* or *ppk1-GAL4*-positive neurons extending from abdominal ganglia arborize concomitantly in the midgut-hindgut junction and the rectum (Cognigni *et al.*, 2011; Miguel-Aliaga *et al.*, 2008; Olds & Xu, 2014; Talsma *et al.*, 2012). Although many GAL4 lines labeled the midgut-hindgut junction, it was difficult to distinguish them from another based on their innervation patterns. Unlike the midgut–hindgut junction, the rectum exhibited more readily recognizable enteric innervation patterns for different GAL4 lines. Thus, we were able to group the hindgut-positive GAL4 lines based on their innervation patterns.

A previous study examining the midgut expression among a subset of Flylight GAL4 lines revealed the existence of progenitor GAL4 lines (Marianes & Spradling, 2013). In this work, 41 identified new progenitor GAL4 lines did not overlap with the previously identified GAL4 drivers. Notably, these progenitor drivers described in both studies exhibited diverse expression patterns among various types of cells (i.e. progenitor cells only, progenitors and ECs, or progenitors and EEs), as well as in specific regions of the midgut (R1–R5), which suggest that progenitor cells found throughout the midgut epithelium with little morphological differences might be more heterogeneous than they appeared to be. For example, *R77G01* was expressed in progenitors and a portion of the EC population, whereas *R29F11* was expressed in progenitors and a portion of EE population. One hypothesis that has been suggested for this phenomenon is that a group of progenitors are biased toward producing a certain type of differentiated cells. This is in

accordance with the finding that progenitors for ECs are distinct from those for EEs (Zeng & Hou, 2015). Furthermore, among the progenitor-specific GAL4 lines, *R59F09* was expressed throughout the midgut, whereas *R28A12* was expressed only in the R2 and R4–5 regions. This suggests that progenitors in the R1 and R3 regions are distinct from those in the R2 and R4–5 regions. This is supported by the observation that ISCs in the R3 region produce copper cells during the morphogenesis of the adult midgut, which mediate distinct functions (Driver & Ohlstein, 2014). The heterogeneity of progenitors in the R1 region versus to other regions remains to be understood, however. Considering the importance of research into gut progenitors and the limited number of progenitor drivers currently available (Micchelli & Perrimon, 2006; Zeng, Chauhan, & Hou, 2010), the novel progenitor GAL4 drivers identified in this study that are specific for region and cell type would be useful for future investigations.

ECs in the gut are known to process the digestion and absorption of various nutrients in *Drosophila* and other animals, particularly macronutrients such as carbohydrate, protein and lipid, and possibly key micronutrients (Miguel-Aliaga *et al*., 2018). ECs are located throughout the midgut and have been observed in the hindgut. It appears, however, that the characteristics of ECs vary with their locations in the gut. The *Drosophila* midgut has been subdivided and categorized by radius (Buchon *et al*., 2013). Soon after these subdivisions were established, Marianes and Spradling speculated that distinct types of ECs exist and mediate different functions (Marianes & Spradling, 2013). Indeed, activation of the insulin-responsive transcription factor Foxo in ECs in the posterior midgut was shown to cause the inhibition of lipase expression in response to dietary changes (Zhao & Karpac, 2020). Recently, Miguel-Aliaga and colleagues demonstrated the expression of a zinc-gated chloride channel in the R3 region of the midgut that acts as a zinc sensor (Redhai *et al*., 2020). Antimicrobial peptides (AMPs) were shown to be expressed in the anterior midgut of *Drosophila* (Tzou *et al*., 2000). Hence, it is reasonable to speculate that many more EC subtypes exist in the midgut that mediate distinct functions in *Drosophila*. The fact that we were able to identify more GAL4 lines with specific patterns of expression in ECs that differ from those in ISCs may support the previous hypothesis that there are more subtypes of ECs that promote distinct functions. The *Drosophila* gut GAL4 lines described in this work would provide a valuable resource for refining investigations of the functions of ECs.

The EEs of the adult midgut can be divided into two subpopulations based on peptide expression with one group expressing allatostatin A, allatostatin C, and orcokinin, and the other expressing tachykinin, neuropeptide F, and diuretic hormone 31 (Chen, Kim, & Kwon, 2016; Veenstra & Ida, 2014). All EE-specific GAL4 lines were expressed in subsets of Prospero-positive cells; none of these lines were expressed in all of the Prospero-positive cells in the gut. This may suggest that EEs can be divided into functionally distinct subpopulations. It is possible that each subpopulation of EE cells acquires a distinct gene expression profile when its fate is decided during the transition from ISCs to EEs. According to single-cell RNA-sequencing data, most EEs in the midgut of adult flies express two to five different types of peptide hormones, and that they can be divided into more than 10 subtypes depending on the combinations of peptide hormones that are present (Guo *et al*., 2019; Hung *et al*., 2020). We were not able to identify GAL4 lines that represent small subsets of EEs; however, we were able to show the potential of intersectional analysis using the split-GAL4 system (Beehler-Evans & Micchelli, 2015; Chen *et al*., 2016). By expanding the split-GAL4 combinations, we should be able to define functionally distinct populations of EEs throughout the entire midgut. These transgenic tools should be useful in such functional analyses in the future. A similar approach should also be informative for other cell types such as ENs or other midgut epithelial cells.

Conclusions

The expression of GAL4 lines in the gastrointestinal tract of adult flies can be classified by cell type and region. The database derived from our study, which is available online (http://kdrc.kr/index.php), may serve as a resource for studies involving the genetic manipulation of specific groups of cells along the gastrointestinal tract. It can be also used to search for the expression of GAL4 lines in any gastrointestinal cells of interest. The split GAL4 system may also be used to target more specific populations of cells in the gut.

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