CHAPTER

Identification of progenitor cells and their progenies in adult *Drosophila* midgut

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Zhen Jin[a], Minhua Che[a,b], and Rongwen Xi[a,c,*]

[a]*National Institute of Biological Sciences, Beijing, China*

[b]*School of Life Science, Peking University, Beijing, China*

[c]*Tsinghua Institute of Multidisciplinary Biomedical Research, Tsinghua University, Beijing, China*

**Corresponding author: e-mail address: xirongwen@nibs.ac.cn*

Chapter outline

Abstract

The intestinal epithelium in the anterior and posterior of the *Drosophila* midgut, which is maintained by intestinal stem cells (ISCs), represents a genetic tractable system for the study of stem cell biology, epithelial homeostasis and intestinal physiology and function. The ISCs self-renew and periodically generate absorptive enterocyte (EC) and secretory enteroendocrine cell (EE) via a committed progenitor stage termed as enteroblast (EB) or enteroendocrine progenitor (EEP), respectively. The progenitors in adult midgut are commonly referred to as

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all of the undifferentiated cells, including ISCs, EBs and EEPs. Under normal conditions, each of the above-mentioned specific type of cells can be reliably identified by a single cell marker or a combination of several cell markers. However, in aged or stressed gut, the increased proliferation and differentiation of ISCs may render many cell markers to be no longer strictly-specific to certain cell types. The self-renewal and differentiation abilities of ISCs or a particular cell of interest can be determined by cell lineage tracing analyses. Here, we provide detailed methods for the identification of ISC, EB and EEP in adult *Drosophila* gut, as well as methods for tracing the progenies of ISCs.

1 Introduction

Intestinal stem cells (ISCs) in adult *Drosophila* midgut were initially identified in 2006, which can be specifically marked by Delta (Dl), a Notch (N) ligand (Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006). When an ISC divides, it self-renews and generates a committed progenitor named enteroblast (EB), which then differentiates into either an enterocyte (EC) (about 90% of chance) or an enteroendocrine cell (EE) (about 10% of chance), depending on the level of Notch activation it receives from ISCs (Ohlstein & Spradling, 2007). Later studies with cell lineage tracing experiments reveal that these Notch-activated EBs invariably differentiate into ECs, but not EEs (Korzelius et al., 2019; Wang, Guo, & Xi, 2014; Zeng & Hou, 2015), and EEs are derived from ISCs via distinct progenitor cells, named as enteroendocrine progenitor cells (EEPs) (Biteau & Jasper, 2014; Chen et al., 2018a; Guo, Lv, & Xi, 2021; Hung et al., 2021; Zeng & Hou, 2015). Moreover, ISC was initially considered the only cell type in the intestinal epithelium that is capable of cell division. However, later studies reveal that EEP is also capable of cell division before terminal differentiation (Chen et al., 2018a; Zeng & Hou, 2015). The generation of EEP is initiated by a pulsed activation of the transcription factor Scute (Sc), which induces asymmetric ISC division, yielding a new ISC and an EEP. The EEP then divides once before differentiation, yielding a pair of EEs (Chen et al., 2018a) (Fig. 1). Interestingly, the two EEs belong to distinct EE subtypes and express different neuropeptides, indicating that the division of EEP is asymmetric (Beehler-Evans & Micchelli, 2015; Chen et al., 2018a; Guo et al., 2019; Ohlstein & Spradling, 2006). As EC and EE are derived from two distinct committed progenitor stages, EB is now commonly referred to as the EC-committed progenitor, whereas EEP as the EE-committed progenitor (Fig. 1). However, those normally-lineage-committed progenitors could change their cellular plasticity and adopt a different cell fate following genetic alternations or under certain stress conditions (Antonello et al., 2015; Korzelius et al., 2019; Reiff et al., 2019; Tauc et al., 2021; Wang et al., 2015).

The progenitors in adult midgut are commonly referred to as all of the undifferentiated cells, which include ISCs, EBs and EEPs. At present, various studies have characterized many cellular markers that can help to distinguish progenitor cells from differentiated epithelial cells during normal intestinal homeostasis in

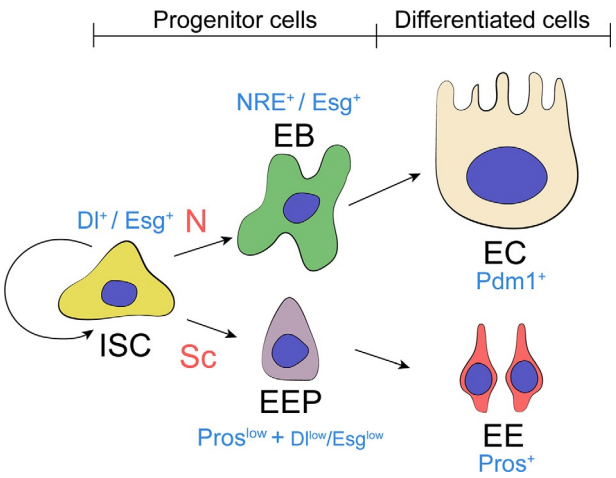

FIG. 1

The ISC lineage and cell type-specific markers. An ISC divides to renew itself and generate a daughter cell, EB or EEP, driven by *Notch* or *Scute* activation, respectively. EBs are immediately post-mitotic and are committed to differentiate into ECs. Each EEP usually divides once before terminal differentiation, yielding a pair of EEs. The progenitor cells are referred to as all the undifferentiated cells that include ISCs, EBs and EEPs. Under normal conditions, ISCs can be specifically marked by Dl, the Notch ligand; EBs by the NRE reporters; EEPs by a combined weak expression of Pros and Dl/Esg; EEs by Pros; and ECs by Pdm1 expression and by their large polyploid nuclei.

Drosophila. Many transgenic reporter lines and antibodies are available and widely used for *in vivo* visualization of progenitor cells. A Snail/Slug family transcription factor Escargot (Esg) is commonly used as a progenitor-specific marker, which marks all the undifferentiated cells, including ISCs, EBs, and EEPs (Fig. 1). Several enhancer trap lines such as *esg-Gal4* and *esg-lacZ* or a GFP trap line *esg-GFP* can be used to reflect Esg expression, but antibodies against Esg is still not available at the moment (Guo, Driver, & Ohlstein, 2013; Korzelius et al., 2014; Loza-Coll et al., 2014; Miao & Hayashi, 2016; Micchelli & Perrimon, 2006; Zeng, Chauhan, & Hou, 2010). Additional progenitor cell-specific transcription factors, such Sox21a, Sox100B, jumu, apt, Fkh and Zfh2, were identified in part through cell type specific and/or single cell transcriptome profiling (Amcheslavsky et al., 2014; Doupe et al., 2018; Hung et al., 2020). Transgenic reporters, such as *Sox21a-GFP* (fosmid vector), *Sox100B-GFP* (Bac vector), and GFP-tagged knock-in lines of jumu and apt, and *Zfh2-Gal4* enhancer trap all show progenitor specific expression patterns (Doupe et al., 2018). Antibodies including *anti-Sox21a*, *anti-Sox100B*, *anti-Zfh2*, *anti-Fkh* as well as enhancer reporter lines *Sox100B-lacZ*, *Sox21a-lacZ* (GMR43E09) and *Sox21a-Gal4(GMR43E09)* are also helpful for labeling of progenitor cells (Chen et al., 2016; Jin et al., 2020; Meng & Biteau, 2015; Zhai et al., 2015). Note that Jumu

or Fkh is also weakly expressed in EEs, Sox21a is expressed in both ISCs and EBs but with a much higher expression levels in EBs that are primed for differentiation (Chen et al., 2016), and Sox100B or Sox21a can also be detected in early ECs especially under stress conditions (Chen et al., 2016; Doupe et al., 2018; Jin et al., 2020; Lan et al., 2018; Zhai et al., 2015). Additional enhancer trap Gal4 lines include *Smv4-Gal4* and *Oatp58Dc-Gal4* also drive gene expression specifically in the progenitor cells in adult midgut (Dutta et al., 2015). As cell division occurs only in the progenitor cell population, it is not surprising that cell cycle related markers, such as *Polo-GFP* and *Cdc2* (by *anti-Cdc2* antibody staining) are specifically expressed in intestinal progenitor cells (Amcheslavsky et al., 2014).

As for individual progenitor cell types, Dl is the most commonly used marker for ISCs (Ohlstein & Spradling, 2007; Zeng et al., 2010). *Dl-Gal4* enhancer trap line, *Dl-lacZ* enhancer trap line and *anti-Dl* antibody are available for labeling ISCs and/or for genetic manipulation in ISCs (Ohlstein & Spradling, 2007; Zeng et al., 2010). In addition to Dl, Sanpodp (Spdo) by *anti-Spdo* staining can also be served as an ISC-specific marker (Perdigoto, Schweisguth, & Bardin, 2011), a *Mira-promoter-GFP* transgenic reporter is also found to be specifically expressed in ISCs (Bardin et al., 2010), and *CG10006-Gal4*, an enhancer trap line, is also considered as an ISC-specific GAL4 driver (Dutta et al., 2015).

EBs, whose Notch is activated, can be marked by a Notch activation reporter *Su(H)GBE-lacZ* (NRE(Notch response element)-lacZ for short) (Fig. 2) (Micchelli & Perrimon, 2006; Zeng et al., 2010). The transcription factor Klumpfuss (Klu) is

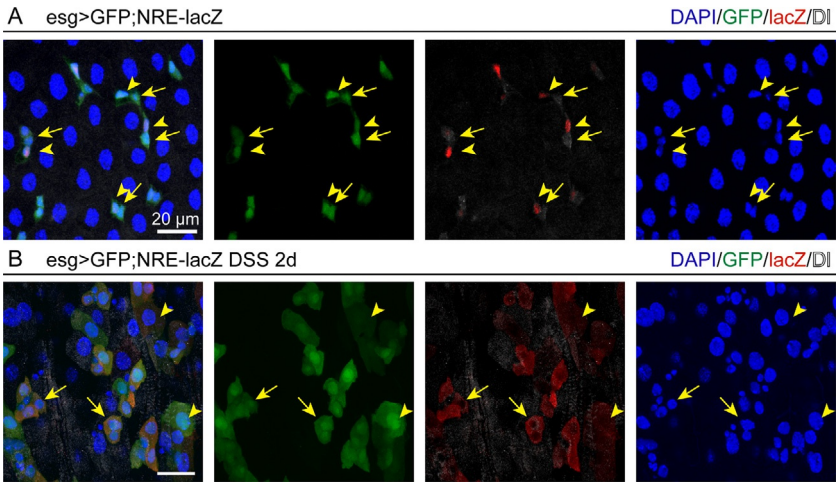

FIG. 2

Immunofluorescence staining of progenitor cell markers. (A and B) The posterior midgut epithelium from control (A) or DSS treated (B) adult females of *esg-GAL4,UAS-GFP;NRE-lacZ* stained with GFP (green) for progenitor cells, anti-Dl (white, arrows) for ISCs, anti-lacZ (red, arrowheads) for EBs. Note that ISCs and EBs are commonly juxtaposed to each other during tissue homeostasis, and the cell type specific markers, such as Dl and NRE-lacZ, become not strictly-specific in stressed epithelium. ECs are large polyploid cells. DAPI (blue).

also specifically expressed in EBs which mimic the expression pattern of *NRE-lacZ* and is required for EC commitment (Hung et al., 2020; Korzelius et al., 2019; Reiff et al., 2019). An enhancer trap line *Klu-Gal4* is available for labeling EBs (Korzelius et al., 2019; Reiff et al., 2019). *Toy-Gal4*, which specifically marks the diploid cells adjacent to ISCs, should also serve as an EB-specific Gal4 driver (Dutta et al., 2015). Two recent studies discussed apoptosis related genes in the regulation of EB cell fate decisions (Arthurton et al., 2020; Reiff et al., 2019). One of the studies described a *Dronc-GFP* transgenic reporter line that shows preferential expression in some but not all *NRE-lacZ* positive cells under normal conditions (Arthurton et al., 2020). Another study described a *Diap1-GFP 4.3* enhancer reporter line and a lacZ enhancer trap line of Diap1 (*Diap1-lacZ*) and both showed EB-specific expression patterns (Reiff et al., 2019).

ECs are large polyploid cells and can be distinguished by their polyploid nuclei (ranging from 8C to 64C under normal conditions) (Xiang et al., 2017) (Fig. 2). The transcription factor Pdm1 (by *anti-Pdm1* staining) is commonly used as an EC marker (Beebe, Lee, & Micchelli, 2010). A Gal4 enhancer trap line *MyoIA-Gal4* (also known as $Myo31DF^{NP0001}$) is a commonly used EC-specific Gal4 line (Jiang et al., 2009). The *Mex1-Gal4* enhancer trap line can also be used as an EC-specific Gal4 driver (Phillips & Thomas, 2006; Reiff et al., 2015).

EEPs are probably relatively hard to be observed in normal intestinal epithelium, as it is rare and always in a transient state. EEP can be indicated by a combined weak expression of Prospero (Pros, a commonly used marker for the committed EEs) and Dl (or Esg). As Sc is transiently activated from ISC to EEP which then committed to EE lineage, a Sc enhancer trap reporter *GMR14C12-Gal4* is found to be expressed in both EEP and the mother ISC that generating EEP (Chen et al., 2018a). Therefore, EEP can be identified by the combination of weak Pros expression and the expression of the *GMR14C12-Gal4* reporter (Chen et al., 2018a). However, *GMR14C12-Gal4* labeled cells are much fewer than Sc^{high} cells, and it is possible that *GMR14C12-Gal4* only marks a subset of EEPs in the midgut. An adaptor protein Phyllopod (Phyl) is a downstream target of Sc and is also transiently expressed in EEPs (Yin & Xi, 2018). However, it is also barely detectable in normal guts using either *anti-Phyl* antibody staining or a GFP-tagged transgenic line unless when Sc is over-expressed in ISCs (Yin & Xi, 2018). A recent study of single cell transcriptome identified an EEP cell cluster marked by Dl and Asense (Ase), and this population shows an age-dependent increase in its size, which is caused by the changes of Polycomb (Pc) activities (Tauc et al., 2021). However, the expression of Ase by antibody staining is undetectable in homeostatic guts and the enhancer trap line *Ase-Gal4* is weakly expressed in both ISCs and EBs (Zeng, Lin, & Hou, 2013).

EEs are heterozygous population of cells and have 10 major subtypes (Guo et al., 2019). The transcription factor Pros is expressed in all types of EEs in the midgut, and is therefore a pan EE marker (Fig. 3) (Guo et al., 2019; Micchelli & Perrimon, 2006; Ohlstein & Spradling, 2006). In addition to *prosV1-Gal4*, *CG32547-Gal4* and *CG5160-Gal4* are also EE-specific Gal4 drivers (Dutta et al., 2015; Guo et al., 2019).

It is worthwhile to note that, in conditions when the proliferation and differentiation processes of progenitor cells are accelerated, many progenitor-specific markers

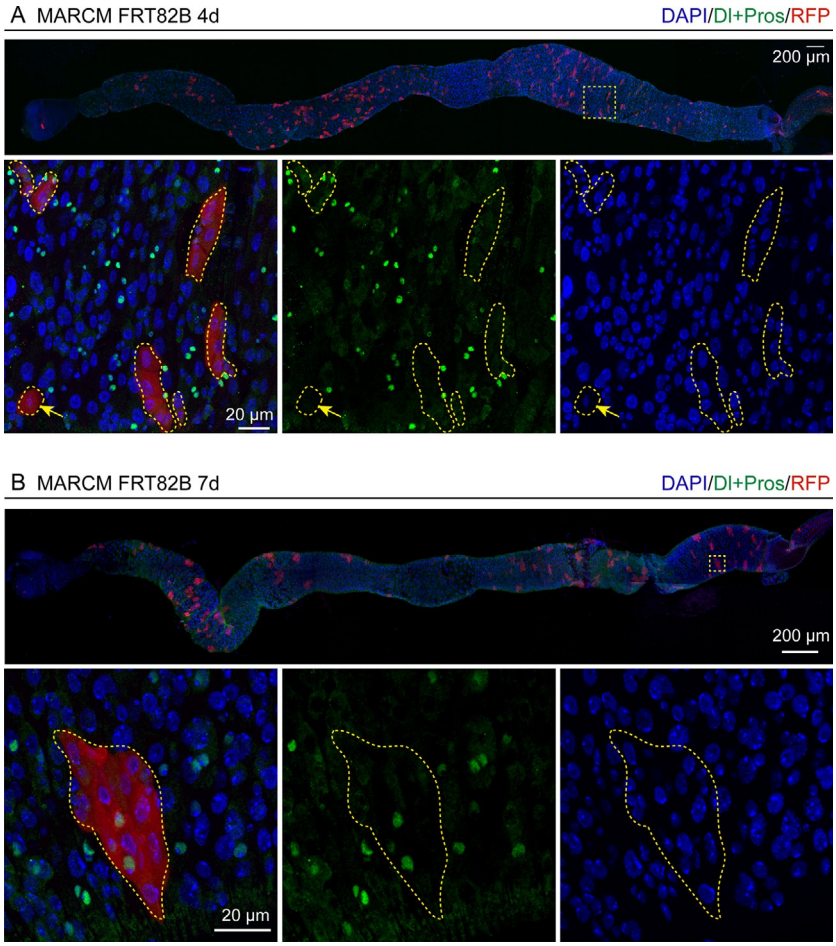

FIG. 3

Clonal analysis using MARCM system for ISCs and their progenies. (A and B) Guts with MARCM 82B RFP marked clones of 4 (A) and 7 days (B) are stained with DAPI (blue) for nuclei, anti-Dl (green, membrane) for ISCs, and anti-Pros (green, nucleus) for EEs. Dashed lines depict the clone margin. The ISC-sustained clones grow larger from day 4 to day 7 after clone induction (insets), and they usually contain multiple cells with all type of intestinal epithelial cells. In contrast, a transient non-stem cell clone usually marks a single differentiated cell (arrow).

may become unspecific, as these markers may not be timely downregulated but instead be carried over to differentiating or differentiated cells. For example, the expression of Esg can be carried into differentiating or newly-differentiated ECs or EEs when the proliferation and differentiation of ISCs accelerate, which commonly

occurs in stressed or aged guts (Fig. 2) (Chen et al., 2018a; Jiang et al., 2009; Jin et al., 2020; Siudeja et al., 2015; Yin & Xi, 2018). Another concern is that, EEs in the middle (R3) region of the midgut also express the Esg (Guo et al., 2019; Hung et al., 2020; Yin & Xi, 2018). Therefore, the progenitor cell markers, such as Esg, are quite conditional, and must be interpreted with caution.

In addition to those cellular markers used for the identification of progenitor cells, a few of genetic tools, such as the Flp-out or the MARCM (Mosaic Analysis with a Repressible Cell Marker) systems, can be used in the *Drosophila* midgut to assess the proliferation, maintenance and differentiation abilities (lineage tracing) of ISCs or a particular type of cells of interest (del Valle Rodriguez, Didiano, & Desplan, 2011; Harrison & Perrimon, 1993; Lee, 2014; Lee & Luo, 2001; Singh et al., 2012; Wu & Luo, 2006).

The FLP-out Gal4 system is built upon the Flippase (Flp)-Flp recognition target (FRT) system and the Gal4/UAS system. The FRT sites are arranged as direct repeats flunking a DNA sequence containing stop signal. After Flp-mediated site-specific recombination between cis-acting FRTs, the intervening DNA containing the stop signal is excised, and the promoter becomes free to drive Gal4 expression. As Flp is induced by heat shock using the hs-flp transgene, the Flp-out Gal4 system can randomly induce clones in any midgut cells, including ECs. The esg^{ts} FLP/Out system (esg^{ts} F/O) was therefore developed to induce FLP-out specifically in progenitor cells (Jiang et al., 2009). In this system, the Flp expression is induced specifically in progenitor cells using *esg-Gal4, UAS-flp*, and with the presence of Tub-$Gal80^{ts}$, the induction of flp can also be temporally controlled by ambient temperature. Similarly, the NRE^{ts} FLP/out system, in which the *esg-Gal4* is replaced by *NRE-Gal4*, can be used to specifically mark, lineage-trace and genetically manipulate EBs (Beehler-Evans & Micchelli, 2015; Wang et al., 2015).

The MARCM (Mosaic Analysis with a Repressible Cell Marker) system combines the Gal4/UAS expression system with the FLP-FRT-mediated mitotic recombination system to mark the daughters of the cells that enter mitosis. With the MARCM system, the sparse labeling of a particular cell allows the labeling and tracing of all its progenies, if any, over time (Fig. 3). As ISCs and their progenies usually show limited migration along the basement membrane, a labeled ISC usually gives rise to a continuous patch of clone over time in which both enterocyte and EEs can be found. In contrast, a labeled non-stem cell cannot give rise to a multiple-cell clone (Fig. 3).

There are additional genetic tools that may serve similar purposes. For examples: G-TRACE (Gal4 Technique for Real-time And Clonal Expression) combines the Gal4/UAS/Gal80 system with the Flp-out LacZ reporter (Duffy, 2002; Evans et al., 2009), with which a certain cell type can be lineage-traced based on cell type-specific driver, such as using the *Dl–Gal4* driver to trace the progeny of ISCs, *esg-Gal4* driver to lineage trace the progeny of the undifferentiated progenitor cells (Fig. 4), and *NRE-Gal4* to trace the progeny of EBs (Jiang et al., 2009; Wang et al., 2015); REDDM (Repressible Dual Differential stability cell Marker) combines Gal4-responsive transgenes encoding fluorescent proteins with short (mCD8-GFP)

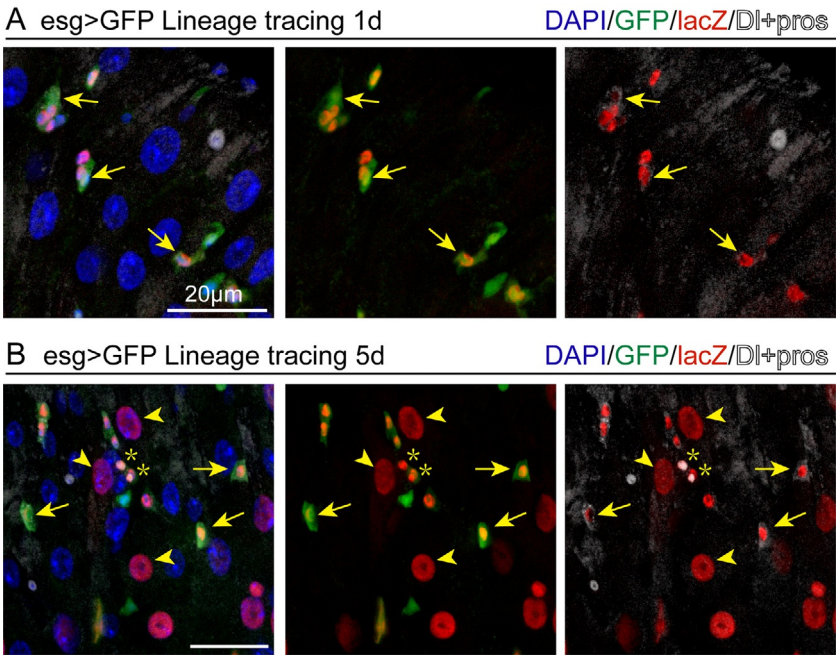

FIG. 4

Cell lineage tracing of intestinal progenitor cells. Adult female flies with genotype of *hs-flp; esg-GAL4, UAS-GFP; act[<stop<]lacZ,tub-Gal80^{ts}* were cultivated at 18 °C and shifted to 29 °C for 1 day (A) or 5 days (B) before dissection. This lineage tracing study demonstrates that the LacZ^{+} (red) large polyploid cells appeared at day 5 are derived from GFP^{+} (green) cells.

and long (H2B-GFP) half-lives and temperature-sensitive Gal80^{ts} that allows simultaneous quantification of precursor cell number and cell renewal for genetic analysis (Antonello et al., 2015); Flybow, which is adapted from the mouse Brainbow system, is a powerful multicolor labeling system. It is consisted of Gal4/UAS, modified inducible Flp-FRT and sequences encoding different membrane-tethered fluorescent proteins that arranged in pairs within cassettes flanked by recombination sites to drive inversions of cassettes for multicolor labeling in the neighboring cells (Antonello et al., 2015; Hadjieconomou et al., 2011).

PH3 (phospho-Histone H3) staining and BrdU (5-bromo-2′-deoxyuridine) incorporation can also be used to assess the proliferation status of intestinal progenitor cells (Chen et al., 2016; Jin et al., 2020; Micchelli & Perrimon, 2006). The former one marks cells at metaphase of mitosis (Fig. 5), and the latter one is indicative of occurrence of DNA synthesis. Be aware that BrdU is also easily incorporated into the developing ECs that undergo endoreplication (Xiang et al., 2017).

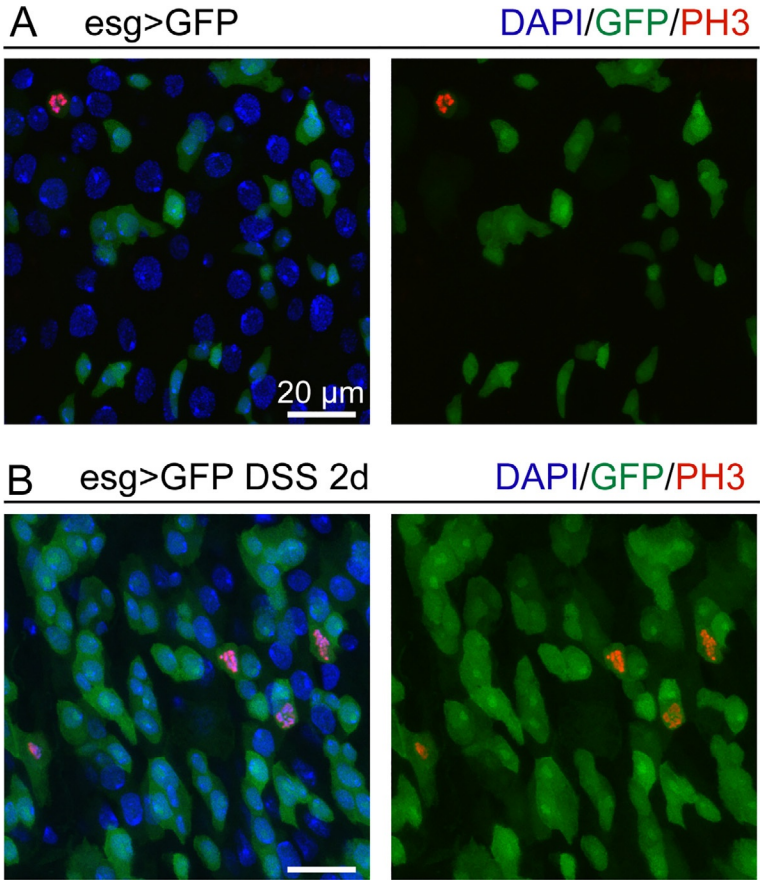

FIG. 5

PH3 staining to mark dividing cells in the midgut. (A and B) Adult female flies of *esg-Gal4, UAS-GFP* with (B) or without (A) DSS treatment. DSS-treatment gut shows increased cell proliferation reflected by increased number of GFP^+ (green) cells and $PH3^+$ (red) cells.

2 Materials

2.1 Common disposables (see Note 1)

- Standard fly cultivation vials and bottles (see *Note 2*)
- Dumont #5 Forceps
- Paintbrushes

2.2 Common reagents (see Note 1)

- Fly standard food (Cornmeal, agar, sucrose, glucose, yeast, and acid medium)
- Yeast paste

- Ringer's solution/Schneider's powdered medium/Grace's insect medium (See *Note 3*)
- 1 × PBS (Gibco, Cat#14190250) (See *Note 3*)
- Triton X-100 (Sigma, Cat# T9248)
- Formaldehyde
- n-heptane
- Methanol
- Normal Goat Serum (NGS, Cell Signaling Technology, Cat#5425)
- DAPI (Thermo Fisher, Cat#D1306)
- Dextran Sulfate Sodium (DSS, MP Biomedicals, Cat#160110) (See *Note 4*)

2.3 Common equipment (see Note 1)

- Fly cultivation cabinet (18, 25, 29 °C)
- Water bath kettle (37 °C)
- Standard CO_2 equipment for anesthetizing the flies (Leica MZ16)
- Stereomicroscope (Leica S6E)
- Confocal microscopes (Leica LP8-DLS/Nikon A1-R)

2.4 Common software

- Prism
- Adobe Photoshop
- ImageJ
- Adobe Illustrator
- Nikon Confocal Software/Leica Confocal Software

3 Methods

3.1 Fly cultivation, DSS treatment and gut dissection

- Flies are cultivated at 25 °C with standard food (Singh et al., 2012) in vials or bottles unless otherwise indicated (see Note 2, 5–7).
- For DSS treatment, 5–10 days old female flies are collected. Flies are cultured in an empty vial with chromatography paper (1 cm^2) added with 5% DSS in 5% sucrose solution (the control group contained only 5% sucrose solution).
- Adult flies are anesthetized by standard source of CO_2 and then put on pre-cold anatomy plate containing pre-cold 1 × PBS or insect medium (see *Note 3*).
- Using two dissection forceps to pull open the abdomen and expose the fly gut under the stereoscope.
- Carefully take out the midguts and put them into 1 × PBS for subsequent use (Table 1).

Table 1 A selected list of cell type-specific reporter lines.

Fly stocks	Marked cell types	Reference
esg-Gal4	Progenitor cells[a]	Goto and Hayashi (1999), Micchelli and Perrimon (2006)
esg-lacZ	Progenitor cells	Micchelli and Perrimon (2006)
esg-GFP	Progenitor cells	Guo et al. (2013)
Sox21a-GFP	Progenitor cells	Doupe et al. (2018)
Sox100B-GFP	Progenitor cells	Jin et al. (2020)
jumo-GFP	Progenitor cells	Doupe et al. (2018)
apt-GFP	Progenitor cells	Doupe et al. (2018)
Zfh2-Gal4	Progenitor cells	Doupe et al. (2018)
Sox100B-lacZ	Progenitor cells	Jin et al. (2020)
Sox21a-lacZ (GMR43E09)	Progenitor cells	Jin et al. (2020)
Sox21a-Gal4 (GMR43E09)	Progenitor cells	Chen et al. (2016)
Smv4-Gal4	Progenitor cells	Dutta et al. (2015)
Oatp58Dc-Gal4	Progenitor cells	Dutta et al. (2015)
Polo-GFP	Progenitor cells	Amcheslavsky et al. (2014)
Ase-Gal4	Progenitor cells	Tauc et al. (2021)
Dl-Gal4	ISC	Zeng et al. (2010)
Dl-lacZ	ISC	Ohlstein and Spradling (2007)
Mira-promoter-GFP	ISC	Bardin et al. (2010)
CG10006-Gal4	ISC	Dutta et al. (2015)
Su(H)GBE-lacZ (NRE-lacZ)	EB	Micchelli and Perrimon (2006)
Klu-Gal4	EB	Korzelius et al. (2019)
Dronc-GFP	EB	Arthurton et al. (2020)
Diap1-GFP 4.3	EB	Reiff et al. (2019)
Diap1-lacZ	EB	Reiff et al. (2019)
Toy-Gal4	EB	Dutta et al. (2015)
MyoIA-Gal4	EC	Jiang et al. (2009)
Mex-Gal4	EC	Reiff et al. (2015)
CG5160-Gal4	EE	Dutta et al. (2015)
CG32547-Gal4	EE	Guo et al. (2019)
ProsV1-Gal4	EE	Zielke et al. (2014)
GMR14C12-Gal4	EEP (Partial)	Chen et al. (2018a)
phyl 4.3n-GFP	EEP (conditional)	Yin and Xi (2018)

[a]*Progenitor cells are referred to as all the undifferentiated cells in the midgut epithelium.*

3.2 ISC/progenitor cell marker detection via immunofluorescence staining

- 10–15 guts are transferred into 1.5 mL tube containing 500 μL 1 × PBS (see *Note 8*).

- Add 4% formaldehyde and 500 μL n-heptane and then put the tubes on a shaker for a 30 min's fixation at room temperature (see *Note 9, 10*).
- Carefully remove the supernatant, add 500 μL n-heptane and 500 μL methanol. Vigorously inverting 30 s (see *Notes 10, 11*).
- Remove the supernatant and add 1 mL methanol, incubate on a shaker for 5 min at room temperature. Repeat once (see *Notes 10, 11*).
- Remove the supernatant and add 1 mL 1 × PBT (1 × PBS apply with 0.1% Triton X-100) to wash the guts, incubate on a shaker for 10 min at room temperature. Repeat twice (see *Notes 10–12*).
- Remove the supernatant, add 300 μL 1 × PBS and 15 μL NGS (Normal goat serum). Incubate on a shaker for 1 h at room temperature to block the guts (see *Note 13*).
- Add primary antibody in 5% NGS-PBT solution with suitable concentration. Incubate on a shaker overnight at 4 °C (see *Notes 14, 15*). Primary antibody used for identification of ISCs and other type of cells are listed below (Table 2):
- Remove the supernatant and add 1 mL 1 × PBT to wash the primary antibody, incubate on a shaker for 10 min at room temperature. Repeat twice.
- Add secondary antibody with suitable concentration. Incubate on a shaker for 2 h at room temperature.
- Remove the supernatant and add 1 mL 1 × PBT to wash the secondary antibody, incubate on a shaker for 10 min at room temperature. Repeat twice.

Table 2 Primary antibodies.

Target genes/reporters	Primary antibody	Marked cell types
Esg-lacZ, Su(H)GBE-lacZ (NRE) or other lacZ reporters	Anti-LacZ (RRID:AB_2334934)	LacZ labeled cells
Dl	Anti-Dl (RRID:AB_528194)	ISC
Cdc2	anti-Cdc2 (Santa Cruz Biotechnology sc-53)	Progenitor cells
Pros	Anti-Pros (RRID:AB_528440)	EE
Pdm1	Anti-Pdm1 (Xiaohang Yang, Zhejiang University, China)	EC
Sox100B	Anti-Sox100B (Jin et al., 2020)	Progenitor cells
Sox21a	Anti-Sox21a (Chen et al., 2016)	Progenitor cells
Zfh2	Anti-Zfh2 (Doupe et al., 2018)	Progenitor cells
Sanpodo	Anti-Sanpodo (Perdigoto et al., 2011)	ISC
Phyllopod	Anti-Phyl (Yin & Xi, 2018)	EEP
PH3	Anti-PH3 (RRID:AB_331535)	Dividing cells

- Add DAPI with suitable concentration. Incubate on a shaker for 5 min at room temperature.
- Remove the supernatant and add 1 mL 1 × PBT to wash the guts, incubate on a shaker for 10 min at room temperature.
- Add 50 μL 70% glycerol to mount the guts. Line guts on microslides and carefully put on coverslips. Store at 4 °C for several days or −20 °C for months (see *Notes 16, 17*).
- Detection of fluorescence signals using confocal microscopes (see *Notes 18–20*).

3.3 Functional analysis via clonal analysis: Flp-out and MARCM systems

- *Flp-out:* Genotype of *hs-flp; act < stop < Gal4,UAS-GFP* flies cross with targeted *UAS-X RNAi/overexpression* transgenic flies or *w1118* wild type flies as control group.
- *MARCM:* Genotype of *hsflp;Act-Gal4,UAS-RFP; FRT 82B Tub-Gal80* flies cross with targeted *UAS-X RNAi/overexpression-FRT 82B* transgenic flies or *FRT82B* wild type flies as control group (see *Note21*).
- Flies are cultivated at 25 °C with standard food.
- Collect 3–5-day-old F1 generation adult female flies of suitable genotypes into an empty vial (10–15 flies/vial) containing a piece of yeast paste.
- Put the vials into 37 °C water bath for 1 h heat-shock (see *Notes 21, 22*).
- Flies are subsequently transfer into standard food and cultivated at 25 °C for another 7 days (see *Notes 23, 24*).
- Flies are dissected and stained with targeted antibodies for subsequently detection for the phenotypes of the generated clones (see Method in Section 3.2 and *Notes 18–20, 25, 26*). Example results are shown in Fig. 3.

3.4 Functional analysis via lineage tracing based on G-TRACE system

- Genotype of *esg-Gal4;UAS-Flp,Tub-gal80^{ts}* flies cross with *UAS-X RNAi; Act [< stop <]lacZ,Tub-gal80^{ts}* flies or *Act[< stop <]lacZ,Tub-gal80^{ts}* flies as control groups. Flies are then cultivated at 18 °C for about 25 days.
- Collect 3–5-day-old F1 generation female flies of suitable genotypes into vials and transfer them to 29 °C for 7 days. Renew food every 2 days (Temperature sensitive Gal80 is able to inhibit Gal4 activation at 18 °C, thus inhibit flippase expression; at 29 °C, Gal80 is inactivated, and Gal4 then drives flippase expression to induce recombination).
- Flies are dissected and stained with the desired antibodies for subsequently detection and analysis of the lacZ^{+} cells (see Method in Section 3.2). Example results are shown in Fig. 4.

4 Assessment of proliferation and differentiation capability of ISCs

There are several common methods for the assessment of the proliferation capability of ISCs. In clonal analysis based on Flp-out, MARCM, or other systems, the labeled wild type ISCs can give rise to multiple-cell clones, usually 8–10 cells per clone after 7-days of clone induction. The labeled non-stem cells cannot form multiple-cell clones (see *Notes 21–26*).

Phosphorylation of histone 3 Ser10 and Ser28 occurs from late G2 phase to late anaphase or early telophase in cell cycle with a peak at metaphase (Jin et al., 2020; Kim et al., 2017), therefore anti-PH3 staining are commonly used for marking dividing cells. A wild type midgut normally contains less than 10 $PH3^+$ cells by average. Under stress conditions, however, each midgut can have tens or hundreds of $PH3^+$ cells (Fig. 5). BrdU is a synthetic analog of thymidine, which can incorporate into newly synthesized DNA instead of thymidine (Ghosh, Mandal, & Mandal, 2018). Therefore, BrdU labeling is also frequently used to detect mitotic cells which need to synthesize DNA at S phase in cell cycle (Ghosh et al., 2018; Singh et al., 2012). Note that BrdU can also be easily incorporated into cells that undergo endoreplication, such as ECs.

As for the differentiation capability, the clonal analysis can demonstrate whether the initially labeled cell is capable to differentiate into enterocyte or EE. Similarly, combined with cell marker analysis, the cell lineage tracing systems can also help to determine whether certain types of cells can be derived from the initially labeled cells.

5 Concluding remarks

There are molecular and genetic tools to either generally mark all undifferentiated cells in the *Drosophila* midgut, or specially mark ISCs, EBs or EEPs. Genetic and molecular tools are also available to assess the self-renewal, proliferation and differentiation abilities of a particular cell or cell type. However, in aged or diseased gut, many cell type specific markers become not strictly specific, and in those cases, a combination of cell marker study and functional analysis becomes important to determine the self-renewal and differentiation abilities of the cells of interest.

6 Notes

1. Catalog number and provider indicated here are as references, any equivalent products suit the experiments are acceptable. The same to those materials without a catalog number and provider, unless otherwise specified.
2. Better to cultivate no more than 20 flies per vial (24×92 mm)-40–80 flies per bottle (50×120 mm) .
3. $1\times$ PBS can be used in the place of Ringer's solution/Schneider's powdered medium/Grace's insect medium.

4 DSS induced gut cell proliferation varied in efficiency when using different DSS products of different molecular weights and/or different working concentrations. DSS with molecular weight as 36,000–50,000 Da (such as from MP Biomedicals) is recommended and the recommended working concentration is 5%. The efficiency for the induction of gut cell proliferation can be up to about 80%.
5 F1 generation flies are collected and cultivated in vials. Make sure to transfer into new vials every 2 days to maintain food freshness and avoid bacteria overload.
6 Yeast paste provides important source for amino acids and can help to keep healthy intestinal conditions.
7 The age of flies or the mating history could impact the experimental results. For experimental consistency, it is advised to use females of similar age and cultivate them with the presence of male flies.
8 During the staining step, too much dissected guts in a single 1.5 mL centrifuge tube will cause tissue intertwine and compromise stain quality.
9 There are several midgut fixation methods. In addition to the methanol method described here, the methanol free method should also be considered if the epitope could be potentially damaged by methanol. In this case, the dissected gut can be fixed in 4% formaldehyde in PBS over night at 4 ℃. The heat fixation method could also be used, especially for the staining of membrane proteins. In this case, samples are fixed in hot 1 × TSS buffer (0.03% Triton X-100, 4 g/L NaCL;95%) for 3 s before transferred to ice-cold 1 × TSS buffer for at least 1 min. Details can be found elsewhere (Chen et al., 2018b; Müller, 2008).
10 Take formaldehyde, n-heptane and methanol at a chemical hood and wear gloves to avoid harm to skin and eyes.
11 Sometimes methanol dehydration can destroy the epitope of certain proteins. If this is the case, the steps with methanol can be skipped.
12 Formaldehyde, n-heptane and methanol residues will lower the efficiency of antibody staining. For better results, wash additional times of these solvent by 1xPBT.
13 Blocking time can be extended to 4 °C overnight for a better reduction of background staining.
14 In many cases, primary antibody incubation can be performed at room temperature for 2 h without obvious effect on staining quality.
15 Primary antibodies can be recycled and reused several times with the same staining quality. For those primary antibodies that have high background staining, pre-absorption using the mutant guts may help to reduce background staining.
16 Low concentration of glycerol cannot protect well of the signals during storage, better to use a concentration higher than 70% or use alternative mounting medium.
17 Slices can be stored at 4 °C for weeks and −20 °C for months and even several years. However, to get better signal quality, acquire images as soon as possible.

18 When using confocal microscopy for image acquisition, 40 × lens is commonly used to detect signals of each cell type in intestinal epithelium.
19 Food content in the gut lumen can sometimes give rise noisy signals. Washing multiple times after fixation can help reduce the luminal content.
20 Non-specific background staining commonly occurs in muscle cells, and ISCs reside at the basement membrane in close proximity to the muscle layers. To get better images, carefully distinguish ISC signals and avoid involvement of muscle layers.
21 Heat-shock in 37 °C water bath sometimes can cause intestinal stress, resulting in increased cell proliferation along the whole gut. Shorten the heat-shock time can reduce this stress effect. Other alternative cell lineage tools, such as G-TRACE and REDDM, can be considered, as well as some heat shock-free genetic tools, such as RU486-inducible GAL4 systems (P[Switch]) (Roman et al., 2001).
22 Repeat one more time of heat-shock after a short interval (half day or the next day) may help increase the induction efficiency of clones.
23 Time course assay are generally used for clonal analysis, that is, examine different time intervals after clone induction (3d, 7d, 14d, and 21d or longer for aging assays, depending on experimental requirements).
24 The Flp-out system sometimes yields leaky clonal signals in the anterior and posterior region of guts, those leaky clones usually do not have clear clonal margins.
25 For cell lineage studies with the Flp-out system, introduction of multiple tub-Gal80^{ts} transgenes can minimize the incidence of leaking clones.
26 For cell lineage studies, be sure that clones are sparsely induced to minimize joint clones.
27 A typical wild type ISC-derived clone of 7 days old contains all type of intestinal epithelial cells. However, not all the induced clones are typical ones. To distinguish ISC-derived clones from transient clones, stain the ISC marker and examine whether the clone contains one or more ISCs.

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