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Generation and Staining of Intestinal Stem Cell Lineage in Adult Midgut

Shree Ram Singh¹, Manoj K. Mishra², Madhuri Kango-Singh³, Steven X. Hou¹

¹Mouse Cancer Genetics Program, National Cancer Institute, Frederick, USA

²Department of Math and Science, Alabama State University, Montgomery, USA

³Department of Biology, University of Dayton, Dayton, USA

Abstract

Stem cell-mediated tissue repair is a promising approach in regenerative medicine. Intestinal epithelium is the most rapidly self-renewing tissue in adult mammals. Recently, using lineage tracing and molecular marker labeling, intestinal stem cells (ISCs) have been identified in *Drosophila* adult midgut. ISCs reside at the basement membrane and are multipotent as they produce both enterocytes and enteroendocrine cells. The adult *Drosophila* midgut provides an excellent in vivo model organ to study ISC behavior during aging, stress, regeneration, and infection. It has been demonstrated that Notch, Janus kinase/signal transducer and activator of transcription, epidermal growth factor receptor/mitogen-activated protein kinase, Hippo, and wingless signaling pathways regulate ISCs proliferation and differentiation. There are plenty of genetic tools and markers developed in recent years in *Drosophila* stem cell studies. These tools and markers are essential in the precise identification of stem cells as well as manipulation of genes in stem cell regulation. Here, we describe the details of genetic tools, markers, and immunolabeling techniques used in identification and characterization of adult midgut stem cells in *Drosophila*.

Keywords

Drosophila; Adult midgut; Intestinal stem cell; Genetic techniques; Immunolabeling

1. Introduction

Stem cells have been identified in many adult tissues. These unspecialized cells are involved in the maintenance of tissue homeostasis throughout the life of an organism by their ability to self-renew and continuously produce differentiated cells to replenish aged or damaged cells. Stem cells are regulated by both intrinsic and extrinsic (niche) factors. The abnormal balance between stem cell proliferation and differentiation results in organ degeneration, premature aging, and cancer (1–7). Elucidating the mechanisms, which regulate this balance, is critical in adult stem cell biology and regenerative medicine.

The intestinal epithelium is the most vigorously self-renewing tissue in adult mammals. Homeostasis in mammalian intestine epithelium is mediated by intestinal stem cells (ISCs), which are located near the base of each crypt (3, 8). It takes only 3–5 days for ISCs at the bottom of the crypt to proliferate and differentiate toward the lumen to become absorptive and secretory cells types and can replenish cell loss from villi. ISCs are intermixed with paneth cells at the bottom of crypt and shown to contain an overlapping population of Lgr5+ and Bmi1+ cells (3, 8). There are several signaling pathways known to regulate ISCs proliferation (3, 8). However, how ISCs and its progenitor cells respond to pathogens and mediate intestinal regeneration needs further investigation (3, 8–10).

Drosophila has been a very attractive model organism to study stem cell biology. Similar to mammalian intestine, the *Drosophila* adult midgut also see the rapid turnover by presence of approximately 1,000 ISCs (11–13). The identification of ISC in the adult *Drosophila* midgut makes it a very useful model organ system to study ISC physiology during aging, stress, regeneration, and infection (14–30). *Drosophila* ISCs are distributed evenly throughout the gut and localized basically to mature enterocytes (Fig. 1a–c). ISCs upon division produce two daughter cells, with one retaining ISC properties and the other becomes an immature daughter cell, enteroblasts (EBs). ISCs are characterized by expression of high levels of Delta, a ligand for the Notch receptor, which triggers Notch signaling in neighboring EBs (13). Escargot (esg), a transcription factor, is expressed in ISCs and EBs. *Su(H)GBE-lacZ*, a transcriptional reporter of Notch signaling, has been used as EBs cells marker (11–13). The EBs do not divide further and directly differentiate into absorptive enterocyte (ECs) (90%) and the secretory enteroendocrine (ee) cells (10%) (Fig. 1d). The two differentiated cell types, EC and ee cells, are more apically localized toward the lumen. The ee cells express the homeodomain transcription factor Prospero (Pros) in the nucleus. The mature ECs can be unambiguously distinguished from other cell types by their polyploid nuclei and large cell bodies as well as by expression of PDM1 (25, 31, 32). The young ECs express ferritin-1 heavy chain homologue (Fer1HCH) (14). As in mammalian intestinal epithelium, the ECs and ee cells are continually migrated from the basal location toward the gut lumen to replace the lost cells on the surface of the epithelium. It has been demonstrated that all the epithelial cells in the adult midgut are generated by adult midgut progenitors (AMPs) from the larval gut (27). Further, it is shown that signaling through the epidermal growth factor receptor (EGFR)/RAS/mitogen-activated protein kinase (MAPK) pathway is necessary and limiting for AMP proliferation (27). AMPs divide asymmetrically, and Notch pathway directs its first daughter to become a PC, which acts as a niche (33). The AMP and its daughters can remain undifferentiated in response to a Dpp signal from the PC and proliferate to form AMP islands (33).

Several signaling pathways and genes are known to regulate the ISCs behavior in *Drosophila* midgut (5, 11–46). Notch is known to promote the gut homeostasis by initiating ISC differentiation and specifying the fates of EBs (13). It has been shown that active Notch inhibits the proliferation of ISC and blocks its turnover (12, 13). Loss of Notch signaling resulted in large, tumorous mosaic clones that contain both Pros+ ee-like cells as well as D1+ ISCs, which suggests that in normal condition, Notch signaling blocks ISC proliferation by inhibiting EBs to ee cells differentiation. It has been demonstrated that E-cad is required for interaction between ISC and EB, and adhesion with ECs (34). E-cad is necessary for Notch

(30, 32, 44, 45). Loss of Hpo signaling or overactivation Yorki (YKi) in midgut induces regenerative response by producing cytokines of the Upd family and multiple EGFR ligands (Spitz, Keren, and Vein), which activate JAK-STAT and EGFR signaling pathways in ISCs to stimulate their proliferation (20, 25, 32, 44). Further, it has been shown that Hpo target, Yki, plays a critical and cell-autonomous role in ISCs, whose activity is suppressed by the upstream Hpo pathway members, Fat and Dachshous (Ds) (44). Moreover, Warts and Yorkie mediate a transition from low- to high-level ISC proliferation to facilitate gut regeneration (45).

Jiang et al. (28) reported that ISC proliferation induced by JAK-STAT signaling is dependent upon EGFR signaling. Their results suggest that EGFR/Ras/MAPK signaling pathway plays a central role in intestinal homeostasis. Xu et al. (5) recently reported that EGFR together with Wg and JAK-STAT signaling regulates ISC maintenance and division. They found that simultaneous disruption of all three signaling leads to rapid and complete ISC elimination, and visceral muscle is a critical component of the ISC niche. Biteau and Jasper (46) have shown that EGF receptor signaling is required to maintain the ISCs proliferation as its ligand Vein is expressed in the visceral muscle, which provides a permissive signal for ISC proliferation. They further found that AP-1 transcription factor FOS acts as a mediator for EGF and JNK to promote ISCs proliferation in response to stress. They emphasize that visceral muscle serves as a functional “niche” for ISCs (46). Amcheslavsky et al. (4) demonstrated that tuberous sclerosis complex (TSC) plays a critical role in balancing ISC growth and division as loss of TSC in the results in large ISCs formation, which show defect in cell cycle. Stem cells have also been reported to present in the other regions of the *Drosophila* gut and are regulated by some of the similar pathways reported in *Drosophila* posterior midgut (47–51).

Drosophila serves as an excellent genetic model system to study adult stem cells. There are several sophisticated techniques that have been used in lineage analysis to identify stem cells as well as to generate mosaic mutant cells in living flies. Most of the techniques used to identify the adult stem cells are based on mitotic recombination. Further, function of specific genes in the stem cells can be manipulated by using the genetic tools such as flipase/flipase recombination target (FLP/FRT) system-based tubulin-lacZ positive-labeling system, (FLP/FRT) and GAL4/UAS system-based positively marked mosaic lineage (PMML), and GAL80-based mosaic analysis with a repressible cell marker (MARCM) (Figs. 2–4). The lineage tracing is the key in the stem cell identification. It has been suggested that most dividing cells marked in the lineage tracing are not stem cells (52). In the marked clones, non-stem cells can generate transient clones, which are short-lived and produce only differentiated cells. However, stem cells clones grow larger, maintain longer, and contain undifferentiated cells, trans-amplifying cells, and terminally differentiated cells (52). Following the stem cell lineage over time is critical in understanding the stem cell potency, replacement, and aging as well as helpful in characterization of the stem cell niches (52). Clonal analysis of stem cells, molecular markers, available phenotypes, sophisticated genetic tools, and immunohistological-staining methods, together with functional genomics and proteomics approach, will be helpful in identifying the genes, and signaling pathways regulate ISCs behavior in adult midgut (53–56). In this chapter, we have presented some of the standard techniques used in the identification and characterization of ISCs in adult

Drosophila. The details of the molecular markers and transgenes expressed in the different cell types in midgut are presented in Tables 1 and 2, respectively.

2. Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature (unless otherwise indicated).

2.1. *Drosophila* Culture

1. Control and transgenic flies.
2. Fly culture incubator (18, 25, and 29°C).
3. Standard *Drosophila* vials.
4. Standard plastic bottles.
5. Foam plugs.
6. *Drosophila* food: Cornmeal, agar, sucrose, yeast, and acid medium.
7. Yeast paste.
8. Autoclave to prepare the food.
9. Morgue for dead flies.
10. Fly trap.

2.2. Lineage Tracing and Gene Manipulation

1. Stocks for generating tubulin-lacZ, PMML, and MARCM are easily available from the Bloomington Stock Center (<http://www.flybase.org>).
2. 37°C water bath tank for heat shock.
3. 18 and 25°C incubator to maintain fly crosses.
4. Useful stock and transgenes required in these experiments are given in Subheading 3.1.

2.3. BrdU Labeling

1. 10 mM BrdU (Sigma) in dH₂O. Store at -20°C (see Note 1).
2. Mouse anti-BrdU monoclonal antibody (Invitrogen).
3. 2 N HCl (8.6 mL concentrated 12 N HCL stock per 50 mL H₂O).
4. DNase (300 µL DNase +700 1×PBS).

¹BrdU is a mutagen and should be handled with care. BrdU solution should be made fresh every time.

2.4. Immunofluorescence Staining of Gut

2.4.1. Gut Dissection

1. *Drosophila* adult flies (3–5 days old).
2. Standard CO₂ source for anesthetizing the flies.
3. Fine paintbrushes.
4. Two fine-tipped dissecting forceps (Roboz) for dissection.
5. Glass microslides (25×75 mm) for dissection.
6. Glass microcoverslips (22×60 mm).
7. Plastic dropper.
8. Kim wipes.
9. Dissecting microscope with attached light source.
10. Dissecting solution (*Drosophila* Ringer's solution): 130 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl₂, and 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 6.9. Dissolve 7.5 g NaCl, 0.35 g KCl, 0.21 g CaCl₂, and 2.38 g HEPES in approximately 1 L distilled water and stir to dissolve. Adjust to pH 7.2 with 1 N HCl and make the final volume of 1 L with distilled water. Store the dissecting solution in glass bottle at 4°C (see Note 2).

2.4.2. Gut Fixation

1. Phosphate-buffered saline (PBS): 130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, and adjust to pH 7.4 with HCl. Store in glass bottles at room temperature. For longer stability of solution, store at 4°C.
2. Triton X-100 (Sigma) (see Note 3).
3. Gloves (Kimberly-Clark).
4. PBX solution: Dissolve the 0.1% Triton X-100 in 1× PBS plus 0.5% BSA. Store in at room temperature. For longer stability of solution, store at 4°C.
5. Formaldehyde (formalin) (37%) solution (Sigma) (see Note 4).
6. Fixation solution: (4% formaldehyde). In a 50-mL tube, add 5.4 mL 37% formaldehyde solution and then add 44.6 mL 1× PBX solution. Mix well at vortex. This solution should be prepared fresh every time but may be stored at 4°C for 1 week or longer at –20°C.
7. Parafilm to seal the Eppendorf tubes.

²1×PBS can be used in the place of *Drosophila* Ringer's solution.

³Use of Triton X-100 allows the cell membrane to be permeable to the antibody. Avoid using more than specified concentration as it can disrupt the epithelial membrane of the gut, which results in no or poor staining of membrane proteins.

⁴Formaldehyde is a fixative and carcinogenic; therefore, skin contact should be avoided. Avoid using bare hands to retrieve the tissue pieces; wear gloves during handling.

2.4.3. Blocking the Gut and Immunostaining

1. Normal goat serum (NGS; Vector laboratories). Store at 4°C.
2. Bovine serum albumin (BSA; Sigma). Use 0.5%.
3. Blocking solution (2% NGS): To make 2% NGS in 50-mL tube, add 1 mL NGS to 49.0 mL of 1× PBX. Mix well in vortex and store at 4°C (see Note 5).
4. Minivortex (VWR Scientific Products).
5. Tube shaker (Labquake).
6. Aluminum foil.
7. Microcentrifuge tube rack (Fisher Scientific).
8. Primary antibodies: The primary antibodies available to study ISCs in adult *Drosophila* are listed in Table 1. We use anti-beta-galactosidase for lacZ reporter lines and anti-GFP for GFP-fusion protein lines (see Table 1) (see Note 6).
9. Secondary antibodies: Secondary antibodies can be purchased from many different companies. We use secondary antibodies of goat anti-mouse, goat anti-rat, goat anti-rabbit, and goat anti-guinea pig IgG conjugated to Alexa Fluor 488 or Alexa Fluor 594 or Texas Red from Invitrogen and Cell Signaling. Store all secondary antibodies in a dark place at 4°C. We used a 1:200–500 dilution of all secondary antibodies in 1× PBX with 0.5% BSA.
10. 4,6-Diamidino-2-phenylidole dihydrochloride (DAPI; Invitrogen) to stain DNA. Dissolve in 1× PBS for counter-staining. Store in the dark at 4°C.

2.4.4. Mounting the Gut, Imaging, and Data Analysis

1. Microscope cover glass.
2. Microscope slides.
3. Quick-dry nail polish.
4. Dissecting microscope.
5. Fluorescence stereomicroscope with GFP filter.
6. Glycerol ultrapure (Sigma).
7. Mounting medium: 50% glycerol in 1× PBS (pH 7.4) containing 1% antifade (1,4-diazabicyclo [2,2,2] octane [DABCO]; Sigma) compounds. Store at room temperature (see Note 7).
8. Waterproof permanent marker to label the slides.

⁵In addition to NGS, calf serum or bovine serum albumin can also be used for blocking the tissues. Blocking solution can be contaminated because it contains serum. It is stable for a week at 4°C and for several months frozen. If the solution is becoming cloudy, that means it is contaminated and should not be used for blocking the tissues.

⁶Store the primary antibodies at 4°C with 0.02% sodium azide. For specificity and quality, primary antibodies can be stored at –20°C with 50% glycerol and at –80°C for long-term storage. Avoid frequent freezing and thawing, which degrade proteins.

⁷As an alternative, Vectashield H-1000 from Vector Laboratories can also be used as a mounting medium.

9. Microslide plastic folder (VWR Scientific Products).
10. Confocal microscope, we use Zeiss LSM510 for imaging.
11. Computer and appropriate software for image processing.

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1. Lineage Tracing and Gene Manipulation

Some of the important stocks will be needed to perform the experiments in this section: *W¹¹¹⁸*; *hs-FLP*, *FRT82B*, *tubP-GAL80*, *FRT19A*, *tubP-GAL80*, *FRTG13*, *tubP-GAL80*, *FRT82B*, *arm-lacZ*; *Arm-lacZ*, *FRT80B*; *FRT42D*, *arm-lacZ*; *Arm-lacZ*, *FRT40A*; *Arm-LacZ*, *FRT19A*; *FRT52B(y)-FRT-Gal4*, *FRT52B(w)-actin5C-FRT*; *X-15-29 (FRT-lacZ)*; *X-15-33 (tub-FRT)*; *UAS-lacZ*; *UAS-EGFP*, and *UAS-mCD8GFP*.

3.1.1. Generation of lacZ-Marked Clones in Adult Gut—The lineage marking system to generate clones of *lacZ*-expressing cells has been developed by Harrison and Perrimon (57). In this labeling system, after mitotic recombination, α -tubulin promoter will be fused to *lacZ* to allow the transcription of the marker. Without heat shock, XI5 flies carry two inactive *tubulin* promoter-*lacZ* (*X-15-33* and *X-15-29*). During mitotic division, randomly cells will go for flipase-mediated recombination at the two-flipase recombination target (FRT) sites, which results in generation of an active *lacZ* transgene (Fig. 3a). When this random recombination occurred in stem cell, the stem cells and its daughter cells will be marked with *lacZ*, and you can see the maintenance of the clones over long time. However, recombination in non-stem cell results in formation of a transient clone with short life span. The *lacZ*-marked lineage system is very efficient because marker genes are activated immediately but no background (see Note 8).

1. Produce the flies with the genotype *yw,hs-FLP/+; X-15-33/X-15-29* by standard crosses.
2. To induce somatic recombination to generate the clones, take the 3–5-day-old adult flies and heat shock in circulating water bath at 37°C for 60 min (see Note 9).
3. After heat shock, return flies to 25°C.
4. Transfer the flies daily to fresh food vials with yeast granules.
5. Dissect the guts at different time intervals to check the changes in clone size over time.
6. Fix, stain, and examine the gut under confocal microscope as per protocol given in Subheading 3.3.

⁸This method has limitation because it is not ideal for manipulating the gene's activity in the clones.

⁹A 37°C bacteria incubator can also be used in the place of circulating water bath. A single heat shock at 37°C for 1 h is enough to induce the clones. After heat shock, transfer the flies to a fresh, dry vial to avoid flies to stick with wet vial as water vapor is formed after heat shock.

3.1.2. Generation of PMML Clones in Adult Gut—This system utilized the heat shock-inducible FLP to reconstitute a functional *actin5C-gal4* gene from two complementary inactive alleles, *actin5C FRT52B* and *FRT52B gal4*. The *actin5C-gal4* gene drives *GFP* expression to mark cells and at the same time activate or knock down the gene function by having UAS constructs in the marked cells (Fig. 4a) (58).

1. To use the PMML system to generate GFP-marked clones that also overexpress their respective genes, cross the *hs-Flp UAS-srcEGFP, FRT^{52B}(w) UAS-EGFP/Cyo* virgin females with males of genotypes *FRT^{52B}(y)/Cyo, UAS-geneAFRT^{52B}(y)/Cyo*.
2. Collect the 3–5-day-old non-*Cyo* females and heat shock them in a water bath at 37°C for 60 min, one time.
3. After heat shock, return flies to room temperature or at 25°C.
4. Transfer the flies daily to fresh food vials with yeast granules.
5. Dissect the guts at different time intervals to check the changes in clone size over time.
6. Fix, stain and examine the gut under confocal microscope as per protocol given in Subheading 3.3.

3.1.3. Generation of MARCM Clones In Adult Gut—MARCM technique has been used to create individually labeled homozygous cells in an otherwise heterozygous background. MARCM relies on recombination during mitosis mediated by FLP-FRT recombination. MARCM contain six transgenes: two transgenes for two homologous FRT sites, one for the FLP recombinase, one for a UAS marker, one for a GAL4 driver, and one for the *tubP-GAL80* transgene. The *tubP-GAL80* has been placed distal to the FRT site in trans to the mutant gene of interest. In heterozygous cells, GAL80 blocks GAL4-induced UAS marker expression; however, in homozygous mutant cells, *tubP-GAL80* transgene is inactive, and therefore, the marker will be expressed (59–62) (Fig. 5a). Here, we describe the brief protocol, for details see (59–62).

1. Cross the flies of genotype *yw, hs-FLP UAS-GFP tub-Gal4+; FRT82B tub-Gal80*, either with *FRT^{82B}* flies or *FRT^{82B}*-mutant flies.
2. Raised the F1 progenies at 18°C.
3. Take the 3–5-day-old flies from 18°C and heat shock at 37°C in a circulating water bath for 60 min, twice a day for 2 days, with an interval of 8 h in each heat shock (see Note 10).
4. After every heat shock, invert the vials and keep the flies at room temperature.
5. Dissect the guts at different time intervals to check the changes in clone size over time (see Note 11).

¹⁰.With this system, clones are activated slowly. The advantage of this system that clones can mis-express a gene or homozygose a mutation (52).

6. Fix, stain, and examine the gut under confocal microscope as per protocol given in Subheading 3.3.

3.2. Brdu Labeling

5-Bromo-2-deoxyuridine (BrdU) serves as marker of proliferation and is a uridine derivative and a structural analog of thymidine. It can be incorporated into DNA during the S-phase of the cell cycle. We used BrdU labeling to see the stem cell proliferation in adult *Drosophila* midgut.

1. Culture the adult 3–5-day-old flies with fly food containing 200 μ L of 6 mg/mL BrdU in 20% sucrose for 2 days.
2. Transfer the flies every 2 days to new fly food vials.
3. For maximum labeling, feed the flies with Brdu 4–6 days.
4. After feeding the flies with BrdU, dissect the gut as per protocol given in Subheading 3.3.
5. Fix the guts as per protocol given in Subheading 3.3.
6. Treat the guts with DNase I for 1 h at 37°C to denature the DNA.
7. Remove the DNase I from the gut.
8. Rinse the guts three times with 1#x00D7;PBX.
9. Block and perform the staining as per protocol given in Subheading 3.3.

3.3. Immunofluorescence Staining of Midgut

The presented staining protocol is similar to one described for *Drosophila* testis with some modifications (53).

3.3.1. Gut Dissection

1. Anesthetize the flies on the CO₂ source surface.
2. Use a clean glass microslide and put under the dissecting microscope.
3. Use a plastic dropper to place a few drops of Ringer's solution on the slide.
4. Take the flies from fine forceps and place flies on a slide containing Ringer's solution.
5. With the help of two fine forceps, dissect the gut in fresh Ringer's solution.
6. Use one pair of forceps, turn the body upside down, hold the top of the abdomen, and pull out the external genitalia with the other pair of forceps.
7. Take off the gut, which is usually cream color, and remove the other fly parts such as ovary (in case of female flies), malpighian tubules, and anterior gut.

¹¹If there are very few clones induced, make sure the heat shock regimes are timed properly. In addition, double-check your cross to verify the genotypes. In *Drosophila* midgut, transient clones are present until 7 days after heat shock; it is advised to examine the clones at different time intervals (3, 6, 12, 20 days) (11).

8. Transfer the dissected gut into a tube with Ringer's solution. Optional: guts after dissection can be transferred directly to the fixation solution.
9. 10–20 flies gut should be ideal for one tube.
10. Wait the gut tissues to settle down at the base and mark the tube for specific genotype.

3.3.2. Fixation of Gut

1. Take out the dissecting solution from the tube and keep the gut in the tube.
2. Prepare 4% formaldehyde (fixing solution) in 1× PBX. Optional: 4% formaldehyde can also be prepared in 1×PBS.
3. Add 200–500 µL 4% formaldehyde in the tube with gut.
4. Fix the gut by incubating the tube on a shaker at room temperature for 20–40 min (see Note 12).
5. After specified time above, stop the shaker and place the tube in the tube rack to allow the gut to settle down in the tube.
6. Remove the fixative solution and rinse the guts three times, for 2 min each in 1× PBX.

3.3.3. Blocking and Staining of Gut

1. To block the gut tissue, prepare the blocking solution in 1× PBX.
2. Add 200–500 µL blocking solution to guts and incubate the guts overnight at 4°C or 30 min at room temperature (see Note 13).
3. Take out the blocking solution.
4. Dilute the primary antibody in 1× PBX containing 0.5% BSA.
5. Mix the tube containing antibodies on minivortex (see Note 14).
6. Add 50–100 µL of diluted primary antibody to the guts and wrap the tube with Parafilm (see Note 15).
7. Incubate the gut tissues with the primary antibody, preferably overnight at 4°C or at room temperature (see Note 16).
8. After specified incubation time above, take out the primary antibodies and save at 4°C for reuse (see Note 17).

¹²For best results, limit the fixation time to 25–40 min as prolonged fixation can cross-link antigens and mask epitopes.

¹³Blocking reduces background staining by preventing nonspecific protein-antibody interactions. The blocking can be done for extended time (over weekend) at 4°C without any visible loss of staining quality.

¹⁴Gut can be stained with several primary antibodies raised in different species in the same tube. Sometimes, mouse and rat antibodies cross-react; avoid longer incubation time of primary antibodies.

¹⁵To prevent the leakage of the antibodies during shaking, wrap the tube with Parafilm.

¹⁶Alternatively incubate the primary antibodies at room temperature for 2 h. Sometimes, it can be incubated for 2 days at 4°C.

However, long incubation times can create background staining.

¹⁷Primary antibodies can be reused many times with same quality of staining.

9. Rinse the gut three times with 1× PBX.
10. Then wash the gut on shaker at room temperature for 15 min (three times) in 1×PBX.
11. Prepare secondary antibody to desired concentration in 1×PBX (see Note 18).
12. Add 200–400 µL of diluted secondary antibody to the gut tissues.
13. Wrap the tube with aluminum foil to avoid the exposure of light.
14. Incubate the gut with secondary antibodies on shaker at room temperature for 2 h or overnight at 4°C (see Note 19).
15. Remove the secondary antibody from the tube.
16. Rinse the gut three times with 1× PBX.
17. Then wash the gut on shaker at room temperature for 15 min (three times) in 1× PBX.
18. After final wash, rinse the gut three times in 1× PBS.
19. To counter stain the gut with DNA, prepare the DAPI (from 1 mg/mL stock) in 11× PBS (see Note 20).
20. Add the DAPI to gut tissues and incubate for 5 min at room temperature.
21. Remove the DAPI solution and store in a dark place at 4°C for to reuse.
22. Rinse the gut with 1× PBS three times for 2 min each.
23. Wait for tissues to settle down and prepare for mounting.

3.3.4. Mounting, Microscopy, and Data Analysis

1. Prepare mounting medium in 1× PBS.
2. Add 20–100 µL of mounting medium to the gut tissues.
3. Place the gut tissues with mounting medium in a dark overnight at 4°C to allow tissues to equilibrate.
4. With the help of pipette, transfer the gut to a glass microslide frosted at one end.
5. Arrange the gut under the dissecting microscope using low levels of light.
6. Carefully place a cover slip to the slide containing the gut (see Note 21).
7. Remove excess mounting medium using Kimwipes.

¹⁸.Store the secondary antibodies in the dark at 4°C. For optimal staining, order new secondary antibodies if they are more than a year old. However, most of the secondary antibodies are good for several years, if stored properly. It can be stored at –20°C.

¹⁹.For best results, incubate the tissues with secondary antibody for 2 h at room temperature. Incubating tissues for overnight at 4°C can produce high background for polyclonal antibodies.

²⁰.DAPI is a potential carcinogen and is a popular nuclear counterstain for *use* in multicolor fluorescent techniques. It may be harmful by inhalation, ingestion, or skin absorption. Wear proper gloves and avoid breathing the dust and vapors. Dispose all tips and tubes in appropriate container.

²¹.Carefully place the edge of a coverslip next to the drop of mounting medium. With the help of forceps, lower the coverslip onto the gut tissues.

8. Seal the edges of the microcoverslip with nail polish (see Note 22).
9. Use a permanent marker to label the slide for the specific genotype.
10. Put the prepared slides in a slide box, wrap the box with aluminum foil, and store the slides in the dark at 4°C until observation (see Note 23).
11. First, confirm the staining by examining the slides by fluorescence microscopy (see Note 24).
12. For best quality imaging, use confocal microscopy. To capture images, we used a Zeiss LSM 510 confocal laser-scanning microscope attached to computer.
13. LSM 5 Image Browser used to download the images (see Note 25).
14. Images processed using Adobe Photoshop.

Some of the examples using the above techniques and staining protocols are presented in Figs. 2–4. However, for best results, optimize the condition as per requirements of the experiments. The above protocols can be used to identify other stem cells in adult *Drosophila*.

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22. To prevent movement of the coverslip and evaporation of the media during imaging, first, put nail polish to the four corners of the cover slip and let it dry. Then, put nail polish to the edges. The edges of the coverslips can also be sealed by using halocarbon oil 27.

23. For best results, store the slides in the dark at 4°C and collect the images within 2–3 days.

24. If there is problem in staining such as no staining and weak or high background in staining, this might be due to missing some steps, primary and/or secondary antibody may be bad due to improper storage, improper dilution, and/or incubation time. To avoid the above problems, use some known antibody with known expression pattern to ensure that the primary and/or secondary antibody is working properly.

25. After capturing the images, one can process the images in several different ways depending upon the available software.

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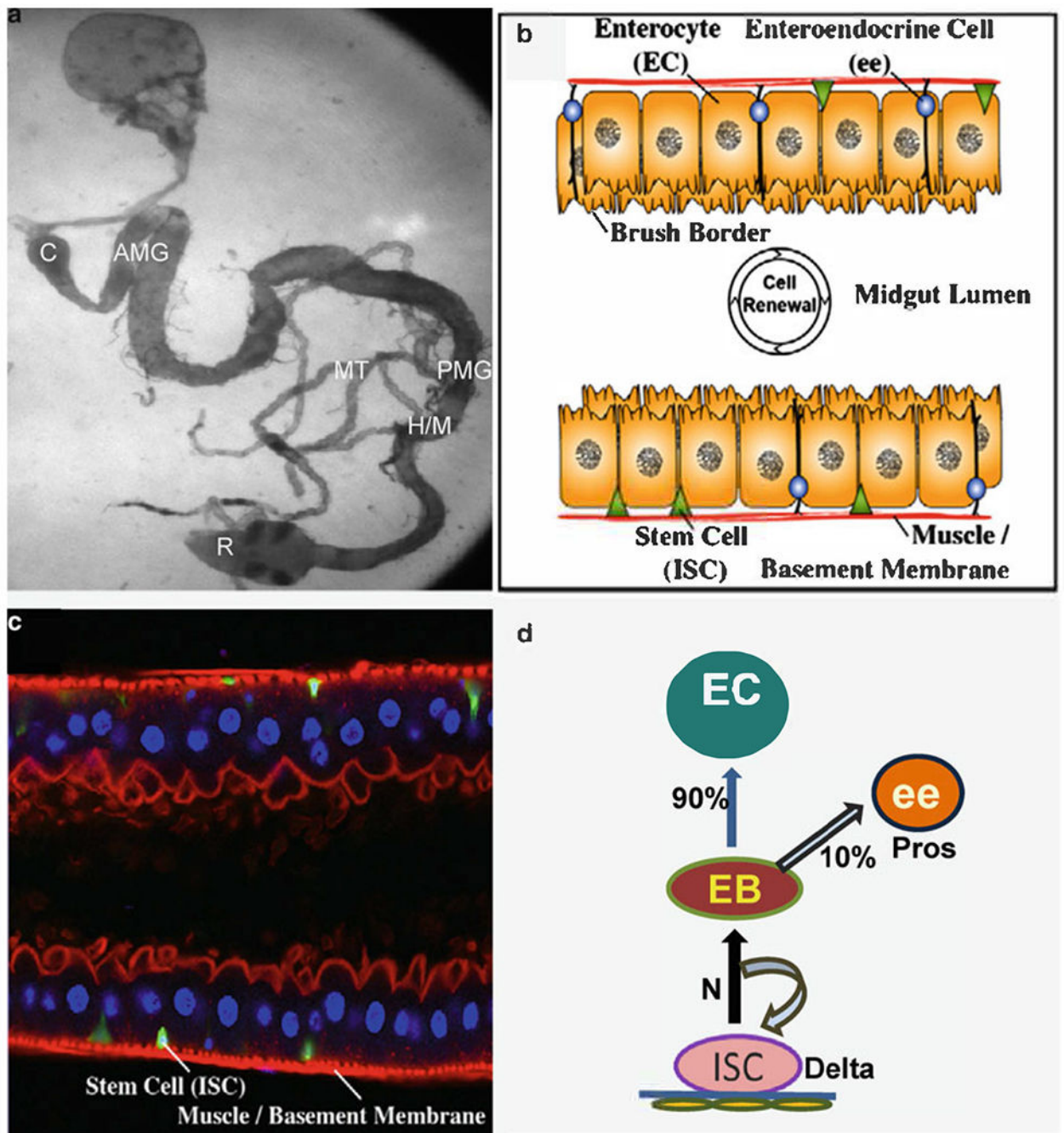


Fig. 1. The adult *Drosophila* midgut is maintained by a population of multipotent intestinal stem cells (ISCs). (a) Wild-type *Drosophila* gastrointestinal tract visualized by phase-contrast microscopy. (b) Diagram of the adult midgut in cross section. ISCs (*green*) occupy a basal position in a niche adjacent to the basement membrane and the visceral muscle (*red*). ISCs give rise to two types of differentiated daughters, enteroendocrine (*ee*) cells (*blue*) and enterocytes (ECs, *orange*). (c) A cross section of the adult midgut showing ISCs marked by *esgGal4, UAS-GFP (greed)*. ECs have large polyploid nuclei (*blue*, DAPI) and form a

polarized cellular monolayer with an actin-rich (*red*, phalloidin) brush border on their luminal surface; ee cells are not marked, **(d)** Summary of ISCs lineage that shows that ISCs give rise to daughters (EB), which become 90% enterocytes (EC) and 10% enteroendocrine (ee) cells. *C* cardia; *AMG* anterior midgut; *PMG* posterior midgut; *H/M* hindgut/midgut junction; *MT* malpighian tubules; *R* rectum. **(b, c)** The figure is adapted with permission from Lee et al. (31).

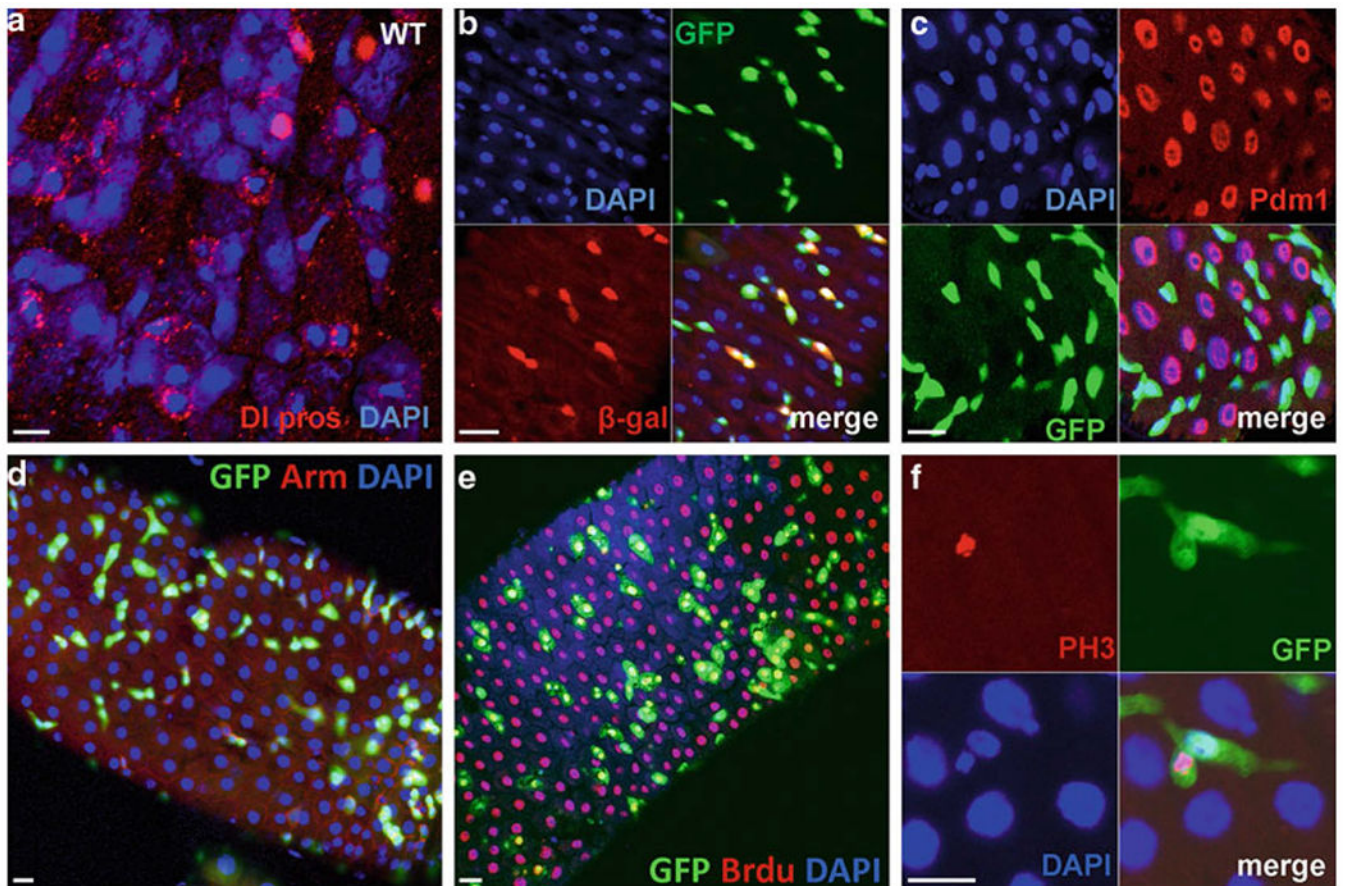


Fig. 2. ISC markers and proliferative cell marker expression in the midgut. **(a)** Wild-type gut with expression of anti-DI (red-punctate structures resembling endocytic vesicles for ISCs), anti-prospero (red-nuclear staining, ee cells); Dapi staining, blue. **(b)** Gut stained for *10X STAT-GFP* (green-labeled the ISCs and EBs) and *Su(H)GBE-LacZ* (red-labeled EBs only), Dapi (blue). **(c)** Gut staining of *esg-GAI4-UAS-GFP* flies with anti-Pdm1. GFP mark the ISCs and EBs (green), Pdm1-mark the EC cells (Red), Dapi (blue). **(d)** *kr-Gal4-UAS-GFP* gut stained for GFP (green-labeled the ISCs and EBs), anti-Arm (red), and Dapi (blue). **(e)** BrdU pulse to detect the DNA synthesis in the gut using *10X STAT-GFP* flies, anti-BrdU (red), GFP (green), Dapi (blue). **(f)** *10X STAT-GFP* (green) gut stained with phospho-histone H3 (red) to detect the mitosis. Scale bars: 10 μ m.

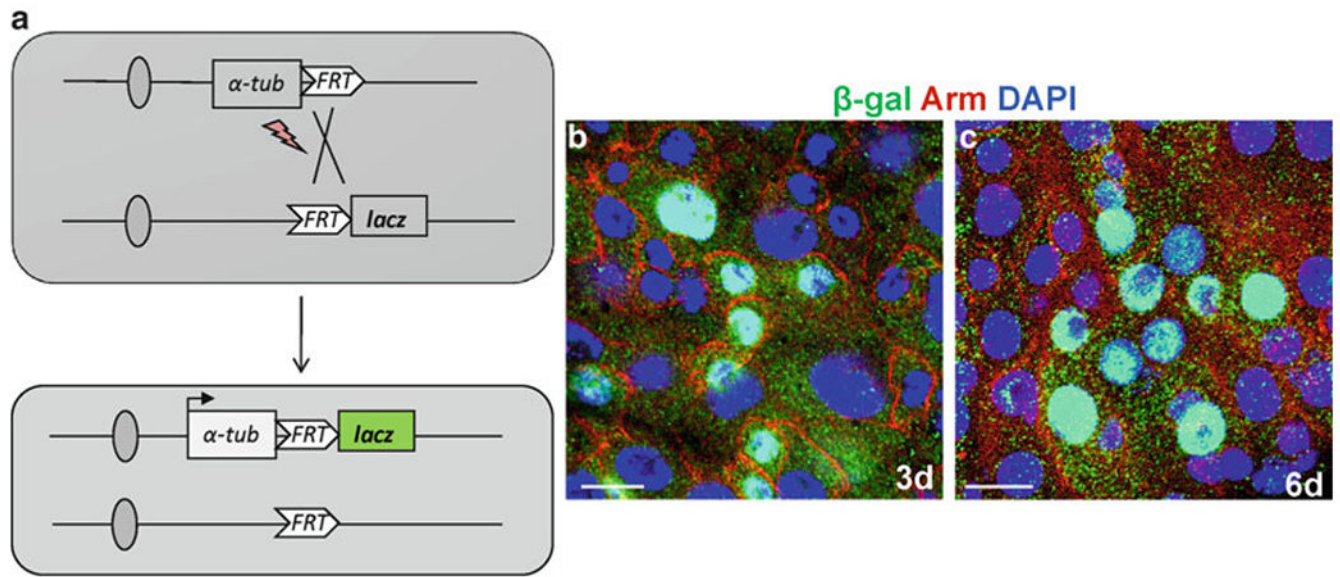


Fig. 3. Tubulin-lacZ positive-labeling to generate ISC clones in the midgut. **(a)** Diagram to show the lineage-tracing scheme to mark the randomly dividing cells by heat shock FLP-catalyzed site-specific recombination. **(b, c)** The representative examples of the induced clones, 3 day **(b)** and 6 day **(c)** in the midgut anti- β -Gal (*green*), anti-arm (*red*), and Dapi (*blue*). Scale bars: 10 μ m.

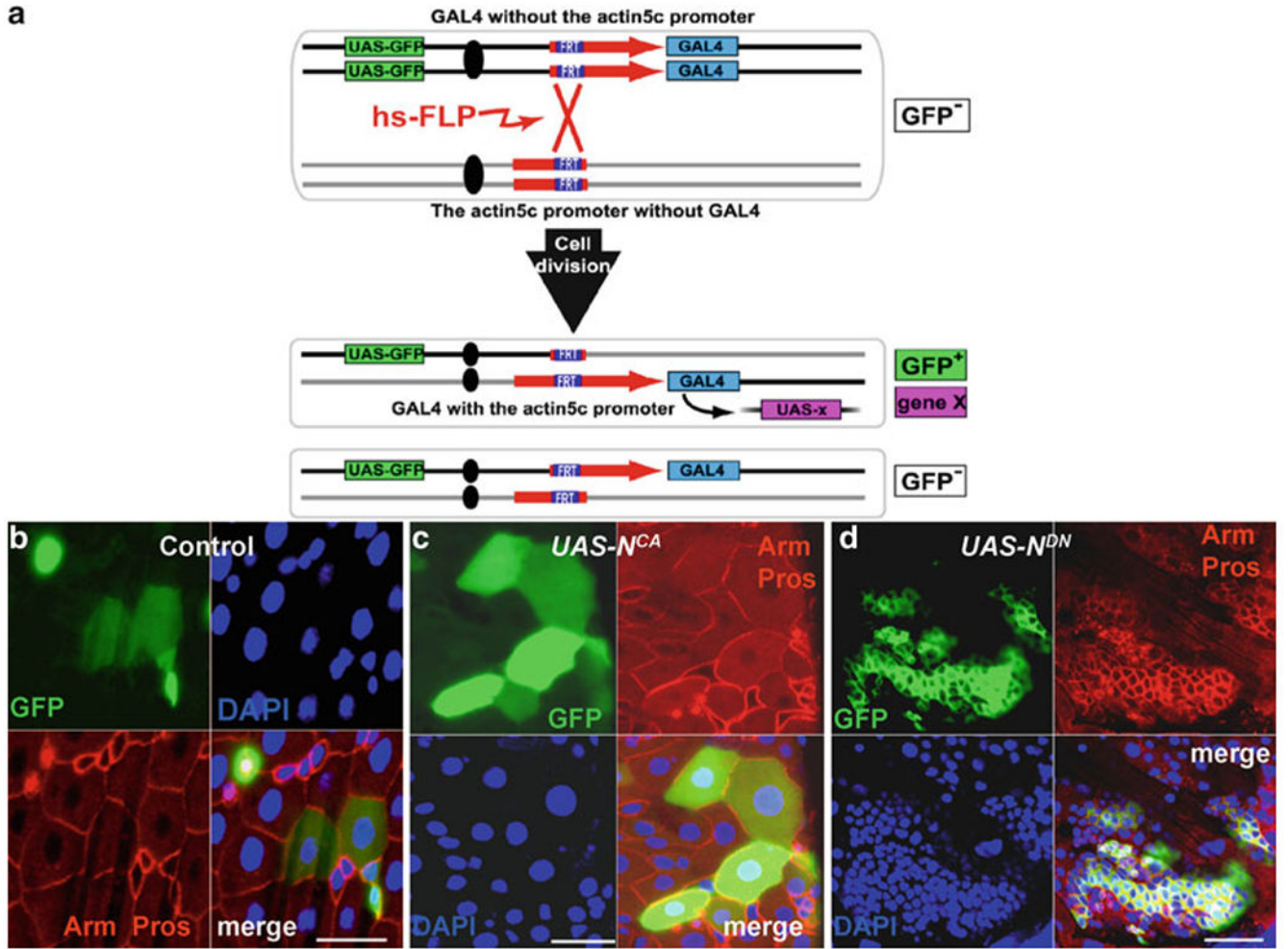


Fig. 4.

PMML labeling system to generate ISC clones in the midgut. **(a)** A schematic diagram showing how to generate a functional *actin5C-gal4* gene by using the FLP-mediated FRT recombination technique. A functional *actin5C-gal4* gene is reconstituted by heat shock-induced FLP-mediated recombination between inactive but complementary alleles, *actin5C FRT* and *FRT gal4*. The daughter cell that inherits the *actin5C-gal4* gene expresses UAS-GFP or any other transgene constructs. **(b)** Gut with GFP-marked wild-type PMML clones. Anti-GFP (*green*), anti-Arm (red-diploid cell nest), anti-Pros (red-nuclei of ee cells), and Dapi (*blue*). **(c)** Gut with GFP-marked UAS-N^{CA} PMML clones. GFP (*green*), Arm (red-diploid cell nest), anti-Pros (red-nuclei of ee cells), and Dapi (*blue*). **(d)** Gut with GFP-marked UAS-N^{DN} PMML clones. Anti-GFP (*green*), anti-Arm (red-diploid cell nest), anti-Pros (red-nuclei of ee cells), and Dapi (*blue*). **(a)** The figure is adapted with permission from Kirilly et al. (58). Scale bars: 10 μ m.

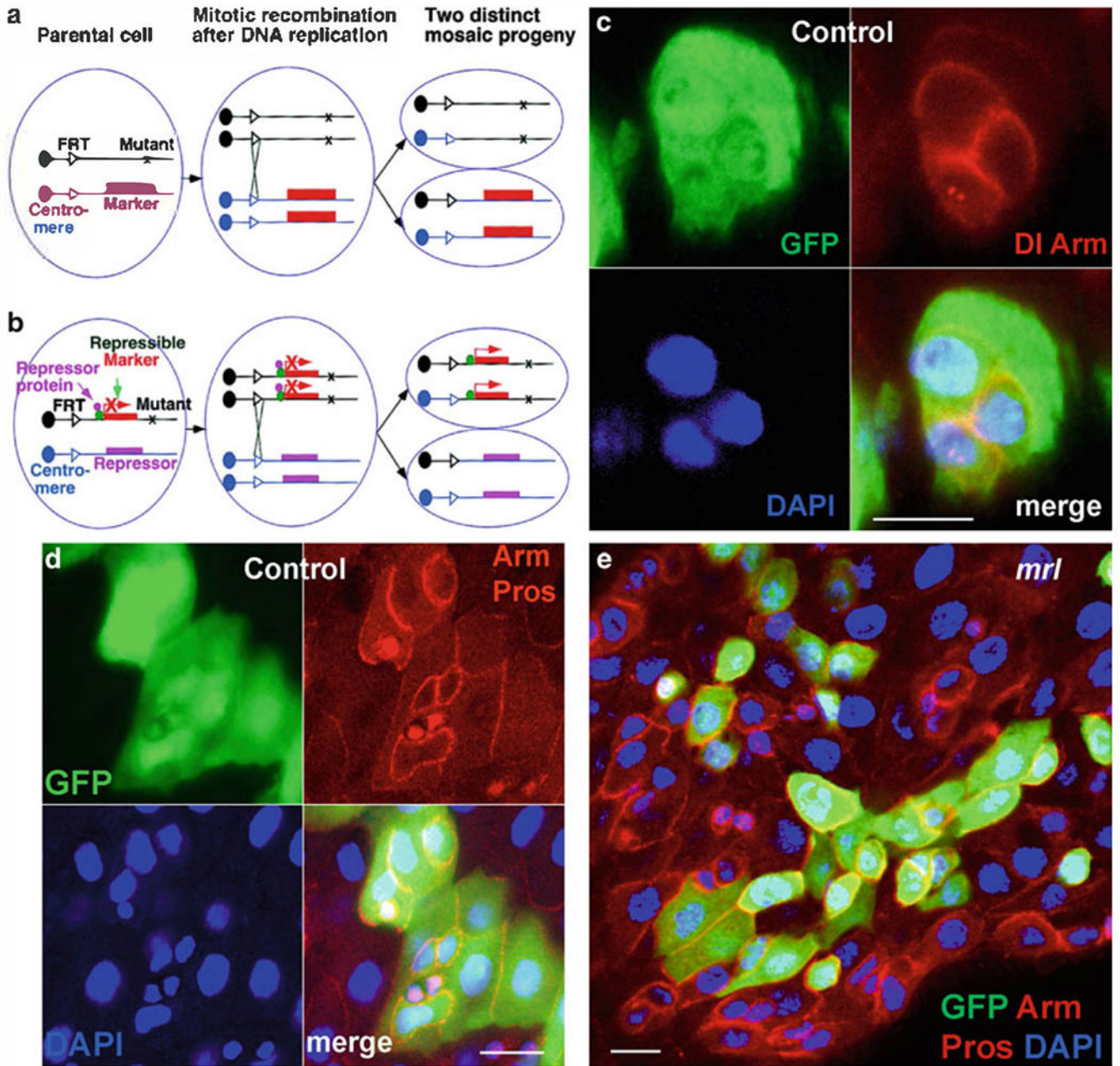


Fig. 5. MARCM labeling system to generate ISCs clones in the midgut. **(a)** In conventional mosaic analysis, homozygous mutant cells are identified as unstained cells if the marker gene is placed distal to the FRT site on the homologous chromosome arm in *trans* to the mutant gene. **(b)** In the MARCM system, a transgene encoding the repressor of marker gene expression is placed distal to the FRT site on the homologous chromosome arm from the mutant gene. Only in homozygous mutant cells can the marker gene be expressed because of the loss of the repressor transgene. **(c, d)** Gut with GFP-marked wild-type MARCM clones. In **(c)** GFP (*green*), anti-delta (red, punctate expression) and anti-Arm (red-cell nest), and Dapi (*blue*). In **(d)** GFP (*green*), anti-Arm (red-cell nest), anti-Pros (red-nuclei of ee cells),

and Dapi (*blue*). (e) Gut with GFP-marked *Stat92E*⁰⁶³⁴⁶ mutant clones. GFP (*green*), anti-Arm (red-cell nest), anti-Pros (red-nuclei of ee cells), and Dapi (*blue*). Panel A in this figure is adapted with permission from Lee and Luo (59). Scale bars: 10 μm .

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Table 1Antibodies used to mark *Drosophila* intestinal stem cell and differentiated cells

| Antibody | Cell type expression | Species | Dilution | Source | References |
|------------------|--|---------------|------------------|---------------------------------------|------------|
| Notch | ISC, EB | Mouse | 1:10 | DSHB | (11) |
| Armadillo | Outer layer of ISC, EB, and EC | Mouse | 1:20 | DSHB | (11) |
| Prospero | ee cells | Mouse | 1:100 | DSHB | (13) |
| Allatostatin | ee cells | Mouse | 1:10 | DSHB | (11) |
| Tachykinin | ee cells | Rabbit | 1:2,000 | D. Nässel | (11) |
| Delta | ISC | Mouse | 1:100 | DSHB | (13) |
| Anilin | Mitotic ISC | Rabbit | 1:1,000 | Chris field | (13) |
| Alpha-tubulin | Microtubule and spindle | Rat | 1:50 | Immunologicals direct | (13) |
| Gamma-tubulin | Centrosomes | Mouse | 1:500 | Sigma | (13) |
| Daughterless | ISC, EB, ee, EC | Rabbit | 1:1,000 | Y.-N. Jan | (25) |
| Cdc2 | ISC, EB | Rabbit | 1:500 | Santa Cruz | (4) |
| AcH3 | ISC, EB | Rabbit | 1:500 | Millipore | (4) |
| Pdml | Mature EC | Rabbit | 1:100 | Xiaohang Yang | (31) |
| pH3 | Dividing ISC | Rabbit | 1:1,000 | Upstate | (12) |
| Hairless | ISC and EB, some differentiated progeny | Guinea pig | 1:500 | A. Preiss | (25) |
| EGFR | ISC and/or EB | Goat | 1:200 | Santa Cruz | (5) |
| Yki | ISC/EB | Rat Rabbit | 1:1,000 1:400 | Helen McNeill Kenneth Irvine | (44, 45) |
| Fat | ISC, EB | Rat | 1:2,000 | Michael Simon | (44) |
| Ds | Borders of EC cells | Rat | 1:5,000 | Michael Simon | (44) |
| Asense | ee cells | Rabbit | 1:5,000 | Y.-N. Jan | (25) |
| <i>scute RNA</i> | ISC, EB and/or ee | – | – | – | (25) |
| <i>km RNA</i> | ISC | – | – | – | (28) |
| dpERK | ISC, EB | Rabbit | 1:1,000 | Cell signaling | (32) |
| STAT92E | ISC, EB | Rabbit | 1:500 | S. Hou | (39) |
| pMoe | Interface membranes between the ISC and EB cells and their corners | Rabbit | 1:500 | Cell signaling | (34) |
| E-cad | Interface membranes between the ISC and EB cells | Rat | 1:20 | DSHB | (34) |
| aPKC | Apical areas of the ISC/EB | Rabbit | 1:100 | Santa Cruz | (34) |
| wg | Circular muscles, basement membrane, and ISCs | Mouse | 1:200 | DSHB | (38) |
| Caudal | All epithelial cells of gut | Guinea Pig | 1:400 | East Asian Distribution Centre, JAPAN | (15,16) |
| Spectrin | Apical side of ECs | Mouse | 1:100 | DSHB | (17) |
| Dapi | Mark the nuclei | – | 1 µg/mL | Sigma | (13) |
| Brdu | S-phase cell marker | Mouse | 1:100 | Invitrogen | (39) |
| β-galactosidase | lacZ expression | Rabbit | 1:2,000 | Cappel | (13) |

| Antibody | Cell type expression | Species | Dilution | Source | References |
|----------|------------------------------|--------------|-------------|------------|------------|
| GFP | For GFP-fusion protein lines | Mouse/rabbit | 1:100/1:500 | Invitrogen | (39) |

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Table 2Transgenes expressed in *Drosophila* midgut stem cells and differentiated cells

| Transgenes | Expression in midgut | References |
|--|---------------------------------|---------------|
| <i>esg-Gal4-UAS-GFP</i> | ISC, EB | (12) |
| <i>Kr-Gal4-UAS-GFP</i> | ISC, EB | Present study |
| <i>Su(H)Gbe-lacZ</i> | EB | (12) |
| <i>Pvf2-lacZ</i> | ISC/EB | (15) |
| <i>DI-LacZ</i> | ISCs | (26, 37) |
| <i>delta-Gal4</i> | ISC | (55) |
| <i>Su(H)Gbe-Gal4</i> | EB | (55) |
| <i>polo-GFP</i> | ISCs metaphase plate | (4) |
| <i>vn-lacZ</i> | Midgut visceral muscle cells | (28) |
| <i>spi (spi-Gal4^{NF0261})</i> | ISC, EB and low in EC | (28) |
| <i>rho^{X81}-lacZ</i> | Midgut visceral muscle cells | (28) |
| <i>upd-lacZ</i> | ISC and EB | (39) |
| <i>upd-3-lacZ</i> | EC | (21,28) |
| <i>10XSTAT-DGFP</i> | ISC, EB | (26, 39) |
| <i>myoIA-Gal4-UAS-GFP</i> | ECs cells | (27) |
| <i>myoIA-lacZ</i> | ECs cells | (27) |
| <i>24B-Gal4 (howGal4)</i> | Visceral muscle-specific | (5,46) |
| <i>ds-LacZ</i> | EC and some ee cells | (44) |
| <i>E(spl)mβ-CD2</i> | EB cells | (34) |
| <i>mira-promoter-GFP</i> | ISC | (25) |
| <i>vkg-GFP</i> | Basement membrane | (17) |
| <i>esg-lacZ</i> | ISC, EB | (12,19) |
| <i>cad-Gal4-UAS-GFP</i> | Posterior midgut cells | (19) |
| <i>wg-lacZ</i> | Circular muscle cells of midgut | (38) |
| <i>collagenIV-GFP</i> | Basement membrane structure | (17) |
| <i>gstDI-lacZ</i> | ISC and ee cells | (43) |
| <i>D-p38b-lacZ</i> | ISC and EB | (41) |
| <i>SCNY-GFP</i> | ISC and EC | (24) |