Enteroendocrine cells are generated from stem cells through a distinct progenitor in the adult Drosophila posterior midgut

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ABSTRACT
Functional mature cells are continually replenished by stem cells to maintain tissue homeostasis. In the adult Drosophila posterior midgut, both terminally differentiated enterocyte (EC) and enteroendocrine (EE) cells are generated from an intestinal stem cell (ISC). However, it is not clear how the two differentiated cells are generated from the ISC. In this study, we found that only ECs are generated through the ISC commitment to ECs. Prospero (Pros) regulates ISC commitment to EEs. Our data provide direct evidence that different differentiated cells are generated by different modes of stem cell lineage specification within the same tissues.

KEY WORDS: Intestinal stem cell, Secretory enteroendocrine cell, Pre-enteroendocrine cell, Prospero, Drosophila

INTRODUCTION
Adult stem cells maintain tissue homeostasis by continuously replenishing damaged, aged and dead cells in any organism (Weissman, 2000). As expected, adult stem cell self-renewal and differentiation are tightly regulated processes, and an imbalance in adult stem cell homeostasis can result in complications such as tumor formation and degenerative diseases. Moreover, adult stem cell behavior must be precisely regulated to ensure a prompt response to tissue damage and stress.

The Drosophila midgut is one of its largest organs, and it serves as the entry site not only for nutrients like food and water, but also for pathogens, such as harmful bacteria, viruses and toxins (Hakim et al., 2010). As a result, the midgut epithelium is constantly exposed to environmental assault and undergoes rapid turnover. The integrity of the epithelium is maintained by replenishing lost cells with intestinal stem cells (ISCs) (Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006). These ISCs lie adjacent to the basement membrane and divide approximately once a day to give rise to either absorptive enterocytes (ECs) or secretory enteroendocrine (EE) cells. Because of the simple cell lineage, ease of performing genetic analysis and availability of abundant mutants, the Drosophila midgut has served as a powerful model system for studying adult stem cell-mediated tissue homeostasis and regeneration.

Previous studies suggest that an ISC divides asymmetrically to produce one new ISC (self-renewal) and one immature daughter cell, an enteroblast (EB), which further differentiates into an EC or an EE cell (Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006). Notch (N) signaling plays a major role in regulating ISC self-renewal and differentiation (Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006, 2007). The ligand of the N pathway, Delta (Dl), is specifically expressed on an ISC and unidirectionally switches on the N-signaling pathway in the neighboring EB to promote the differentiation of an EB to an EC and to inhibit the differentiation of an EB to an EE (Bardin et al., 2010; Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006, 2007). In addition, commitment and terminal differentiation of ISCs require distinct levels of N activity; commitment requires high activity and terminal differentiation requires low activity (Perdigoto et al., 2011). Furthermore, the overexpression of Dl in ISCs does not affect ISC proliferation, but promotes EC fate at the expense of EE cells, suggesting that N signaling is not only necessary, but also sufficient, for specifying EC cell fate (Beebe et al., 2010; Jiang et al., 2009).

The differentiated ECs can also regulate ISCs through a feedback mechanism to maintain tissue homeostasis (Biteau et al., 2011; Jiang and Edgar, 2011). Cells in the intestine are constantly exposed to numerous insults, from tissue damage to bacterial infection. It was reported that these events initially affect differentiated ECs, causing either ablation or activated JNK-mediated stress signaling in the ECs (Jiang and Edgar, 2011). The affected ECs would upregulate unpaired (Upd) ligands (Upd2 and Upd3) of the JAK-STAT signal transduction pathway, which would activate the JAK-STAT signal transduction pathway in ISCs. This activation would induce ISC division and differentiation to generate new ECs that would replenish the damaged epithelium.

Compared with the extent of knowledge on EC specification and regulation, relatively little is known about EE cell fate specification and regulation. In this study, we performed lineage-tracing experiments using a newly developed tracing system and found that EE was generated from ISCs through a distinct progenitor. We further found that Prospero (Pros) functions downstream of or parallel to the achaete-scute complex (AS-C) members to determine ISC commitment to EE.

RESULTS
Su(H)GBE+ EBs are progenitors of ECs but not EEs
Different cell types in the adult Drosophila posterior midgut can be identified morphologically as well as by their expression of different markers. ISCs are diploid, have a small nucleus, and express DI and Sanpodo (Spdo). EBs are diploid and have a small nucleus; Su(H)GBE-lacZ, a transcriptional reporter of N signaling, was found to be highly expressed in the daughter cell (EB). ECs are polyploid, have a large nucleus and express the transcriptional factor Pdm-1. EEs are diploid, have a small nucleus, and express the transcriptional reporter of N signaling.
factor Pros and synaptic protein Bruchpilot (Brp/nc82) (Jiang et al., 2009; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006, 2007; Perdigoto et al., 2011; Zeng et al., 2013). However, it is not clear whether all ISCs are Dl positive (Dl+), whether all EBs are Su(H)GBE+, whether all ECs are Pdm-1+ and whether all EEs are Pros+. In this study, we only examined Dl+ ISCs, Su(H)GBE+ EBs, Pdm-1+ ECs and Pros+ EEs.

Both ISCs and EBs in the midgut express the transcriptional factor escargot (Esg) (Micchelli and Perrimon, 2006). Esg-Gal4 is widely used to manipulate gene expression in ISCs and EBs of the posterior midgut. During a routine staining of esg-Gal4, UAS-GFP midguts with the Pros antibody, we unexpectedly found that 3.3% of Esg+ diploid cells express the EE marker Pros (n=45 guts; Fig. 1A,B). Interestingly, these Esg+ Pros+ cells do not express the EB marker Su(H)GBE-lacZ (n=45 guts; Fig. 1A′). These Esg+ Su(H)GBE− Pros+ cells may be EE progenitors (or pre-EEs). We reasoned that some of the newly generated ECs and EEs might inherit weak green fluorescent protein (GFP) from Su(H)GBE+ EBs if they were developed from EBs. Indeed, we found that, among all GFP+ cells, 7.6% (32/472, n=12; Fig. 1C-D) of the cells are ECs that express weak GFP inherited from Su(H)GBE+ EBs; but surprisingly, we did not find any EEs that have inherited GFP from Su(H)GBE+ EBs (0 Pros+ cells expressed GFP among 153 GFP+ cells; supplementary material Fig. S1A-A′). Additionally, we generated MARCM clones using Su(H)GBE-Gal4 instead of Tub-Gal4. Consistently, we found that some Pdm−1+ ECs inherited weak GFP from Su(H)GBE+ EB (14 Pdm−1+ cells expressing GFP among 153 GFP+ cells; supplementary material Fig. S1A-A′). These data again indicate that ECs, but not EEs, are developed from Su(H)GBE+ EBs. The above data suggest that EEs may not be developed from Su(H)GBE+ EBs, but may be generated from ISCs through another mechanism.

To further test this possibility, we developed a novel lineage-tracing system to trace cell lineage in the posterior midgut.

Developing a new lineage-tracing system

The lineage-tracing system that combines a tamoxifen-inducible Cre knock-in allele with the Rosa26-lacZ reporter strain has been successfully used to identify cell (particularly stem cell) lineages in adult mice (Barker et al., 2007; Takeda et al., 2011). In Drosophila melanogaster, FLP/FRT, an alternative site-specific recombination system originally identified in yeast, has been predominantly used, with great success, to generate a genetic mosaic for clonal analysis

Fig. 1. Su(H)GBE+ EBs are not EE progenitors. (A,A′) Esg+ (green) Pros+ (red) cells (arrows) do not express the EB marker Su(H)GBE-lacZ (arrowheads, purple in A). (B) The quantification of Esg+ Pros+ Su(H)GBE+ and Esg+ Pros− Su(H)GBE+ cells among all Esg+ cells. Data are represented as means±s.e.m. (C,C′) Some of the ECs (arrowheads) inherited weak GFP from Su(H)GBE+ EBs; none of the EEs (arrow) inherited GFP from Su(H)GBE+ EBs, suggesting that ECs, but not EEs, are developed from Su(H)GBE+ EBs. (D) The quantification of GFP+ ECs and EEs among all GFP+ cells in Su(H)GBE>GFP posterior midguts. Data are represented as means±s.e.m. (E-E′) Wild-type MARCM clones with Su(H)GBE-lacZ. Some polyploid ECs (asterisks) express β-Gal inherited from Su(H)GBE-lacZ+ EBs (arrows). However, none of the EEs (arrowhead) expresses β-Gal, suggesting that ECs, but not EEs, are developed from Su(H)GBE+ EBs. (F-F′) Wild-type MARCM clones with Su(H)GBE-lacZ. The arrows indicate a β-Gal+ Pdm−1+ EB. The asterisks indicate a β-Gal+ Pdm−1+ EC. The arrowheads indicate a β-Gal− Pdm−1+ EC. Scale bars: 10 μm.
(Chou and Perrimon, 1996; Golic and Lindquist, 1989; Lee and Luo, 1999; Xu and Rubin, 1993). These experiments depend on inducing recombination in a few isolated progenitor cells, followed by clonal expansion of these recombined cells (Decotto and Spradling, 2005; Michell and Perrimon, 2006; Ohlstein and Spradling, 2006; Singh et al., 2007, 2011; Strand and Michell, 2011; Tulina and Matunis, 2001; Xie and Spradling, 1998). The mosaic clonal systems are effective for identifying a marked cell lineage, but they are not ideal for accurately identifying the origin of individual marked cells. A Gal4 technique for real-time and clonal expression (G-TRACE) was developed to identify more precisely the origin of individual cells (Evans et al., 2009). The TARGET system, in which Gal4 is combined with the temperature-sensitive GAL80 protein (Gal80ts), can be used for inducible control of Gal4 activity (McGuire et al., 2003). Theoretically, a combination of the G-TRACE and TARGET systems, which is akin to the mouse-inducible Cre/Rosa26-lacZ reporter system, would enable a precise lineage-tracing experiment to be performed. However, in our experience, the TARGET system always had a low level of leakage that complicated the interpretation of the lineage-tracing results. The Gal4s used in these experiments were all expressed in various cell types in embryos or during other earlier developmental stages. The marked progeny cells might come from other labeled cells during the earlier developmental stages instead of coming from the adult stem cells, if the leakage occurred during the earlier developmental stages. To overcome these complications, we developed a new (to our knowledge) lineage-tracing system (Fig. 2A,B), which combines the main features of the TARGET, G-TRACE and inducible Cre/loxP (Heidmann and Lehner, 2001) systems. We call this new system the TARGET technique for real-time and clonal expression system, or, for simplicity, the T-TRACE system.

We tested the T-TRACE system by using esg-Gal4 (in both stem cell ISCs and EBs) and Su(H)GBE-Gal4 [in Su(H)GBE+ EBs only] in the adult Drosophila posterior midgut (Micchelli and Perrimon, 2006; Zeng et al., 2010). When cultured on food without estrogen at 18°C, the flies of both Esg-Gal4/T-TRACE (supplementary material Fig. S2D) and Su(H)GBE-Gal4/T-TRACE (supplementary material Fig. S2H) grew to adulthood with no obvious phenotype, and they did not express GFP in their midguts; when cultured on food with estrogen at 29°C, or on food with estrogen at 18°C, the flies of both Esg-Gal4/T-TRACE (supplementary material Fig. S2B,C) and Su(H)GBE-Gal4/T-TRACE (supplementary material Fig. S2F,G) expressed GFP in a few cells in their midguts, indicating that either condition is causing partial leakage; when cultured on food...
with estrogen at 29°C, the flies of both Esg-Gal4/T-TRACE (supplementary material Fig. S2A) and Su(H)GBE-Gal4/T-TRACE (supplementary material Fig. S2E) expressed GFP in the cells of the respective Gal4 driver and daughter cells. These results suggest that the T-TRACE system is a reliable and leakage-proof system.

**ECs, but not EEs, develop from Su(H)GBE⁺ EBs**

To clarify cell lineage in the posterior midgut, we performed lineage-tracing experiments with the T-TRACE system on esg-Gal4, Di-Gal4 (in ISCs only) and Su(H)GBE-Gal4 in the adult Drosophila posterior midgut (Micchelli and Perrimon, 2006; Zeng et al., 2010). We cultured the adult flies on food with estrogen at a restrictive temperature (29°C). After 1 week, the flies were dissected, stained and examined for expression of GFP, Dl (ISC marker) and Pros (EE cell marker) under a confocal microscope. We found that EE cells represent 7.5% (80/1067) of the GFP-positive cells from the Dl-Gal4/T-TRACE (Figs 2D,D′ and 3B). Interestingly, Pros-positive EE cells are totally absent in GFP-positive cells from the Su(H)GBE-Gal4/T-TRACE (Fig. 3A-B). These data suggest that EE cells may be generated from absent in GFP-positive cells from the Su(H)GBE-Gal4/T-TRACE (Figs 2D,D′ and 3B). Similarly, Pros-positive EE cells are totally absent in GFP-positive cells from the Su(H)GBE-Gal4/T-TRACE (Fig. 3A-B). These data suggest that EE cells may be generated from the Su(H)GBE⁺ EBs through another way. This information suggests that there may be some transit cells expressing both the ISC marker DI and EE marker Pros that exist during EE maturation from ISCs in the posterior midgut. To test this, we stained posterior midguts with the ISC marker DI and the EE marker Pros and looked for some transit cells expressing both DI and Pros. Indeed, about 1.9% of ISCs (11/566, n=11) expressed both DI and Pros (Fig. 4A-A″) in wild-type posterior midguts. We renamed the DI⁺ Pros⁺ cells ‘pre-EEs’ or (EE progenitor) and the DI⁺ Pros⁺ cells ‘mature EEs’ or just ‘EEs’.

We unexpectedly found that Pros was expressed in some of the dividing phospho-Histone H3 (pH3⁺) positive ISC-like cells in N_{DN} (esg⁻<N_{DN}) midguts (supplementary material Fig. S3A-A″) when we were checking the mitotic index of N_{DN} (esg<_{N_{DN}}) midguts. This encouraged us to further examine the expression of Pros in dividing pH3⁺ ISCs in the wild-type posterior midgut. Strikingly, we found that 6.7% (42/628) of pH3⁺ ISCs express

![Fig. 3. EEs are not developed from Su(H)GBE⁺ and 5966GS⁺ EBs.](image-url)
few of Pros+ EEs can undergo S-phase and mitotic division type posterior midguts. Consistently, a recent study shows that a...s; supplementary material Fig. S4) in the wild-type midguts (supplementary material Fig. S6A-A‴). We also found one-cell ISC lineages in which the single cell is a ProsEE (n=32 from seven guts; Fig. 5C,C‴), indicating that an ISC may directly convert to an EE through a post-mitotic EE progenitor. The data above suggest that EE cells can be generated from ISCs in one of three ways: an ISC directly becoming one EE cell (32/49=65%; Fig. 5C-D); an ISC becoming two EE cells through symmetric division (12/49=24%; Fig. 5A,A‴; D); or an ISC becoming one new ISC and one EE cell through asymmetric division (5/49=10%; Fig. 5B,B‴; D). An EE cell occurs in either two-cell pairs (Ohlstein and Spradling, 2006) or in a single cell in the wild-type Drosophila midgut (supplementary material Fig. S5); the ratio of an EE cell pair to a single cell is 1:4.5. These EE cell pairs may be generated through symmetric ISC division, whereas the single EE cells may be generated through either asymmetric ISC division or direct conversion of an ISC into an EE cell.

Pros determines ISCs commitment to EEs

The expression of Pros in pre-EEs suggests that Pros may play an important role in the conversion of ISCs into EEs. To understand the function of pros in EE formation, we generated GFP-marked ISC clones that were homozygous for the loss-of-function allele pros17 (Cook et al., 2003), using the MARCM technique (Lee and Luo, 1999). Seven days after clone induction, we found that GFP-marked clones of pros17 were completely devoid of nc82 EE cells (Fig. 6B,D,D‴) compared with their wild-type counterparts (Fig. 6B-C″), whereas the ISCs in the GFP-marked clones showed normal proliferation (Fig. 6A). Consistently, the EE population dramatically decreased when we knocked down pros in both ISCs and EBs by expressing UAS-prosRNAi in these cells using esgA′ driver (Fig. 6E). To rule out the possibility that Pros directly regulates the expression of the EE cell marker Bp (the antigen of nc82) in contexts other than in EE cell formation, we identified another EE cell-specific marker, Dachshund, which is a transcriptional regulator required for eye and leg development (Mardon et al., 1994). The anti-Dac antibody Mabdac1-1 specifically labeled ProsEEs in wild-type midguts (supplementary material Fig. S4); as well as excessive EEs in UAS-Pros′RNAi (supplementary material Fig. S6B) and UAS-ace- (supplementary material Fig. S6C) overexpressing midguts. Consistently, we did not detect Dac′EEs in GFP-marked pros17 clones (supplementary material Fig. S6D-E″) compared with their wild-type counterparts (supplementary material Fig. S6D-E′). Together, these data suggest that Pros regulates EE cell fate determination.

To further clarify whether Pros functions in ISCs or Su(H)GAL80EBs to regulate EE cell fate determination, we generated an ISC-specific Gal4 system, in which Su(H)GAL4-Gal80 blocks the activation by Gal4 of Esg-Gal4 in Su(H)GAL80EBs, but keeps it active in ISCs only (this study; Wang et al., 2014). We named this Gal4 ‘ISC-Gal4’ and found that it is much stronger than the previously published DI-Gal4 (Zeng et al., 2010). Knockdown of pros in ISCs using the ISC-Gal4 system combined with tub-Gal80A′′ (esg-Gal4, UAS-GFP; Su(H)GAL80, tub-Gal80A′′; referred to as ISC′) resulted in fewer nc82 EE cells (Fig. 6E), whereas knockdown of pros in Su(H)GAL80 EBs using Su(H)GAL4-UAS-mCD8-GFP; tub-Gal80A′′ (referred to as Su(H)GAL80EBs) generated a phenotype that was no different from that of the wild-type control (Fig. 6E).
Surprisingly, however, the overexpression of pros in ISCs and EBs did not increase EEs in comparison with the wild-type control in the posterior midgut epithelium (data not shown).

These data suggest that Pros is necessary, but not sufficient, for EE cell fate determination in ISCs.

**Pros functions downstream of or parallel to the achaete-scute complex to determine EE fate in ISCs**

The members of the achaete-scute complex (AS-C), Scute (Sc) and Asense (Ase), have been shown to play a major role in EE cell fate determination and to be upregulated in the midgut that expressed a dominant-negative form of N (N\textsuperscript{DN}) (Bardin et al., 2010). The overexpression of Sc or Ase in both ISCs and EBs by Esg-Gal4 resulted in a dramatic increase in EEs (Bardin et al., 2010). To further clarify whether Sc and Ase function in ISCs or EBs to regulate EE cell fate determination, we expressed UAS-sc specifically in ISCs or Su(H)GBE+ EBs using ISC\textsuperscript{ts} or Su(H)GBE\textsuperscript{ts}. Compared with the wild-type control (supplementary material Fig. S7A,D,I), the expression of UAS-sc in ISCs generated an excess number of EE cells (supplementary material Fig. S7B,I), which were indistinguishable from those generated previously by the expression of UAS-sc in both ISCs and EBs using Esg-Gal4 (Bardin et al., 2010); however, the expression of UAS-sc in Su(H) GBE+ EBs (supplementary material Fig. S7B,I) generated a small number of EE cells (supplementary material Fig. S7E,I).

**Fig. 5. EEs are generated by symmetric and asymmetric division of ISCs and are directly converted from ISCs.** Fluorescence images showing T-TRACE analysis of the Di-Gal4 line at 29°C on food with estrogen. The adult fly posterior midguts were stained with anti-GFP (green), anti-Dl (cytoplasmic, red), anti-Pros (nuclear, red) and DAPI (blue). Scale bars: 5 μm. (A,B) An ISC becomes two EEs (arrows) through symmetric division. (B,B') An ISC becomes a new ISC (arrowhead) and an EE (arrow) through asymmetric division. (C,C') An ISC directly becomes an EE (arrow).

**Fig. 6. Pros regulates EE cell fate determination in ISCs.** (A) The quantitative cell numbers per clone in C-D'. (B) The quantitative percentages of nc82\textsuperscript{+} EEs in GFP+ clones in C-D'. Data are represented as means s.e.m. (C-D') MARCM clones of wild-type control (C,C') and pros\textsuperscript{17} (D,D'). Seven days after clone induction, GFP-marked clones of pros\textsuperscript{17} (D,D') were completely devoid of nc82\textsuperscript{+} EEs (arrow) compared with their wild-type counterparts (C,C'), whereas the clone sizes are similar (A). (E) The numbers of nc82\textsuperscript{+} EE cells in the posterior midguts of pros\textsuperscript{RNAi} driven by indicated Gal4, compared with wild-type controls. Data are represented as means s.e.m. *P<0.01. (F,F') The overexpression of sc and pros\textsuperscript{RNAi} in ISCs and EBs (esg\textsuperscript{ts}>sc+pros\textsuperscript{RNAi}). The overexpression of pros\textsuperscript{RNAi} suppressed the excess EE phenotype associated with sc overexpression, indicating that Pros functions either downstream of or parallel to Sc. The adult fly posterior midguts were stained with anti-GFP (green), anti-nc82 (red) and DAPI (blue). Scale bars: 10 μm.
phenotype that was no different from the wild-type control (supplementary material Fig. S7D,I). Likewise, the overexpression of the other three members of As-C, including *ase*, *I/l* and *achaete* (*ac*), in ISCs but not in Su(H)GBE+ EBs, resulted in a dramatic increase in EEs (data not shown). Conversely, we knocked down *sc* in ISCs or EBs by expressing UAS-scRNAi in these cells, and we found that the knockdown of *sc* in ISCs resulted in reduced EEs (supplementary material Fig. S7C,I), whereas the knockdown of *sc* in Su(H)GBE+ EBs did not change the EE cell population (supplementary material Fig. S8J).

Similarly, we expressed UAS-*N* in ISCs using cell type-specific Gal4 and found that the expression of UAS-*N* in ISCs caused both ISC and EE cell expansion phenotype, which is indistinguishable from those generated previously by the expression of UAS-*N* in both ISCs and EBs using Esg-Gal4 (supplementary material Fig. S8A,B; Bardin et al., 2010; Ohlstein and Spradling, 2007; Zeng et al., 2013). To explicitly evaluate the function of *N* in Su(H)GBE+ EBs, we expressed UAS-*N* in Su(H)GBE+ EBs using Su(H)GBE-Gal4 along with the T-TRACE system. Interestingly, compared with the wild-type control (supplementary material Fig. S8C), the expression of UAS-*N* in Su(H)GBE+ EBs completely blocked the differentiation of Su(H)GBE+ EBs into ECs (supplementary material Fig. S8D), whereas the EE cell density was not changed at all (supplementary material Fig. S8E). These data, plus previously published data, suggest that *N* regulates EE cell types through Sc and Ase only in ISCs.

To further determine an epistatic relationship between As-C and Pros, we expressed UAS-*sc* and UAS-prosRNAi in ISCs at the same time using *esg*46. Interestingly, compared with a dramatic increase in *nc82*+ EEs in the *sc* overexpression (esg*46*+sc) midgut, the expression of prosRNAi in the *sc* overexpression midgut (esg*46*+sc+ prosRNAi; Fig. 6G) suppressed the excess EE cell phenotypes associated with overexpressing *sc* (esg*46*+sc; Fig. 6F,F'). Likewise, the expression of prosRNAi in the *ase* midgut (esg*46*+ase+prosRNAi; supplementary material Fig. S7H) also suppressed the excess EE cell phenotypes associated with overexpressing *ase* (esg*46*+ase; supplementary material Fig. S7G). Together, Pros functions either downstream of or parallel to As-C to regulate EE cell fate in ISCs.

**DISCUSSION**

In the adult *Drosophila* posterior midgut, both terminally differentiated EC and EE cells are generated from the ISC. However, it is not clear how the differentiated cells are generated from the ISC. In this study, we found that only EEs are generated through the Su(H)GBE- immature progenitor EBs, whereas EEs are generated from ISCs through a distinct type of progenitors by an ISC converting to an EE, an ISC becoming a new ISC and an EE through asymmetric division, or an ISC becoming two EEs through symmetric division. The transcriptional factor Pros functions specifically in ISCs to guide the commitment of ISCs into EEs.

The anatomy and cell types in the *Drosophila* midgut are similar to those in the mammalian small intestine: both systems are a tubular epithelium composed of absorptive and secretory cells, which are constantly replenished by ISCs (Wang and Hou, 2010). We demonstrated that EEs are generated from ISCs through a distinct type of progenitor in the *Drosophila*, based on the following evidence. First, we found that some Esg+ cells express the EE marker Pros, but none of the Su(H)GBE+ cells expresses Pros. Second, we identified that Su(H)GBE+ EBs are the progenitors of EEs, instead of EEs, by using a novel lineage-tracing system: T-TRACE. To rule out the possibility that some Su(H)GBElow EBs may be the progenitors of EEs, we performed a lineage-tracing experiment using another EB Gal4 driver, 5966GS (Guo et al., 2014; Hur et al., 2013; Kapuria et al., 2012; Mathur et al., 2010), and further confirmed that 5966GS+ EBs are also not the progenitors of EE cells. Third, we identified a transit cell type called pre-EE (or EE progenitor), which expresses both the ISC marker DI and the EE marker Pros. Finally, we demonstrated that ISCs can convert into EE cells without cell division, by which 65% of EE cells are generated. In addition to direct conversion of an ISC into an EE cell, ISCs can undergo mitotic division to generate two EE cells by symmetric division, and an EE cell and a new ISC by asymmetric division, by which 35% of EEs are generated. Consistently, quiescent ISCs in mice have been recently identified as direct precursors of secretory Paneth cells and EE cells (Buczacki et al., 2013). These quiescent ISCs can differentiate into secretory Paneth cells and EE cells with minimal cell division (Buczacki et al., 2013). Recent studies in ISCs of mice also suggest that stem cell pools are maintained through population asymmetry. Some stem cells are lost due to differentiation or damage and their positions are filled by other stem cells using symmetric division (Snippert et al., 2010; Simons and Clevers, 2011). Further insight into the mechanism of mouse ISC regulation has been provided by a combination of *in vivo* and *in vitro* assays (Sato et al., 2011). It was found that the Lgr5+ stem cells are closely associated with the niche-supporting Paneth cells. Lgr5+ cells undergo neutral competition for contact with Paneth cell surface after symmetrical division. The detached stem cells are unable to maintain stem cell competence without access to short-range signals and progressively differentiate. In *Drosophila* posterior midgut, the predominance of ISC symmetric division is one of the main mechanisms for increasing the gut cell number during adaptive growth (O’Brien et al., 2011). The method of EE generation by ISCs may also change during environmental and dietary stresses to meet physiological need. For example, symmetric division of an ISC into two EEs may predominate when more secreting EEs are needed.

Based on the results of this study and previously published information (Bardin et al., 2010; Beebe et al., 2010; Ohlstein and Spradling, 2007; Perdigoto et al., 2011), we proposed a model of ISC lineage specification (Fig. 7). An ISC can generate an Su(H)GBE+ EB for EC lineage differentiation or a pre-EE for EE cell lineage differentiation. The N signaling promotes EC lineage differentiation and inhibits EE lineage differentiation through promoting ISC to Su(H)GBE+ EB commitment and blocking ISC to pre-EE commitment by repressing expression of *sc* and *ase* in ISCs, whereas N signaling in Su(H)GBE+ EBs is required for promoting Su(H)GBE+ EB to EC differentiation. The transcriptional
factor Pros functions specifically in ISCs to determine commitment of ISCs into EEs. Both pre-EEs and Su(H)GBE+ EBs are intermediate diploid cells, but they are different from each other. First, Su(H)GBE+ EBs do not express the ISC marker Di, but pre-EEs do. Second, Su(H)GBE+ EBs are post-mitotic cells (Ohlstein and Spradling, 2007), but some pre-EEs are able to undergo mitotic division. Our understanding of the flexibility of stem cell commitment is of the utmost importance for not only basic stem cell biology, but also targeted tumor therapy.

The T-TRACE lineage-tracing method
In mouse, the combination of a tamoxifen-inducible Cre knock-in allele with the Rosa26-lacZ reporter strain has been successfully used to identify stem cell lineages in adult mice (Barker et al., 2007; Takeda et al., 2011). In Drosophila, the FLP/FRT-based site-specific recombination system and its derivatives (including MARCM system) have been successfully used to identify cell (particularly stem cell) lineages. The FLP/FRT-based mosaic clonal systems are very effective for identifying a marked cell lineage, but they are not ideal for accurately identifying the origin of individual marked cells. In this study, we developed a new lineage-tracing system: T-TRACE. Similar to the mouse lineage-tracing system, the T-TRACE lineage-tracing method is a gene-based system and allows the accurate identification of all cells derived from one marked cell. However, the T-TRACE method does not require chromosome segregation at mitosis to label cells and may have limitation in some applications. Additionally, as the T-TRACE system is estrogen inducible, the variable efficiency of estrogen delivery to different tissue may obscure the tracing result. Thus, the optimal concentration of estrogen has to be determined for T-TRACE for different tissue. The combination of the FLP/FRT-based site-specific recombination system and the T-TRACE system, such as first using the FLP/FRT-based site-specific recombination system to identify general cell (particularly stem cell) lineages and then using the T-TRACE system to accurately identify the origin of individually marked cells, will give more precise and accurate information.

MATERIALS AND METHODS
Fly stocks
The following fly strains were used: esg-Gal4 line (from Shigeko Hayashi, RIKEN, Japan); Di-Gal4 and Su(H)GBE-Gal4 were generated in our laboratory and have been described previously (Zeng et al., 2010); UAS-NprD (from Mark Fortini, Thomas Jefferson University, Philadelphia, PA, USA); UAS-ase (from Yuh-Nung Jan, UCSF, CA, USA); UAS-Cre-EBD104 (from Christian Lehner, University of Zurich, Switzerland); FRT102b-pros17 and UAS-prosRNS1 (from Tiffany Cook, University of Cincinnati, OH, USA); 5966 GS (from Haig Keshishian, Yale University, New Haven, CT, USA); and Su(H)GBE-lacZ (from Sarah Bray, University of Cambridge, UK). The following strains were obtained from the Bloomington Drosophila Stock Center: UAS-sc; tub-Gal80/SM6, hs-flp; FRT102b, pim. The flies were cultured on food containing 150 μg/ml of estrogen at 29°C for 7 days before dissection. Then the stop cassette with flanking loxP sites was amplified from the UAS-dBrainbow construct (Hampel et al., 2011) using the following primers: 5’-AGTTGATACATACTTCGTATAAGATCCC-3’ and 5’-AGTTGATACATACTTCGTATAAGATCCC-3’.

The PCR product was then cloned into Ubi-pStiner at the KpnI sites to produce the Ubi-loxP-Stop-loxP-GFP. The above construct was sequenced, purified, and microinjected into embryos using the standard method.

Lineage tracing T-TRACE
The lineage-tracing system is doubly controlled by both temperature shift and estrogen induction. To perform lineage-tracing experiments, we used the following flies: Ubi-p63E<Stop<EGFP, esg-Gal4; tub-Gal80, UAS-cre-EBD104, Ubi-p63E<Stop<EGFP, Di-Gal4/tub-Gal80, UAS-cre-EBD104, and Ubi-p63E<Stop<EGFP, Su(H)GBE-Gal4; tub-Gal80, UAS-cre-EBD104. These flies were cultured on food without estrogen at 18°C. To initiate the lineage-tracing experiment, 3- to 5-day-old flies were transferred to new food containing 150 μg/ml of estrogen at 29°C for 7 days before dissection.

Pswitch tracing
UAS-flp/5966GS (PswitchC)-Gal4/act>y+<EGFP flies were cultured on food containing RU-486 (10 μg/ml) (Sigma) at 29°C for 7 days before dissection.

MARCM clonal analysis
To induce MARCM clones of FRT102b-pim (as a wild-type control) and FRT102b-pros17, we generated the following flies: act>y+>Gal4, UAS-GFP/SM6, hs-flp; FRT102b tub-Gal80/pim (or -pros17). Three- or four-day-old adult female flies were heat-shocked for 45 min twice, at an interval of 8-12 h, at 37°C. The flies were transferred to fresh food daily after the final heat shock, and their posterior midguts were processed for staining at the indicated times.

RNAi-mediated gene depletion
Male UAS-RNAi transgene flies were crossed with female virgins of esg-Gal4, UAS-GFP; tub-Gal80, esg-Gal4, UAS-GFP; Su(H)GBE-Gal4, tub-Gal80 (for ISC-specific expression) or of Su(H)GBE-Gal4, UAS-GFP; tub-Gal80 [for Su(H)GBE EB-specific expression]. The flies were cultured at 29°C. Three- to five-day-old adult flies with the appropriate genotype were transferred to new vials at 29°C for 7 days before dissection.

Fly genotypes
Fig. 1A: Su(H)GBE-lacZ, esg-Gal4, UAS-GFP.
Fig. 1E-F*: Su(H)GBE-lacZ/hs-flp, tub-Gal4, UAS-GFP; FRT82b, tub-Gal80/FRT82b, pim.
Fig. 2C: Ubi-p63E<Stop<EGFP, esg-Gal4; tub-Gal80, UAS-cre-EBD104.
Fig. 2D: Ubi-p63E<Stop<EGFP, Di-Gal4/tub-Gal80, UAS-cre-EBD104.
Fig. 3A: Ubi-p63E<Stop<EGFP, Su(H)GBE-Gal4; tub-Gal80, UAS-cre-EBD104.
Fig. 3B: UAS-flp/5966GS (PswitchC)-Gal4/act>y+<EGFP.
Fig. 5: Ubi-p63E<Stop<EGFP, Di-Gal4/tub-Gal80, UAS-cre-EBD104.
Fig. 6C: act>y+>Gal4, UAS-GFP/SM6, hs-flp; FRT102b tub-Gal80/FRT102b, pim.
Fig. 6D: act>y+>Gal4, UAS-GFP/SM6, hs-flp; FRT102b tub-Gal80/FRT102b, pros17.
Fig. 6F*: esg-Gal4, UAS-GFP/UAS-scute; Tub-Gal80.
Fig. 6G*: esg-Gal4, UAS-GFP/UAS-scute; Tub-Gal80ts/UAS-ProRNS1.

Histology and image capture
The fly intestines were dissected in PBS and fixed in PBS containing 4% formaldehyde for 20 min. After three 5-min rinses with PBT (PBS 0.1% Triton X-100), the samples were blocked with PBT containing 5% normal goat serum and kept overnight at 4°C. Then the samples were incubated with the primary antibody at room temperature for 2 h and incubated with the fluorescence-conjugated secondary antibody for 1 h at room temperature. Samples were mounted in the Vectashield mounting medium with DAPI (Vector Laboratories). We used the following antibodies: mouse anti-β-Gal


