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A Transient Niche Regulates the Specification of *Drosophila* Intestinal Stem Cells

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Abstract

Stem cell niches are locations where stem cells reside and self-renew. Although studies have shown how niches maintain stem cell fate during tissue homeostasis, less is known about their roles in establishing stem cells. The adult *Drosophila* midgut is maintained by intestinal stem cells (ISCs); however, how they are established is unknown. Here, we show that an ISC progenitor generates a niche cell via Notch signaling. This niche uses the bone morphogenetic protein 2/4 homolog, decapentaplegic, to allow progenitors to divide in an undifferentiated state and subsequently breaks down and dies, resulting in the specification of ISCs in the adult midgut. Our results demonstrate a paradigm for stem cell–niche biology, where progenitors generate transient niches that determine stem cell fate and may give insights into stem cell specification in other tissues.

Intercellular factors regulate stem cell proliferation and maintenance in stem cell niches in the *Drosophila* ovary (1) and testis (2,3), as well as in mammalian systems, such as the hematopoietic system (4–6), skin (7), and neural cells (8). These niches, which are generally fixed stromal locations, signal to prevent stem cell differentiation (9,10). However, even though the role of niches in the maintenance of tissue homeostasis has been well examined, relatively little is known about their function in establishing stem cell lineages during organogenesis.

The lineage of intestinal stem cells (ISCs) in the adult *Drosophila* midgut (11,12) can be tracked to determine how progenitors establish different intestinal cells during development. Adult midgut progenitors (AMPs) from the three larval stages generate all epithelial cells in the adult midgut, including ISCs, enterocytes, and enteroendocrine cells (table S1) (13,14). In the first two instars (L1 and L2), AMPs proliferate and disperse throughout the midgut. Dispersal stops by the third instar (L3), when AMPs proliferate and form clusters known as midgut imaginal islands. During metamorphosis, when the larval gut histolyzes, the islands merge and generate the adult midgut epithelium, including ISCs, enterocytes, and

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Supporting Online Material

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Materials and Methods

Figs. S1 to S10

Table S1

References

enteroendocrine cells (fig. S1). Here we analyze the mechanism by which these cells are established from this pool of AMPs.

Because Notch signaling determines stem cell–daughter identity in the adult midgut (11,12,15), we asked if it plays a similar role during larval intestinal development. We examined the expression of the Notch ligand, Delta (DI), which is expressed in adult ISCs, and the Notch reporter, *Gbe⁺Su(H)LacZ*, which marks ISC daughters, called enteroblasts (table S1), in the process of differentiation. We identified enteroendocrine cells by staining for the nuclear protein Prospero, and we distinguished enterocytes by their polyploid nuclei with 4',6'-diamidino-2-phenylindole (DAPI) staining (11,12,15). In late L1 (16), AMPs could be identified as single DI-positive cells dispersed throughout the midgut, although no Notch activity could be detected at this time by staining for *Gbe⁺Su(H)LacZ* (Fig. 1A). Toward the late L2, when AMP islands contained two cells, one cell was DI-positive, and the other was *lacZ*-positive (Fig. 1B). Because it has been shown that all cells in an AMP island arise from a founder AMP (14), the first AMP division after the dispersion phase must be asymmetric, from which one daughter became a *lacZ*-positive cell. Thereafter, as the island grew, all newly generated AMPs expressed DI but were *lacZ*-negative (Fig. 1, C and D).

We observed that by mid L3, the *Gbe⁺Su(H)lacZ*-positive cell extended processes that encased all AMPs in the island (Fig. 1C). The *lacZ*-positive cells were similar to those described more than 50 years ago by El Shatoury and Waddington, who called them peripheral cells (PCs) (table S1); however, the function of these cells was not determined (17). As the island grew, the PC processes extended even further (Fig. 1D). Additionally, the PC nucleus appeared to be larger than AMP nuclei and often had a bent shape (Fig. 1C). The PC processes could also be outlined by staining for the *Drosophila* β -catenin homolog Armadillo (Arm) and appeared to extend loosely around AMP clusters, which were tightly packed as indicated by Arm expression between AMPs (fig. S2). Although in most islands there was only one PC, by late L3 ~20% of the islands ($n = 328$) had a second PC, which was at the opposite end from the first one (fig. S2). We made MARCM (mosaic analysis of a repressible cell marker) clones to elucidate the origin of the second PC. In clusters where two PCs were present, our clonal analyses indicated that the second one was generated by AMPs, and, therefore, PCs did not undergo any divisions (fig. S3).

Because AMPs express DI and PCs express *Gbe⁺Su(H)lacZ*, we investigated the role of Notch signaling in the generation of a PC. Loss-of-function clones of *Notch* induced in early L1 larvae and analyzed at late L3 lacked a discernible PC, suggesting that Notch signaling is required for PC generation (Fig. 1, E and F). Furthermore, *Notch* mutant islands tended to merge together, indicating that PCs may be required to keep islands separated throughout the midgut (Fig. 1E). However, the lack of a PC did not seem to affect AMP divisions, implying that the activity of known AMP proliferation signals, the epidermal growth factor receptor ligands (14) emanating from the overlying muscle and surrounding AMPs, was not affected. In a screen to identify cell-type-specific, RU-486 (mifepristone)-inducible GAL4 lines (Pswitch) (16, 18) expressed in the larval midgut, we found one that was specifically expressed in the midgut in AMP islands (Pswitch^{AMP}) (Fig. 1G). Pswitch^{AMP} was recombined with UAS—mCD8::GFP (GFP, green fluorescent protein), which localizes to cell membranes, and used to induce expression of activated Notch (N^{act}) in AMPs of early L1 larvae. N^{act} expression directed AMPs to differentiate into cells that morphologically resembled PCs, as indicated by their long processes (Fig. 1H). Moreover, these processes lacked directionality, suggesting that they are normally attracted by an unknown signal secreted by AMPs. These experiments suggest that Notch signaling in AMPs is both necessary and sufficient for PC generation.

From our Pswitch screen, we also identified a GAL4 line that was expressed specifically in PCs throughout the larval midgut (Fig. 2A and fig. S4A) and enteroblasts in the adult posterior midgut (fig. S4B). When we recombined this GAL4 line (Pswitch^{PC}) with UAS-mCD8::GFP, we were able to clearly visualize the long processes that encase AMP islands. Furthermore, Pswitch^{PC}-mediated expression of actin5C-GFP showed that the cytoskeleton of these processes was actin-rich (fig. S5).

Pswitch^{PC} UAS-GFP was used to trace the development of PCs and AMPs into the early hours of metamorphosis, when the adult midgut is formed (19). At 0 hours after puparium formation (APF), AMP islands with surrounding PCs could be detected (Fig. 2B). However, between 2 and 3 hours APF, PC processes appeared to open up and spread out, AMPs began to be released from the islands (Fig. 2, C and D), and two pools of AMPs emerged. The larger pool consisted of AMPs that expressed both GFP, like that seen with enteroblasts in the adult midgut, and Pdm1, a marker for adult (20) and larval enterocytes (fig. S6), indicating that these AMPs were differentiating into enterocytes. Intermingled within these differentiating AMPs were fewer AMPs consisting of cells negative for both GFP and Pdm1 expression, some of which expressed DI, similar to the expression pattern of ISCs in the adult midgut. However, because both PCs and differentiating AMPs were GFP-positive at this stage, we followed the expression of Stat92E-GFP, a GFP reporter for JAK/STAT (JAK, Janus kinase; STAT, signal transducers and activators of transcription) signaling (21), as a marker that we identified was expressed specifically in PCs in the larval and pupal midguts (Fig. 2E). At 2 hours APF, we observed breakdown of processes in Stat92E-GFP-positive PCs (Fig. 2, F and G). Consistent with this fragmentation, PC nuclei stained positive for active caspase-3, indicating that these cells were undergoing programmed cell death (Fig. 2H). By 4 hours APF, they were no longer visible in the pupal midgut (fig. S7). Following PC breakdown (after 4 hours APF), most AMPs continued to differentiate into Pdm1-positive cells and started losing their enteroblast-like Pswitch^{PC} expression. They accumulated membrane-bound DI, suggesting that they were no longer able to traffic DI into endocytic vesicles, which is essential for DI-Notch signaling (Fig. 2, I and J) (22). By 14 hours APF, Pdm1-positive cells in the pupal gut were no longer DI-positive, whereas all cells with vesicular DI remained Pdm1-negative. Based on DI expression in these Pdm1 negative cells, they probably correspond to the ISCs of the adult midgut (Fig. 2, K and L). The number of these cells at this stage (767 ± 142.7 , $n = 4$ guts) correlated approximately with the number of islands in late L3 (820 ± 91.1 , $n = 10$ guts), suggesting that, on average, one AMP from each island becomes a future ISC. In support of this hypothesis, these numbers also correspond with the number of ISCs (~1000) previously reported to be present in an adult midgut (11).

The observation that the breakdown of PC processes during metamorphosis correlates with the appearance of Pdm1-positive cells in AMP islands suggests that PCs may regulate AMP differentiation and establishment of ISCs. To determine the function of PCs, we crossed Pswitch^{PC} UAS-GFP to a programmed cell death-inducing line, *UAS-reaper*, and fed larvae RU-486 at early L3 to induce *reaper* expression in PCs. By mid L3, PC nuclei stained for active caspase-3, indicating that these cells were dying (fig. S8). Subsequently, by late L3, AMPs differentiated into polyploid enterocyte-like cells, as established by positive staining for GFP, Pdm1, and DAPI (Fig. 3, A and B). This result suggests that PCs are required for maintaining AMPs in an undifferentiated state until the onset of metamorphosis. In further support of this hypothesis, at 4 hours APF, when PCs have normally disappeared and AMPs have scattered throughout the midgut, Pswitch^{PC} UAS-GFP-induced expression of the cell death-blocking line, *UAS-p35*, delayed the disappearance of PCs and differentiation of AMPs (Fig. 3, C and D). Consequently, PC-encased AMP islands with multiple DI-positive cells could be detected at this stage, further suggesting that the loss of PCs is required for directing AMPs to generate ISCs and differentiated cells in the adult midgut.

These experiments demonstrate that in the larval midgut, the PC acts as a niche for AMPs to keep them undifferentiated until metamorphosis. Stem cell niches in the *Drosophila* ovary and testis send molecular signals to stem cells to keep them undifferentiated (23–25). Because both these niches use bone morphogenetic protein signaling to maintain stem cells, we tested the relevance of this pathway in maintaining the differentiation state of AMPs. *Decapentaplegic* (*Dpp*) RNA interference (RNAi) induced specifically in PCs using Pswitch^{PC} UAS-GFP resulted in the appearance of positive, polyploid cells in the islands, which started to break away from other AMPs in the island, as indicated by a decrease in Arm staining between the cells (Fig. 4, A and B). Similar to differentiating enteroblasts, these cells also expressed GFP, suggesting that a Dpp signal from the PC maintains AMPs in an undifferentiated state. However, these differentiating cells were Pdm1-negative, suggesting that other signals that repress differentiation are present. In accordance with the *dpp* RNAi results, loss-of-function MARCM clones of a Dpp receptor, *thickveins* (*tkv*) (26), and a downstream effector molecule, mothers against decapentaplegic (*Mad*) (27), both resulted in premature differentiation of AMPs into large, polyploid, enterocyte-like cells (Fig. 4, C and D) compared with wild-type (WT) MARCM clones (fig. S9).

Our studies suggest how stem cells might be determined during intestinal organogenesis (fig. S10). After symmetric divisions and dispersal during early larval development, a founder AMP undergoes an asymmetric division and signals via the Notch pathway to direct its first daughter to become a PC that acts as a niche, where the AMP and its subsequent daughters can remain undifferentiated in response to a Dpp signal from the PC, and proliferate to form AMP islands. During metamorphosis, the PC breaks down, allowing AMPs in the island to respond to Notch signaling and differentiate into enterocytes. However, one AMP per island, on average, remains undifferentiated and becomes a future ISC. The mechanism that allows this AMP to stay undifferentiated remains to be determined.

Unlike other characterized niches, where a stem or progenitor cell moves away from its niche to differentiate (1,7,28), the PC niche is a holding pen, which does not allow its cells to escape or to differentiate until the niche breaks down. The transition to a functionally homeostatic adult niche that maintains ISCs would require a separate step. Our observations indicate a paradigm that other stem cell systems may also use: The progenitor cell divides to form both niche and stem cells. Such a mechanism that lends greater autonomy to stem cells might exist in other epithelial cell populations during development or tissue homeostasis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Characterization of AMP islands during larval development. **(A)** During late L1, AMPs exist as single, DI-positive cells (arrowheads). Larval enterocytes are polyploid (arrow), and enteroendocrine cells are Prospero-positive (asterisk). **(B)** By late L2, an AMP island contains one DI-positive AMP (arrowhead), and another cell that is *Gbe⁺Su(H)lacZ*-positive (arrow). **(C)** The *Gbe⁺Su(H)lacZ*-positive cell (asterisk) extends processes (arrows) around AMPs, throughout mid L3 and **(D)** late L3, and islands contain multiple Delta-positive AMPs (arrowheads). (A to D) DAPI, nuclear blue; DI, cytoplasmic red; Prospero, nuclear red; and β -galactosidase, green. **(E and F)** Notch mutant MARCM clones (green, asterisk) lack a discernible PC [(inset in (E))]. Sometimes, mutant clones merge with each other (dashed line). They also have increased DI staining at the membranes [inset in (F)], whereas WT islands (arrow) have predominantly vesicular DI. **(G)** Pswitch^{AMP} UAS-GFP (green), expressed in all cells of an AMP island (late L3 shown) used to **(H)** ectopically express N^{act} at early L1, results in differentiation into a PC-like cell (green) with long extensions (arrows) when analyzed at late L3. (E, G, and H) DAPI, nuclear blue; DI, cytoplasmic red; Prospero, nuclear red; and GFP, green. Scale bars, 10 μ m.

Fig. 2.

Developmental fate of AMPs and PCs during metamorphosis. (A) Pswitch^{PC} UAS-GFP labels PCs (arrows) surrounding DI-positive AMPs (arrowheads) during late L3 and (B) 0 hours APF. (C and D) Between 2 and 3 hours APF, PC (asterisk) extensions appear to spread out (arrows), and most AMPs (stars) express GFP and Pdm1, indicating differentiation into enterocytes. A smaller AMP population (arrowheads) remains Pdm1-negative, whereas some cells are Delta-positive. (E) Stat92E-GFP is expressed only in PCs (arrows) at 0 hours APF. (F and G) At 2 hours APF, PC (asterisk) processes break apart (arrow) and AMPs express Pdm1 (arrowheads). (H) PCs stain positive for active caspase-3 (arrows). (I and J) At 4 hours APF, Pswitch^{PC} UAS-GFP expression is diminished in differentiating AMPs. Most Pdm1-positive cells accumulate membrane-bound DI (arrows). Pdm1-negative cells with vesicular DI (arrowheads) are present at this stage. (K and L) By 14h APF, Pdm1-positive cells lose GFP and DI expression (arrows), whereas Pdm1-negative cells are DI-positive (arrowheads). (A to C, F, I, and K) GFP, green; DI, cytoplasmic red; Prospero, nuclear red; and DAPI, nuclear blue. (D, G, and L) Pdm1, nuclear grayscale, unmerged. (H) GFP, green; active caspase-3, nuclear red; and DAPI, nuclear blue. Scale bars, 10 μ m.

Fig. 3. The PC acts as a niche that regulates AMP differentiation. (**A** and **B**) $Pswitch^{PC}$ UAS-GFP-mediated expression of *UAS-reaper* at early L3 results in differentiation of AMPs into polyploid, GFP-positive, Pdm1-positive, enterocyte-like cells with a large cytoplasm (arrows indicate PDM1-positive, polyploid nuclei; dashed lines denote enterocyte-like cells). DAPI, blue; GFP, green; and Pdm1, red, nuclear. (**C** and **D**) (DI channel) $Pswitch^{PC}$, UAS-GFP-driven expression of UAS-P35 results in prolonged PC survival (arrowhead) and delayed AMP differentiation (arrows). DAPI, nuclear blue; GFP, green; DI, cytoplasmic red; and Prospero, nuclear red. Scale bars, 10 μ m.

Fig. 4. PCs signal via the Dpp pathway to prevent AMP differentiation. (A and B) Pswitch^{PC} UAS-GFP-driven expression of *dpp* RNAi results in (A) AMP differentiation into GFP-positive, polyploid, enterocyte-like cells (arrow, dashed line) that dissociate from other AMPs, indicated by diminished Arm staining, as compared with the Arm expression (arrowhead) between GFP-negative, undifferentiated AMPs. (B) Arm is expressed between cells in islands with GFP UAS-GFP-negative AMPs (arrowhead). MARCM clones of (C) *tkv* and (D) *Mad* result in differentiation of AMPs into polyploid enterocyte-like cells (arrows). (A to D) GFP, green; DAPI, nuclear blue. (A and B) Arm, red. Scale bars, 10 μ m.