# Intestinal stem cell function in *Drosophila* and Mice

**Huaqi Jiang**[a] and **Bruce A. Edgar**[b]

[a]Department of Developmental Biology, UT Southwestern Medical Center, 6000 Harry Hines Blvd., Dallas, TX, 75235 USA

[b]German Cancer Research Center (DKFZ)-Zentrum für Molekulare Biologie der Universität Heidelberg Alliance (ZMBH), Im Neuenheimer Feld 282, 69120 Heidelberg, Germany

## Abstract

Epithelial cells of the digestive tracts of most animals are short-lived, and are constantly replenished by the progeny of long-lived, resident intestinal stem cells. Proper regulation of intestinal stem cell maintenance, proliferation and differentiation is critical for maintaining gut homeostasis. Here we review recent genetic studies of stem cell-mediated homeostatic growth in the *Drosophila* midgut and the mouse small intestine, highlighting similarities and differences in the mechanisms that control stem cell proliferation and differentiation.

## Introduction

The endodermal portion of the insect intestine, termed the midgut, and its mammalian counterpart, comprising the stomach, small intestine and colon, serve as the animal's principal organs for digestion and nutrient absorption. In the mammalian small intestine, absorptive enterocytes and secretory goblet and enteroendocrine cells reside in finger-like protrusions known as villi. These cells are short-lived, being constantly shed from the villi and replaced by new cells generated in neighboring invaginations called the Crypts of Lieberkühn. The intestinal epithelium is perhaps the most rapidly turned over tissue in mammals, with enterocyte lifespans averaging a week or less. Intestinal stem cells (ISCs) reside at the basal ends of the crypts, intermingled with long-lived Paneth cells of the secretory lineage (Figure 1A). ISCs proliferate to self-renew and also give rise to transient progeny that amplify through further divisions. As cells exit the crypts and move apically, they differentiate into either absorptive enterocytes or one of three types of secretory cells: Paneth, enteroendocrine, or goblet. The mammalian colon is similarly maintained by ISCs located in crypts, but villi are absent and replaced by a smooth epithelium. In addition to these endodermal cells produced by ISCs, the mammalian intestine has stromal cells of several types – mesenchymal fibroblasts, immune cells and others – and is surrounded by mesodermally derived visceral muscle.

The endodermal portion of the *Drosophila* intestine, termed the midgut, undergoes similar dynamic cell turnover, also mediated by long-lived intestinal stem cells [1,2]. The fly midgut however lacks crypts and villi, instead comprising a cellular monolayer ensheathed by two orthogonal layers of visceral muscle. Intestinal stem cells reside at the basal side of

Correspondence to: Huaqi Jiang; Bruce A. Edgar.

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this epithelium, sandwiched between enterocytes and basement membrane produced in part by visceral muscle (Figure 1B). They divide to self-renew and to give rise to committed progenitors (called enteroblasts), which directly differentiate, without cell division, into two functional cell lineages similar to those found in vertebrates: absorptive enterocytes and enteroendocrine cells. Differentiating enterocytes endoreplicate their genome 2–3 times to increase their size and develop a brush border similar to that in mammals. *Drosophila* lacks the Paneth, Goblet, Stromal, and Dentritic cells found in mammals, but some of their functions – such as immunity and barrier production - are fulfilled by enterocytes. Instead of the thick mucosa produced by mammalian goblet cells, insect intestines have a tough but relatively thin (~200μm) membrane called the peritrophic matrix. This matrix is comprised of the exoskeletal protein chitin and glycoproteins including mucins related to those found in vertebrate mucosa, and it provides an essential barrier against infection by enteric pathogens [3].

## Intestinal stem cell niches

The location and number of intestinal stem cells in the crypts of the mammalian intestine have long been debated. Studies using improved stem cell markers and elegant cell lineage tracing techniques, however, suggest that there may be two inter-convertible stem cell types present in the crypts [4,5]. One cell type, located at the "+4" position in the crypt (see Figure 1A), is marked by *Bmi, Tert* and *Hopx* expression. These slow-cycling, label-retaining cells can produce entire intestinal cell lineages [6,7]. Another group of cells with stem properties, located at the bottom of the crypts and interdigitated with Paneth cells, have been called crypt basal columnar cells (CBCs). CBCs are marked by *Lgr5, CD133* and *Sox9* expression. These are fast-cycling cells that are also capable of giving rise to entire crypts and villi within 3 days [8–10]. Cell ablation and lineage tracing demonstrated that these two stem cell types could interconvert, suggesting that slow-cycling +4 Bmi^{+} stem cells might function as a reserve stem cell pool for fast-cycling, Lgr5^{+} CBCs [5]. In the mouse small intestine, the Lgr5^{+} stem cells reside between Paneth cells and the +4 Bmi^{+} cells reside just above the Paneth cell zone (Figure 1A). Gene expression profiling of Paneth cells revealed that they express essential regulators of ISC growth and survival, including EGF, TGF-α, Wnt3 and Dll4 [11]. Although early models invoked the intestinal stroma as a source of such niche factors, recent *in vitro* organoid culture experiments as well as *in vivo* genetic tests indicate that Paneth cells serve an essential supportive niche role for the ISCs [11,12]. As Paneth cells are long-lived secretory cells of the ISC lineage, it can be concluded that ISCs generate an important component of their own niche. Paneth cells have been reported to be absent from the colon, however, indicating that there may be more than one way to build a niche for ISCs.

The midgut epithelium of *Drosophila* lacks any cell type obviously analogous to a Paneth cell, but ISCs adhere via integrins to the visceral muscle. The muscle expresses several potential niche factors that are capable of promoting ISC growth, including *wingless* (a Wnt), *vein* (an Egfr ligand), and *dilp3* (an insulin-like peptide) [13–15]. Hence the visceral muscle has been proposed to serve as the stem cell niche [13–15]. However, cells within the ISC lineage -differentiated enterocytes and transient enteroblasts - also produce survival and growth factors that support *Drosophila's* ISCs. As detailed below these factors are especially important during gut epithelial regeneration following damage, but they are also likely to be used for stem cell maintenance. Hence, in so far as stem cell progeny comprise an important part of the niche, insects look somewhat similar to vertebrates. Interesting in this light is that, during the midgut's development, stem-like progenitor cells proliferate to form clusters, and some cells within these clusters differentiate into peripheral cells that function as a transient niche for the progenitors that build the adult gut and contribute the adult ISCs [16,17]. These peripheral cells produce Dpp, a BMP-type signal that suppresses

differentiation, and the EGFR ligands Spitz and Keren [17], potent ISC growth factors. Thus, during midgut development, stem-like progenitor cells also generate an essential part of their own niche. The *in vitro* self-assembly of intestinal organoids composed of crypts and villi from isolated murine ISCs and Paneth cells [11,12] suggests a similar capability in mice.

## Stem cell proliferation, differentiation, and the regenerative response

Genetic analyses identified canonical Wnt signaling as a primary force in maintaining tissue homeostasis in the murine intestine. Mice lacking the Tcf4 transcription factor or β-catenin, positive effectors in Wnt signaling, have reduced proliferation in the intestinal epithelium that depletes transient amplifying cells, and consequent loss of crypts and villi [18–20]. Conversely, activating Wnt signaling drives hyperproliferation in the crypts, as do loss-of-function mutations in *APC* (*adenomatous polyposis coli*), a negative effector of Wnt signaling. Such mutations are associated with a majority of CRC, and their relevance as causal initiators of adenoma formation has been confirmed in several mouse models [19,21–24].

In contrast to the central role played by Wnt signaling in maintaining the stem/progenitor cell compartment in the murine intestine, alterations in *Drosophila* Wnt signaling show relatively mild phenotypes. Although the fly's visceral muscle produces a Wnt ligand, *wingless (wg)*, that is important in ISC maintenance [14,15], loss of both fly *APC* homologs or ectopic expression of Wg or Arm$^{S10}$ (activated *β-Catenin*) lead only to slow onset dysplasia and modest increases in intestinal stem cell proliferation. In these cases, both differentiated cell types were still produced indicating that, *wingless* signaling does not control self-renewal or differentiation as in the mouse [14,25]. Further studies are needed, however, to fully appreciate the role of Wnt signaling in the *Drosophila* midgut.

In mammals, *Notch* signaling is also essential to maintain cells in the crypt compartment in their undifferentiated, proliferative state [26]. Activation of Notch signaling impairs secretory cell differentiation and leads to an increase in Wnt-dependent proliferating cells that expand to the crypt-villus boundary [27,28]. Consistent with a pro-proliferative function, mice that are mutant for CSL, a transcription factor required together with Notch for target gene activation, display the reciprocal phenotype: the proliferative compartment decreases at the expense of an expanded population of secretory (goblet) cells. Similar phenotypes were obtained with the γ-secretase inhibitor dibenzazepine (DBZ), when the *Notch1* and *Notch2* receptors were deleted [29], or when *Dll1* and *Dll4* ligands were deleted [30]. Further supporting Notch's role in maintaining progenitor cells, the Notch ligand *Dll4* is expressed by Paneth cells [11], and lineage tracing of Notch activity has indicated that Notch signaling is active in intestinal stem cells [31].

Notch is also a central regulator in the fly's intestine, but its precise functions only partially overlap with those in the mouse. In the fly's midgut, *Notch* is expressed in progenitor cells, including ISCs and their undifferentiated sisters, the enteroblasts. The Notch ligand, Delta (*Dl*) is expressed only in stem cells, however, resulting in asymmetric Notch activation only in enteroblasts [32]. Loss of *Notch, Dl*, or other pathway components such as *Su(H)* or *Neuralized*, or treatment with the γ-secretase inhibitor DAPT, all result in defective lineage commitment and a rapid, exponential expansion of stem-like cells that produce endocrine cells, but not enterocytes [1,2,32–34]. Conversely, ectopic expression of an activated Notch (N$^{intra}$) drives the rapid, direct differentiation of ISCs into enterocyte-like cells and consequently depletes the gut of stem cells [1]. Thus, although the role for *Notch* signaling in suppressing secretory cell fate is seen in both systems, Notch signaling has no apparent pro-proliferative function in the fly's midgut. In considering how to reconcile this striking

difference, Fre et al. [26] have noted that in both systems Notch activation may promote the transient cell fate, and that the principle difference may be that the fly's transient cells, the enteroblasts, are post-mitotic.

Cytokines related to leptins and interleukins (termed Unpaireds, Upd) and ligands for the EGF receptor (Spi, Krn, Vn) act as growth, mitogenic, and survival factors for *Drosophila* intestinal stem cells [15,35–40]. Ectopic activation of Jak/Stat or EGFR signaling promotes rampant stem cell proliferation, leading to severe midgut hyperplasia, whereas midguts defective in either pathway suffer reduced rates of epithelial renewal, leading in some cases to atrophy. These functions are particularly prominent during regenerative growth following injury or enteric infection. Multiple ligands in each pathway are expressed by different midgut cell types, with some (Upd2, Upd3, Vn) being strongly induced following damage and others (Spi) more constitutive. Positive feedback between cells types and the EGFR, JAK-Stat, and JNK pathways generates a robust response akin to inflammatory signaling in mammals and capable of driving rapid epithelial repair. The activation of negative feedback inhibitors (e.g. SOCS, Puckered) helps to downregulate the response as repair is completed. These same cell- and signaling interactions appear to act at lower levels to maintain basal rates of epithelial replacement. From these observations, a feedback mechanism for regulating midgut homeostasis and regeneration was proposed, in which signaling from spent enterocytes promotes their replacement by activating the ISCs.

Although a similar sort of retrograde feedback from villi to crypts has not yet been rigorously elucidated in mammals, the mouse intestine regenerates rapidly following damage and appears to depend on Cytokines (*e.g.* IL-6) and Stat signaling to repair itself [41] [42]. Murine ISCs are also known to require EGFR/ErbB signaling for their growth [11,12]. Indeed a recent report has shows that a negative feedback regulator of ErbB/EGFR signaling, Lrig1, is highly expressed in the crypts, and that its deletion leads to excessive ISC proliferation, crypt expansion, and longer villi in the small intestine [43]. Thus Cytokine/Jak/Stat and Receptor Tyrosine Kinase/Ras/MapK signaling appear to have important roles in controlling ISC growth in the mouse. However, other signals such as Bone Morphogenetic Proteins (BMPs), Sonic Hedgehog (Shh) [44] or Ephs and ephrins [45] are also plausible regulators of crypt/villus balance. In any case it is difficult to imagine how the mouse intestine, being subject to extremely dynamic changes in nutrients and enteric ecology, could maintain homeostasis without a sophisticated system of feedback between differentiated cells and stem cells.

Recent work from both *Drosophila* and the mouse has also highlighted a role for *Hippo* signaling in intestinal epithelial homeostasis. In the mouse, loss of negative effectors (Mst1, Mst2, Sav) or gain of a positive downstream transcription co-activator (Yap1) in the Hippo signaling pathway results in small intestinal hyperplasia and adenoma formation. Furthermore, Yap was demonstrated to be induced and essential for effective intestinal regeneration following damage [46–48]. Similar results were obtained in *Drosophila*, with the additional insight that Hippo signaling in differentiated enterocytes was essential to restrain the proliferation of ISCs [49–53]. This surprising finding led to the proposal that *Hippo* signaling, as a known sensor of cell adhesion and cytoskeletal integrity, might be part of the first-line damage sensing mechanism to control expression of the cytokines and growth factors that regulate ISC proliferation. Such damage sensing functions could be relevant to diseases involving chronic inflammation in the intestine, such as ulcerative colitis and Crohn's disease, which are risk factors for CRC. Indeed, mutations in the *Hippo* pathway are now being recognized in CRC. Nevertheless, the fly work showed that Hippo signaling is unlikely to be the only damage sensor in the intestinal epithelium, and other mechanisms, for instance JNK signaling, are believed to play major roles.

## Symmetric *vs.* asymmetric stem cell division

To maintain tissue homeostasis, stem cells must balance self-renewal with differentiation. Early models proposed that stem cells are immortal and always divide asymmetrically, replacing themselves at each division, but quantitative lineage analysis in the murine intestine has ruled out this classical model for tissue maintenance [54]. Rather, lineage tracing showed that fast-cycling intestinal stem cells divide symmetrically and are lost stochastically, mostly to differentiation. At the population level, this loss rate averages 50%, and so a constant stem cell pool is maintained [54,55]. To explain the exact balance of ISC loss and duplication, it was postulated that $Lgr5^+$ stem cells at the crypt base undergo "neutral" competition for niche signals provided by a limited number of Paneth cells, which thereby define the number of stem cells each crypt can support. The mechanisms determining the number of Paneth cells, and how new crypts emerge through crypt fission during regeneration, thus become interesting questions.

For *Drosophila*, initial clonal assays suggested that nearly all ISC divisions were functionally asymmetric [2,32], but a second look revealed a picture more similar to the mouse, in which stem cells can either be duplicated or lost to differentiation following symmetric divisions [13,56]. In this case it has been proposed that proximity of an ISC to the visceral muscle, which produces growth and survival factors such as an EGFR ligand (Vein), a Wnt (Wingless), and an insulin like peptide (dILP3), may promote stemness or at least ISC survival and maintenance [13,14,40]. It is noteworthy that *Drosophila's* enteroblasts (EBs, Fig 1B), transient stem cell daughters that adhere to ISCs, also make an EGFR ligand (Spitz) and a cytokine (Upd) that promote ISC growth. This suggests that fly enteroblasts may function analogously to the mouse's Paneth cells, as a limiting niche component that controls ISC number. In addition to this, any factor that tips the balance of lateral inhibition during Notch/Delta signaling between ISC/EB pairs will have a critical impact on ISC number in the fly's midgut. The rate of ISC division is one such factor, since this determines the number of cells in the clusters that participate in Delta/Notch interactions. Also quite interesting in this regard is the finding [13] that increased insulin signaling, a response to feeding that stimulates ISC growth and division, can increase the likelihood of functionally symmetric, duplicative stem cell divisions. This can expand the stem cell pool, and consequently the size of the whole gut. Starvation, which reduces insulin signaling, was found to have the opposite effect, allowing gut shrinkage during fasting [13]. Effects of nutrition on the stem cell lineage have not yet been documented in a mammal, though a similar phenomenon was reported to occur during the establishment of crypts and villi in newborn mice [57]. Further advances in understanding the *in vivo* rules governing ISC duplication should be highly relevant to regenerative medicine, and also promise to help explain known connections between inflammation, nutrition, and stem cell derived cancers such as colorectal carcinoma.

## Acknowledgments

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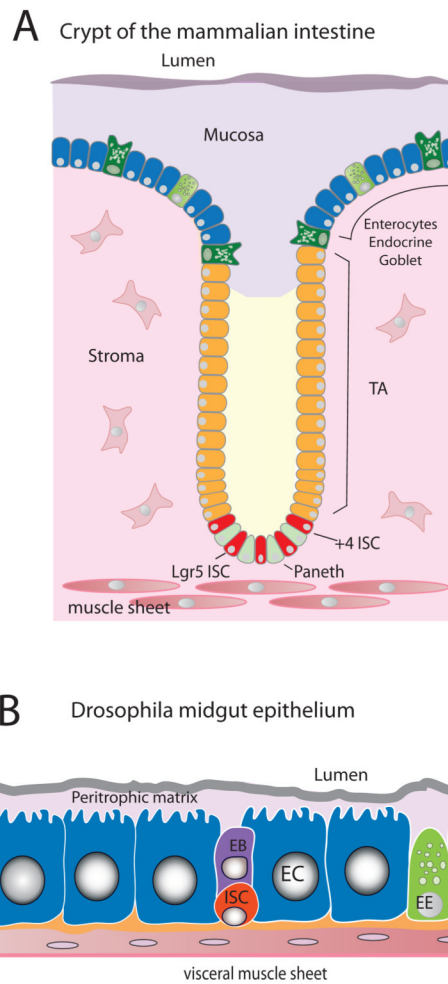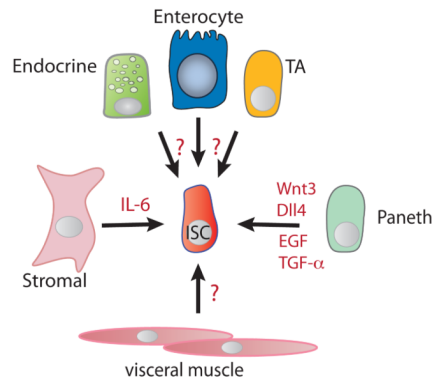
**Figure 1.**
A) Schematic of a crypt of the adult mammalian small intestine, with villi omitted. B) Schematic of the midgut epithelium of adult *Drosophila*. EC: enterocyte; EE: Enteroendocrine cell; EB: enteroblast (transient undifferentiated cell); ISC: intestinal stem cell; TA: transient amplifying cells.

A   Mammalian intestinal stem cell niche
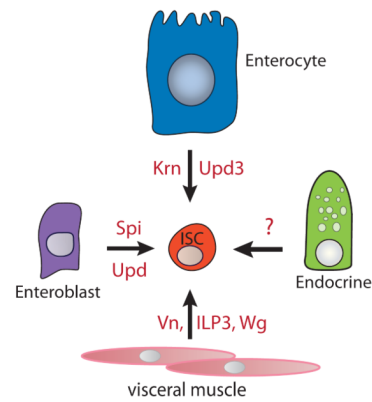


B   *Drosophila* intestinal stem cell niche



**Figure 2.**
Factors that are known to stimulate ISC maintenance and proliferation in the A) mouse small intestine and B) *Drosophila* midgut and their cellular sources.