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Intestinal stem cell response to injury: lessons from *Drosophila*

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Abstract

Many adult tissues and organs are maintained by resident stem cells that are activated in response to injury but the mechanisms that regulate stem cell activity during regeneration are still poorly understood. An emerging system to study such problem is the *Drosophila* adult midgut. Recent studies have identified both intrinsic factors and extrinsic niche signals that control the proliferation, self-renewal, and lineage differentiation of *Drosophila* adult intestinal stem cells (ISCs). These findings set up the stage to interrogate how niche signals are regulated and how they are integrated with cell-intrinsic factors to control ISC activity during normal homeostasis and regeneration. Here we review the current understanding of the mechanisms that control ISC self-renewal, proliferation, and lineage differentiation in *Drosophila* adult midgut with a focus on the niche signaling network that governs ISC activity in response to injury.

Keywords

ISC; midgut; Wnt; Wg; Hedgehog; Hh; BMP; Dpp; Gbb; EGFR; Ras; Hippo; Yki; Yap; JAK-STAT; JNK; Notch; N; Insulin; InR; Calcium; microRNA; Aging; Tissue damage; Self-renewal; Symmetric division; Asymmetric division; Proliferation; Regeneration; Tumor

Introduction

Drosophila adult midgut is the functional equivalent of mammalian small intestine where food is digested and the majority of nutrients are absorbed [1]. Recent studies have identified stem cells, called intestinal stem cells (ISCs), in the midgut [2, 3]. They are derived from the larval progenitors called adult midgut progenitors (AMPs) [4–7]. Structurally, the *Drosophila* midgut is comprised of a single layer of epithelial cells and lacks the well-known crypt/villus structure of the mammalian intestinal epithelium. Thus, the *Drosophila* ISCs do not reside in an obvious anatomical crypt-like niche structure as their mammalian counterparts but instead scatter along the basal side of the midgut epithelium (Fig. 1). Neighboring cells including differentiated midgut epithelial cells, visceral muscle (VMs) and trachea cells serve as niches to secrete factors that control ISC self-renewal, proliferation,

and differentiation (Table 1; Fig. 2). ISC divisions are mostly asymmetric and occur apical-basally, with the basal daughter cells remaining as renewed ISCs while the more apical daughter cells becoming enteroblasts (EBs) committed to terminal differentiation in most cases [2, 3, 8]. However, symmetric ISC divisions have been observed at lower frequency, which produce two ISCs or two EBs [9–12]. Different from the transient amplifying progenitors in the murine ISC lineage, the *Drosophila* EBs do not proliferate; instead, they directly differentiate into two functionally conserved ISC lineages: the absorptive enterocyte (EC) and a single secretory cell type called enteroendocrine cell (EE) [2, 3]. EB committed to the EC fate undergoes several rounds of endoreplication to significantly increase its size to form the bulk of the gut epithelium [2, 3]. Several markers have been widely used to identify the *Drosophila* ISC lineage (Fig. 1a). The ISCs are marked by the Notch (N) pathway ligand Delta (Dl), which activates the N signaling in the neighboring EB that will differentiate into EC [8]. The majority of EBs can thus be specifically identified by a reporter of N signaling, *Su(H)Gbe-lacZ* (referred to as *Su(H)-Z* for simplicity) [2, 3]. The enhancer trap in the *Drosophila* snail family gene *escargot* (*esg*) marks both ISC and EB, which are commonly known as midgut precursor cells/progenitors [3]. As for the mature gut cells, Nubbin (Pdm1) and Brush Border Myosin (MyoIA) mark the ECs, and Prospero (Pros) marks the EEs [2, 3, 13].

Unlike mammalian intestines that undergo fast turnover under physiological conditions, *Drosophila* midguts turn over slowly during normal homeostasis. Lineage tracing experiments show that the posterior region of adult female midguts turns over in about 2–4 weeks, which varies likely due to both the sensitivity of the tracing methods and culturing conditions [13, 14]. However, in response to tissue damage elicited by genetic cell ablation, chemical feeding, or bacterial infection, the midguts can mount regenerative programs to accelerate stem cell division and lineage differentiation to effectively replenish damaged cells [13, 15–19]. A number of signaling pathways have been implicated in the control of ISC activity in response to injury (Table 1; Fig. 2). In the following, we review recent progress toward understanding the genetic pathways and molecular mechanisms that control ISC/EB fate determination and ISC proliferation with a focus on the signaling network that mediates the regenerative responses to injury. Studies exploring the regulation of ISC activity and function in aging guts have been extensively reviewed elsewhere [20].

N signaling regulates the ISC fate and lineage differentiation

N signaling plays a critical role in ISC fate determination and inhibits ISC self-renewal by promoting its differentiation into EB in the EC lineage (Fig. 1c) [2, 3]. Loss of N signaling leads to symmetric ISC divisions and the formation of intestinal tumors consisting of large clusters of ISC and EE-like cells [2, 3, 21]. Indeed the same neoplasia occurs spontaneously in aging guts due to frequent somatic mutations that inactivate N in ISCs [22]. On the contrary, activating the N pathway induces ectopic differentiation of midgut progenitors into mature ECs, resulting in their rapid loss from the midgut [3, 8, 23]. At the conclusion of ISC division, the N ligand Dl is initially segregated into both ISC daughter cells; however, shortly after that, one daughter cell (usually the basally localized one) maintains high levels of Dl that signals to the apically localized daughter cell; as a consequence, the N signaling

pathway is activated in the apical daughter cell, where it downregulates DI and promotes committed EB fate [8].

How asymmetric N signaling is established and maintained has remained poorly understood but recent studies suggest that both cell intrinsic and extrinsic mechanisms may exist (Fig. 1c). One study showed that adhesion of an ISC to the basement membrane, which is mediated by integrins, induces cell-intrinsic polarity within the ISC and promotes cell division along the apical-basal axis, resulting in asymmetric segregation of Par proteins (Par-3 and Par-6) and aPKC into the apical daughter cell [10]. This study further showed that aPKC promotes the EB fate likely by regulating N activity. Another study showed that Sara endosomes are asymmetrically segregated into the future EB during an ISC division, and that the N ligand DI is associated with the Sara endosomes and rapidly degraded by lysosomes, leading to the biased N activation in the EB [24]. The observed ISC-specific DI expression is likely the result of its continued expression in the ISCs whereas it is repressed by N signaling in the EBs. However, perturbation of either aPKC or Sara function only slightly skews fate choice between ISC and EB, raising the possibility for the involvement of additional mechanism(s) [10, 24]. Indeed, a recent study showed that after ISC division, the basal daughter cell transduces higher levels of BMP signaling than the apical one, and that BMP signaling promotes ISC fate by antagonizing N signaling (Fig. 1c) [11]. The profound ISC phenotypes associated with either loss- or gain-of-BMP signaling suggest that this cell extrinsic mechanism plays a critical role in initiating and/or maintaining the asymmetric N signaling. It is likely that BMP acts in conjunction with aPKC and Sara endosome to regulate the asymmetric N signaling (Fig. 1c). Further study is needed to determine the relationship between the cell extrinsic and intrinsic mechanisms in ISC/EB fate determination.

Under homeostatic conditions, the majority of ISCs (~80 %) undergo asymmetric division (ISC/EB) whereas a smaller fraction of ISCs (~20 %) undergo either symmetric self-renewing division (ISC/ISC) or symmetric differentiating division (EB/EB) [9–12]. However, ISC division modes are not fixed and can be skewed in response to environmental stimuli. In newly emerged adult flies, the majority of ISCs in the posterior midguts undergo symmetric self-renewing division under fed condition whereas starvation switches the ISC division mode to mostly asymmetric division [9]. In addition, feeding increases ISC proliferation rate, leading to increased number of progenitors and differentiated cells, which drives rapid growth of midguts during the early stage of adult life [9]. At later stage, re-feeding increases ISC proliferation to compensate for the loss of intestine cells after prolonged starvation. Nutrient-stimulated intestinal growth is mediated by the local production of the *Drosophila* insulin-like peptide 3 (dILP3) in the muscle cells, which is thought to directly act on the ISCs to promote their proliferation through the *Drosophila* insulin-like receptor (InR) pathway [9]. Other studies revealed that the InR pathway is also required for injury-stimulated ISC division [15], and can act in both ISCs and EBs to regulate ISC proliferation [25, 26]. Whether muscle-derived dILP3 mediates the effect of feeding on ISC division mode, i.e., asymmetric (ISC/EB) vs. symmetric (ISC/ISC) division has not been directly tested; however, a recent study revealed that a conserved RNA-binding protein Lin-28 promotes symmetric self-renewing division to drive adaptive growth of young adult midgut by regulating the expression of InR [27]. Another study showed that the

insulin pathway acts through the microRNA miR-305 to control N pathway activity [28], thereby providing a mechanistic insight into how the insulin/InR pathway may balance between ISC self-renewal and differentiation in adaptive homeostasis.

It has been proposed that EBs, which are marked by Su(H)-Z⁺, produce either ECs or EEs depending on the levels of DI they receive and thus the levels of N signaling they transduce: EBs adjacent to ISCs expressing high levels of DI differentiate into ECs whereas those adjacent to ISCs expressing low levels of DI differentiate into EEs [8]. However, recent studies challenged the model that Su(H)-Z⁺ EBs are the common progenitors for both EC and EE in the midgut [29–31]. Specifically, their lineage tracing experiments indicate that the Su(H)-Z⁺ EBs only give rise to ECs and a small subset of EEs whereas most EEs are directly derived from a rare population of proliferative Pros⁺ progenitors. In addition, these studies show that Pros⁺ progenitors are directly generated following a small subset of ISC divisions. Genetic analyses indicate that the induction of *Achaete-Scute Complex* genes (*AS-C*), *ase* and *sc*, and the subsequent induction of *pros* in these rare progenitors are key for determining their EE fate [29]. Interestingly, a recent study revealed that during asymmetric divisions that produce ISC/EE pairs in pupal as well as adult midguts, EEs rather than ISCs are the source of DI whereas ISCs transduce low levels of N signaling activity that prevent them from adopting the EE fate (Fig. 1d) [32]. How this asymmetric N signaling is established remains to be explored.

A number of pathways and factors have been shown to regulate the EE fate, including N and Slit/Robo pathways [2, 3, 8, 23, 30], the SWI/SNF chromatin-remodeling complex [33], and the transcriptional repressor Ttk69 [34]. In particular, N signaling plays an evolutionary conserved role in determining the fate of ISC progenies. In both mouse intestine and *Drosophila* midgut, high levels of N activation lead to EC differentiation while low levels or loss of N activity lead to the secretory cell fate, such as EEs in the fly midgut [3, 8, 35, 36]. Mechanistically, the activation of the N pathway represses the activity of a bHLH transcription factor (TF) Daughterless (Da) to promote the EC fate [23]. However, as mentioned above, both positive and negative TFs have been identified to play a role in determining the EE fate, thus the mechanism appears to be complex. In addition, how the correct ratio of EC/EE is determined in the midgut is still largely unknown.

Recent gene expression profiling studies indicate that the *Drosophila* midgut can be functionally divided into several different regions [1, 37, 38]. In particular, the middle region of *Drosophila* midgut, commonly known as the copper cell region, actually functions as the equivalent of mammalian stomach with its special cell types and physiological features, such as extremely low pH, and cells in this region are maintained by relatively quiescent ISCs known as gastric stem cells [39, 40]. High levels of BMP signaling activity appears to promote the regional identity of gastric stem cells [41, 42].

Transcription factors maintain midgut progenitor fates

As discussed previously, N signaling induces the expression of repressive E(spl)-C transcription factors that suppress the activity of Da to promote EC differentiation [23]. In fact, loss-of-*da* phenocopies the N gain-of-function phenotype: the ISCs ectopically

differentiated into ECs [23]. Interestingly, the murine homologs of Da, E2A and HEB, also play an important role in maintaining ISCs and their activities are regulated by a different niche pathway: both of them are specifically expressed in the crypts, and their heterodimerization partner Achaete-scute like 2 (Ascl2) is specifically induced in the mouse *lgr5*⁺ ISCs by the critical niche signal Wnt and required for the maintenance of ISCs [43]. Thus, while the niche signals responsible for the long-term maintenance of ISCs differ in the *Drosophila* midgut and mouse intestine, their key downstream transcription factors seem to be conserved: in both systems, the niche signaling pathways regulate the activity of E-protein/Da transcription factors to maintain the ISCs.

While EBs are committed to differentiate into ECs under the influence of N signaling, they do not immediately differentiate into mature gut cells. Their transient undifferentiated fates are maintained by a fly Snail family transcription factor Esg [14, 44, 45]. Loss of Esg leads to ectopic differentiation of midgut progenitors similar to gain of N. However, unlike loss of N, ectopic induction of Esg in the midgut progenitors does not lead to the formation of ISC/EE tumors. Instead, Esg suppresses their differentiation during midgut regeneration. Mechanistically, Esg appears to directly repress mature gene expression, including *pdm1*, to maintain the progenitor fates [45]. In addition, Esg likely also mediates an epithelial–mesenchymal transition (EMT) program in the midgut progenitors. In particular, it confers EB's invasive and migratory properties that are potentially important for its ability to sense cell loss/defect within the midgut epithelium [14]. In response to local tissue needs, the conserved microRNA *miR-8/miR-200* is induced in the differentiating EBs, which suppresses Esg to allow their terminal differentiation into mature gut cells [14]. Interestingly, both Esg and Da bind E-Box consensus site [46], further studies should focus on whether they bind similar genomic loci to specify the ISC fate.

EGFR and Jak-Stat pathways are the major mitogenic signals for ISCs

In the adult midgut, EGFR and Jak-Stat signaling pathways have been reported to be the main mitogenic signaling pathways [13, 16, 19, 47–55]. Both signaling pathways are required for the activation of ISC division during regeneration [13, 49]. On the other hand, ectopic activation of either pathway induces dramatic ISC proliferation, resulting in midgut hyperplasia [13, 49]. Both pathways are activated in the progenitors by several functionally redundant EGF-like ligands or IL-6-like cytokines. The sources of these ligands appear to be diverse in the midgut (Table 1). For the EGFR ligands, the weak ligand Vein (Vn) is expressed in the visceral muscle cells (VMs) [40, 49, 50]. The strong ligands Keren (Krn) and Spitz are expressed in the EC and midgut progenitors, respectively [49, 50]. Similarly, the *Drosophila* cytokines are also expressed in diverse midgut epithelial cells [19, 49, 51, 53, 56]: Upd is specifically expressed in the progenitors; Upd2 is expressed in both progenitors and mature ECs; Upd3 is mainly expressed in the mature ECs. After midgut damage, all these ligands are induced in their respective midgut cell types, resulting in the dramatic activation of both pathways in the midgut progenitors to promote ISC proliferation.

Consistent with the diverse sources of ligands, multiple signaling pathways have been shown to regulate their induction in the regenerating midgut (Table 1; Fig. 2). The induction of Vn in the VMs has been shown to be regulated by two pathways: the Bursicon/Rickets (Rks)

and Jak-Stat pathways [13, 53, 57]. A subset of EEs in the posterior midgut secrete Bursicon, which activates the receptor Rks/dLGR2, the ortholog of mammalian leucine-rich repeat-containing G protein-coupled receptors (LGR4-6), to suppress ISC proliferation by inhibiting Vn in the VMs [57]. In addition, upon midgut damage, Jak-Stat signaling is activated in the VMs by the elevated Upds from the epithelial cells, which is capable of inducing Vn there [53]. However, it is not clear how these two pathways interact to regulate Vn during midgut regeneration. It is possible that Jak-Stat signaling functions to relieve the suppression of Vn by the Bursicon/Rk pathway. Multiple signaling pathways have also been reported to regulate Spi, Krn and Upd cytokines in the midgut epithelial cells, including the Hippo (Hpo), BMP, Hedgehog (Hh), Wingless (Wg) and JNK pathways (Table 1; Fig. 2). In general, activating the Wg, Hh and JNK pathways or suppressing the Hpo and BMP pathways induces these ligands in the midgut to promote ISC proliferation [11, 13, 18, 19, 41, 58–64]. The functions of the representative pathways are discussed in detail below. However, it is unknown whether these pathways directly or indirectly regulate the production of these ligands.

Besides its role as a potent mitogenic signal for ISCs, Jak-Stat pathway is also required for the differentiation of EBs into mature gut cells [13, 52, 54]. In fact, during midgut homeostasis, the pathway is not required for ISC proliferation and functions solely to promote lineage differentiation. As a result, loss of Jak-Stat signaling leads to the accumulation of EBs during midgut homeostasis. However, the mechanism of how Jak-Stat pathway regulates the differentiation of midgut progenitors is still not known. Since the suppression of Da or Esg is sufficient to promote the differentiation of midgut progenitors, the Jak-Stat pathway might function to inhibit either or both of them to promote midgut differentiation.

Wnt and Hh signaling pathways regulate the regenerative proliferation of ISCs

The mammalian Wnt signaling pathway plays an essential role in maintaining the ISC fate [65]. An early study seems to show a similar function for the *Drosophila* Wnt/Wingless (Wg) pathway in specifying the ISC fate [66]. Specifically, clonal studies indicated that ISCs defective in the Wg signaling were gradually lost in the adult midgut, and ectopically activating the Wg pathway led to ISC expansion [66]. However, a couple of follow-up studies showed that the Wg pathway most likely functions to promote ISC proliferation in the midgut [58, 59]. One strong evidence is that the ISC clones defective in the *Drosophila* homologs of *Adenomatous polyposis coli* (Apc) and thus constitutively activating the Wnt/Wg pathway proliferate faster and grew larger than the control clones; however, the number of ISCs within these mutant clones did not increase appreciatively [58].

Like the ligands for the EGFR and Jak-Stat pathways, the *Drosophila* Wnt pathway ligand Wg also appears to come from diverse cell types in the midgut. It has been shown that Wg is expressed in the VM and this source of Wg signal is thought to be required for promoting ISC fate during midgut homeostasis [66]. Following midgut damage, Wg is also reported to be induced in the midgut progenitors, especially in the committed progenitor EBs, where Wg

signaling increases the expression of proto-oncogene *dMyc* to drive ISC proliferation [59]. However, like several other ISC signaling pathways discussed below, the Wg signaling does not appear to regulate ISC division in a strictly cell-autonomous manner as it also appears to promote ISC proliferation indirectly by inducing the ligands of EGFR and Jak-Stat pathways [67]. Another study revealed that Wg signaling acts in ECs to prevent aberrant JAK-STAT pathway ligand production, thereby restricting ISC proliferation non-cell-autonomously during homeostasis [68]. Interestingly, this study also revealed that Wg is highly expressed in the epithelium and surrounding visceral muscle at adult intestinal compartment boundaries and that cell-autonomous Wg signaling at midgut/hindgut junction influences cell fate specification and prevents lineage mixing during development [68].

The Hh signaling pathway is another major developmental signaling pathway that plays important roles in adult tissue homeostasis and regeneration [69, 70]. Recent studies suggest that Hh signaling controls ISC proliferation in *Drosophila* midgut [61, 71, 72]. ISC lineage clones lacking the Hh signal transducer Smoothened (Smo) grow normally, suggesting that loss of Hh signaling in the ISC lineage does not affect normal homeostasis [61]. However, ectopic activation of Hh pathway in precursor cells either by overexpression of a constitutively active form of the transcription factor Cubitus interruptus (Ci) or inactivation of the Hh pathway inhibitors Patched (Ptc) or Debra promoted ISC proliferation [61, 71]. These observations suggest that under normal homeostasis, Hh pathway activity is kept low and the basal pathway activity is not essential for the homeostatic ISC proliferation.

Feeding flies with dextran sulfate sodium (DSS) increased the production of Hh ligand in multiple cell types including precursor cells and ECs through the JNK pathway; however, DSS stimulated the Hh pathway activity in precursor cells but not in ECs, likely due to the lack of Ci expression in these differentiated cells. Surprisingly, blocking Hh pathway activity in EBs but not in ISCs affected DSS-induced ISC proliferation, suggesting that Hh signaling did not drive ISC proliferation by acting directly on the stem cells themselves but rather by acting on the neighboring cells [61]. Hh signaling in EBs upregulated several growth factors, most notably, the JAK-STAT pathway ligand Upd2 [61]. Indeed, inactivation of Upd2 in EBs blocked ISC proliferation induced by either DSS feeding or ectopic Hh signaling [61], suggesting that DSS stimulates Hh signaling in EBs where it increases the production of Upd2, which in turn activates the JAK-STAT pathway in stem cells to fuel their proliferation. Interestingly, Hh ligand production is also elevated in aging guts and inactivation of Hh signaling partially suppressed aging-related phenotypes [71]. Taken together, these studies suggest that Hh signaling regulates ISC proliferation in response to stress induced either by tissue damaging agents or by aging.

Multi-functional BMP signaling regulates midgut homeostasis and regeneration

Several recent studies revealed that BMP signaling is involved in midgut homeostasis and regeneration and appears to play diverse functions including ISC self-renewal [11], proliferation [11, 41, 60, 73], lineage differentiation [11, 56], and regional specification [41, 42]. Using a phospho-Mad (pMad) antibody to monitor BMP pathway activity in the

midgut, Tian et al. found that BMP signaling activity is asymmetric between ISCs and EBs with ISCs transducing higher levels of BMP signal than EBs [11]. Strikingly, inactivation of BMP signaling resulted in a rapid loss of ISCs due to their precocious differentiation into EBs whereas ectopic BMP signaling by overexpressing a constitutively active form of type I receptor Tkv led to a dramatic expansion of ISCs at the expense of EB fate, suggesting that BMP signaling plays an instructive role in promoting ISC self-renewal [11]. Genetic epistasis experiments revealed that BMP signaling acts upstream of N signaling to promote ISC fate although the molecular mechanism by which BMP signaling antagonizes N pathway activity remains to be determined [11].

Clonal analysis of mutations of various BMP pathway components produced a spectrum of phenotypes ranging from overproliferation of the mutant clones, i.e., increased clone size compared with control clones [11, 41], no obvious change of clone size [42], to reduced clone size coupled with stem cell loss [11]. The overproliferation phenotype associated with BMP pathway mutations echoed those described for juvenile polyposis syndrome in which mutations in BMP pathway components cause intestinal cancers [74–76]. Using cell type specific Gal4 driver to knockdown BMP pathway components, it was found that inactivation of BMP signaling in EC [11, 60], ISC [41], or EB [56] resulted in ISC proliferation, suggesting that BMP signaling acts in multiple cell types to directly or indirectly control ISC proliferation. As BMP signaling regulates multiple aspects of ISC activity that may depend on distinct thresholds of the pathway activity, different degrees of BMP pathway inactivation may produce distinct phenotypes. For example, partial loss of BMP signaling resulted in ISC overproliferation whereas more complete loss of BMP pathway activity led to ISC loss [11].

Another controversial issue regarding BMP signaling is where the BMP ligands come from. Using various *dpp* reporters including enhancer trap line and promoter fusion constructs, it was found that *dpp* is expressed in trachea cells [60], VMs [41, 56], and ECs [11, 42]. A transcriptome study using sorted midgut cells revealed that *dpp* is expressed in VMs [37]. On the other hand, RNA in situ hybridization detected *dpp* expression in ECs [11, 42]. Therefore, it is likely that *dpp* is expressed in multiple cell types and different *dpp* reporters only pick up a subset of expression pattern. In addition, homeostatic expression of *dpp* is likely to be low and different studies might have missed *dpp* expression in certain cell types due to technical limitation. A better reporter for *dpp* expression, for example, knock-in of eGFP into the *dpp* locus, is needed to precisely determine where Dpp ligand is produced during homeostasis and regeneration. On the other hand, *glass bottom boat* (*gbb*), which encodes another BMP ligand, is exclusively expressed in the ECs [11, 37, 56]. Consistent with this, EC-specific knockdown of Gbb resulted in ISC loss [11]. Furthermore, depletion of Dpp from ECs but not from other cell types also resulted in ISC loss [11], suggesting that EC-derived BMPs serve as niche signal for ISC self-renewal. Dpp and Gbb appear to act in a heterodimer to promote ISC self-renewal because overexpression of either Dpp or Gbb alone did not significantly alter ISC/EB fate choice but their combined overexpression resulted in the formation of ISC tumors [11]. Furthermore, Dpp/Gbb heterodimers appear to be secreted basally and trapped on the basement membrane via interaction with the type IV collagen [11], which may explain why basally localized ISC daughters receive higher levels of BMP than their apically localized siblings.

Both Dpp and Gbb production are induced in response to injury [41, 56, 73], suggesting that altered BMP signaling may play a role(s) in midgut regeneration. Guo et al. suggested that upregulation of Dpp production in VMs may serve as a brake to restrict ISC proliferation [41] whereas Zhou et al. argued that an important role that elevated BMP signaling plays during regeneration is to accelerate EB differentiation [56]. Another recent study showed that upon bacterial infection, haemocytes were recruited to the midgut and secreted Dpp to promote ISC proliferation during the early phase of regenerative responses but inhibited ISC proliferation during the recovery stage through distinct type 1 receptors and Smads [73]. Our own study suggests that injury can transiently expand ISC population through increasing BMP production in ECs, which speeds up the production of new cells during midgut regeneration (Fig. 1c; Tian et al., in revision). It is unclear how Dpp derived from different sources could influence ISC in different ways but it is possible that EC-derived Dpp forms a heterodimer with Gbb to regulate ISC self-renewal whereas Dpp derived from other cells that do not express *gbb* forms a homodimer to regulate ISC proliferation.

Cell-autonomous and non-cell-autonomous roles of Hpo pathway in regulating ISC proliferation

The Hpo pathway is a newly emerged and conserved signaling pathway that controls tissue growth and organ size during development [77, 78]. The core Hpo pathway contains a kinase cascade with the upstream kinase Hpo (the homolog of mammalian MST1 and MST2) phosphorylating and activating the downstream kinase Wts (the homolog of mammalian Lats1 and Lats2). Wts phosphorylates and inactivates the transcriptional coactivator Yorkie (Yki), the homolog of mammalian Yap/Taz. Several studies have implicated Hpo signaling in the control of stem cell activity in a number of contexts including *Drosophila* adult midguts [79]. Inactivation of Hpo/Wts or overexpression of Yki either in precursor cells or ECs resulted in ISC overproliferation, suggesting that Hpo signaling restricts ISC proliferation through both cell-autonomous and non-cell-autonomous mechanisms [62–64, 80]. The non-cell-autonomous function of Hpo is mediated by the JAK-STAT pathway ligands including Upd, Upd2, and Upd3 [62, 63, 80], whereas the cell-autonomous function appears to be mediated by dMyc and bantam microRNA [81, 82].

Although inactivation of Yki does not affect homeostatic ISC proliferation, loss of Yki in precursor cells blocks DSS-stimulated ISC proliferation and its inactivation in ECs partially blocks infection- or bleomycin-stimulated ISC proliferation [62–64, 80]. In addition, Yki levels and Hpo pathway reporter genes are upregulated in response to injury. Interestingly, in mammalian intestine, inactivation of Yap does not affect homeostatic turnover but severely impairs DSS-stimulated regenerative proliferation [83], suggesting a conserved role of Hpo signaling in gut homeostasis and regeneration. It is not clear how Yki is stimulated in response to injury but one possible mechanism is through the JNK pathway as activation of JNK can induce Yki activation [63]. Another possibility is that cell loss due to tissue damage may alleviate cell–cell contact mediated inhibition of Yki. Further study is needed to determine the exact mechanism by which Hpo signaling pathway is regulated in response to injury.

It has been generally thought that the core Hpo pathway is invariant; however, recent studies revealed that Yki/Yap can be regulated in Hpo/Mst1/2-independent but Wts/Lats1/2-dependent manner [84, 85], raising the possibility that additional kinase(s) may act in parallel with Hpo/MST1/2 to regulate Wts/Lats1/2 and Yki/Yap. In an RNAi screen, Li et al. found that inactivation of Misshapen (Msn), a member of the MAP4 K family kinases, in precursor cells resulted in ISC overproliferation [86]. Biochemical study revealed that Msn formed a complex with Wts and phosphorylated Wts but not Hpo when these proteins were coexpressed in S2 cells. Further analysis indicated that Msn acts specifically in differentiating EBs and functionally substitutes Hpo in these cells to regulate the production of Upd3 and ISC proliferation whereas ECs mainly rely on Hpo to control the activity of Wts and Yki [86]. It remains to be determined whether mammalian homologs of Msn also participate in ISC regulation.

Integration of niche signals with cell intrinsic factors

While some signaling pathways such as the Hh pathway act on neighboring cells to regulate the production of growth factors to fuel ISC proliferation, others such as the EGFR pathway appears to act directly on ISCs to regulate their proliferation. A third category, including JAK-STAT, Hpo, Wg/Wnt, BMP, InR and JNK, appear to act both in stem cells and in surrounding cells to control ISC proliferation (Table 1; Fig. 2). These observations raise an important question of how niche signals are integrated with cell intrinsic factors to control stem cell proliferation.

The Myc family of transcription factors control multiple cellular process, including cell growth and proliferation, cell survival and cell competition, and their abnormal activation is associated with many types of cancer [87]. Interestingly, the *Drosophila* Myc homolog, dMyc, is upregulated in adult midgut progenitors in response to injury elicited by feeding with chemicals such as DSS and bleomycin or by bacterial infection, and upregulation of dMyc is required for injury-stimulated ISC proliferation [59, 67, 81]. Injury induces dMyc expression in precursor cells through multiple signaling pathways including JAK-STAT, EGFR, Hpo and Wg. In addition, dMyc is required for ISC proliferation induced by ectopic activation of these pathways [59, 81]. The dMyc promoter region contains binding sites for transcription factors in the JAK-STAT, EGFR, and Hpo pathways, implying that dMyc is a direct target of these pathways [59, 81] (Fig. 3). In mammalian intestines, *c-myc* is a target of Wnt pathway and its deletion rescued the neoplastic phenotypes associated with loss of APC. Because Hpo, JAK-STAT, and EGFR pathways have also been implicated in colon cancers, it would be interesting to determine whether these pathways also regulate tumorigenesis in mammalian intestines through the *myc* oncogene.

The Sox family member Sox21a is specifically expressed in midgut progenitors but is required specifically in ISCs to promote their proliferation in the posterior midgut [88, 89]. Sox21a expression is upregulated in response to stress and transgenic overexpression of Sox21a in progenitor cells is sufficient to drive ISC proliferation [88]. Sox21a is regulated by the AP1 family of transcription factor Fos, which is activated by both JNK and EGFR pathways [88]. Furthermore, Sox21a is required for ISC overproliferation induced by these

pathways [88], suggesting that Sox21a is a common mediator of JNK and EGFR pathways in the regulation of ISC proliferation (Fig. 3).

Another downstream effector of the EGFR pathway is the HMG-box transcriptional repressor, Capicua (Cic) [90]. Inactivation of Cic in ISCs promoted their proliferation whereas overexpression Cic inhibited ISC proliferation in response to injury or EGFR pathway activation. Both injury and EGFR pathway activation relocated Cic from the nucleus to cytoplasm, therefore inhibiting its activity. By progenitor-specific gene expression profiling and DNA binding mapping, it was found that Cic directly regulates genes encoding cell cycle regulators such as String and Cyclin E as well as ETS domain transcription factor Ets21C and Pointed (Pnt). Functional study validated that Pnt acts downstream of Cic to regulate ISC proliferation. A previous study revealed that overexpression of an active form of Pnt promoted the transcription of *dMyc* [81]. These observations suggest that EGFR signaling promotes ISC proliferation through a transcriptional cascade that regulates genes involved in cell growth and cell cycle progression (Fig. 3).

Conclusion and future prospective

In summary, multiple developmental signaling pathways have been employed to regulate ISC activity in midgut homeostasis and regeneration. These pathways form a hierarchical and cross-regulatory signaling network and their activities are tightly controlled in response to tissue needs. In general, the ligands for the various pathways are produced by multiple cell types and their expression is stimulated upon injury. Interestingly, some ligands are specifically regulated by certain types of tissue damage reagents but not by the others. For example, Hh is stimulated by DSS but not by bleomycin feeding whereas BMP is stimulated by bleomycin but not by DSS (Tian et al., in revision) [61]. Overall, the mechanisms by which various pathway ligands are activated upon injury remain largely unknown. So are the mechanisms that are responsible for resetting the normal homeostasis after tissue repair is accomplished. Several extracellular matrix proteins have been implicated in regulating ISC but how they are integrated with various signaling pathways remains to be explored [91, 92]. How the extrinsic signals are integrated with the intrinsic factors to coordinate ISC proliferation, self-renewal and lineage differentiation during normal homeostasis and regeneration is still poorly understood and remains another important area to explore in the future. Additional signaling pathways and transcriptional factors are likely to be involved in regulating stem cell activity in various conditions. For example, a recent study suggested EE also functions as a niche to regulate homeostatic ISC proliferation by secreting the neuroendocrine hormones Tachykinin [93]. Another recent study revealed that oscillated Ca^{2+} signaling regulates ISC activity in response to dietary and stress stimuli [94]. Cell type specific transcriptome profiling and genome-wide RNAi screen have the potential to identify new genes and pathways that control ISC activity in normal homeostasis and regeneration [37, 95]. Finally, it will be interesting to compare and contrast the regulatory mechanisms utilized in *Drosophila* midgut and mammalian intestines to learn what are evolutionarily conserved, and hopefully to be able to extend many of the novel findings in *Drosophila* into the mammalian system.

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Fig. 1.

Intestinal stem cell lineage and self-renewal mechanism. **a** ISC lineages in *Drosophila* adult midguts. DI marks ISC. $Su(H)-Z$ marks EB of the EC lineage whereas the EB of the EE lineage is marked by DI and $Pros$. $Pdm1$ and $Pros$ are the markers for EC and EE, respectively. *esg* is expressed in both ISC and EB, which are collectively called progenitors or precursor cells. **b** Sagittal view of *Drosophila* midgut epithelium immunostained with GFP driven by *esg-Gal4* (green), which marks the precursor cells, Phalloidin (red), which highlights visceral muscle (VM) underneath the basement membrane (BM indicated by arrows) and apical cell membrane of ECs, and DRAQ5 (blue), a nuclear dye that highlights the large polyploid EC nuclei. **c** Cell extrinsic and intrinsic mechanisms for ISC self-renewal in the EC lineage. *Left* basal secretion coupled with basement membrane trapping sets up a “basal high and apical low” BMP activity gradient consisting of Dpp–Gbb heterodimers. *Right* the majority of ISCs undergo asymmetric division in which aPKC and Sara endosome (red dots) are inherited by the apical daughter cells. Basally localized ISC daughter cells transduce higher levels of BMP signaling than the apical ones. Asymmetric BMP signaling coupled with aPKC activity bias N signaling in the apical daughter cells. Sara endosome promotes the degradation of DI while N inhibits the expression of DI in the apical daughter cells. N inhibits ISC self-renewal by promoting EB/EC lineage differentiation. Injury can influence ISC division mode through regulating BMP production during tissue regeneration

whereas nutrient can alter ISC division mode through the InR pathway during adaptive growth. **d** ISC self-renewal in the EE lineage. In asymmetric divisions that generate ISC/EE pairs, EE progenitors expressing Pros and Dl activate low levels of N signaling in ISCs to prevent them from adopting EE fate

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Fig. 2. Signaling network that regulates ISC activity in response to injury. **a–b** Diagrams of the signaling network regulating ISC activity in the *Drosophila* adult midgut. EGFR and Jak-Stat pathways are the major mitogenic pathways that drive ISC proliferation during midgut regeneration. Many signaling pathways including Hh, Wnt, BMP, JNK, and Hpo pathways regulate ISC proliferation by controlling the production of EGFR and Jak-Stat pathway ligands during midgut homeostasis and in response to injury. BMP and insulin pathways regulate both ISC self-renewal and proliferation. *BL*, bleomycin; *PE*, *Pseudomonas entomophila*; *Ecc15*, *Erwinia carotovora*; DSS, dextran sulfate sodium

Fig. 3.
Integration of niche signals with cell intrinsic factors in the regulation of ISC proliferation.
Multiple signaling pathways act through cell-intrinsic factors to regulate ISC proliferation.
See text for details

Table 1

The sources of ligands for the ISC niche pathways

Receptors (pathway)	Ligands	Sources of ligands during homeostasis	Sources of ligands during regeneration	Regulated by	Induced by
Domeless (Jak-Stat)	Upd	Progenitors [51] VMs [54]	Progenitors [13]	JNK [13] Hpo [62–64, 80]	<i>Pe</i> [13] <i>Ecc15</i> [16, 19] EC Apoptosis [13]
	Upd2	ECs [13]	ECs [13]	JNK [13] Hpo [62–64, 80] Hh [61]	<i>Pe</i> [13] <i>Ecc15</i> [16, 19] EC Apoptosis [13]
	Upd3	ECs [13]	ECs [13, 53] EBs [53, 86]	JNK [13] Hpo [62–64, 80] BMP [11, 56, 60] Hh [61]	<i>Pe</i> [13] Bleomycin [81] <i>Ecc15</i> [16, 19, 53] EC apoptosis [13]
Egfr (EGFR)	Vn	VMs [13, 40]	VMs [13] ECs [13]	Jak-Stat [13, 48, 53] Burs/Rks [57] Hh [61]	<i>Pe</i> [49] EC apoptosis [13] <i>Ecc15</i> [16, 19]
	Spi	Progenitors [13]	Progenitors [13]	Wg [59] Jak-Stat [53]	<i>Pe</i> [49] EC apoptosis [13] <i>Ecc15</i> [16, 19]
	Km	ECs [13]	ECs [13]	JNK [13] Hpo [62, 80] Hh [61]	<i>Pe</i> [49] EC apoptosis [13] <i>Ecc15</i> [16, 19]
Tkv/Put (BMP)	Dpp	Trachea cells [60] ECs [11, 42] VMs [37, 41, 56]	VMs [41, 56] Hemocytes [73]	Jak-Stat [41]	Bleomycin [41] <i>Ecc15</i> [56, 73]
	Gbb	ECs [11, 56]	ECs [56]	?	<i>Ecc15</i> [56]
	Fz1/Fz2 (Wg/Wnt)	VMs [66, 68]	Progenitors [59]	JNK [59]	<i>Pe</i> , DSS [59]
Ptc/Smo (Hh)	Hh	Progenitors, ECs [61, 71] VMs [71]	Progenitors, ECs [61]	JNK [61]	DSS [61]
InR	dILP3	VMs [9]	?	?	Nutrient [9]

Pe, *Pseudomonas entomophila*; *Ecc15*, *Erwinia carotovora*; DSS, dextran sulfate sodium