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# Insect Gut Regeneration

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In adult insects, as in vertebrates, the gut epithelium is a highly regenerative tissue that can renew itself rapidly in response to changing inputs from nutrition, the gut microbiota, ingested toxins, and signals from other organs. Because of its cellular and genetic similarities to the mammalian intestine, and its relevance as a target for the control of insect pests and disease vectors, many researchers have used insect intestines to address fundamental questions about stem cell functions during tissue maintenance and regeneration. In *Drosophila*, where most of the experimental work has been performed, not only are intestinal cell types and behaviors well characterized, but numerous cell signaling interactions have been detailed that mediate gut epithelial regeneration. A prevailing model for regenerative responses in the insect gut invokes stress sensing by damaged enterocytes (ECs) as a principal source for signaling that activates the division of intestinal stem cells (ISCs) and the growth and differentiation of their progeny. However, extant data also reveal alternative mechanisms for regeneration that involve ISC-intrinsic functions, active culling of healthy epithelial cells, enhanced EC growth, and even cytoplasmic shedding by infected ECs. This article reviews current knowledge of the molecular mechanisms involved in gut regeneration in several insect models (*Drosophila* and *Aedes* of the order Diptera, and several Lepidoptera).

In vertebrates, many tissues and organs undergo dynamic self-renewal, wherein aged or damaged cells are replaced as needed by the offspring of resident stem cells. Examples include the skin, blood, and gastrointestinal (GI) tract, each of which produce millions of cells every day in humans. This regenerative growth is essential for longevity. Tissue self-renewal is less ubiquitous in shorter-lived invertebrates, but nevertheless some tissues retain cells with stem or progenitor properties that confer a capacity to not only heal wounds, but to replace spent or damaged cells with the correct types of differentiated cells, as needed. In adult insects, the gut epithelium is one of only a few tissues that is highly regenerative. Researchers have used several insect models to study the basis of gut regeneration. Because of the powerful genetic tools available, the adult midgut of *Drosophila melanogaster*, in particular, has proven to be an excellent model for studies of stem-cell-mediated regeneration and tissue homeostasis. Lepidoptera (moths and butterflies) have also received attention because some are agricultural pests, and mosquitoes (order *Aedes*) are highly researched as vectors of human and animal diseases, includ-

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ing malaria, dengue fever, chikungunya, zika fever, etc. (Bonizzoni et al. 2013). The bacterium *Bacillus thuringiensis* (Bt), which attacks its host via the gut, has been widely used for the biological control of both agricultural pests and disease vectors since the 1960s. However, the evolution of resistance threatens the continued success of Bt toxin as an insecticide (Schünemann et al. 2014), and so understanding the regenerative response of the host gut after damage (e.g., to Bt toxin ingestion) should help inform the development of novel bioinsecticides for pest control.

Damage to the adult insect digestive system activates defense responses that are expected to be conserved among different insect groups. In the case of attack by pathogens, these responses include not only activation of the innate immune system (Ferrandon 2013), but also the activation of gut epithelial replacement to maintain gut integrity and function. Changes in gut epithelial cell turnover rates can also occur in response to alterations in nutrition, dietary exposures to chemical toxins, mechanical injury, and changes in the commensal microbiota. These environmental perturbations alter rates of gut epithelial cell loss, and intestinal stem cells (ISCs) respond in turn with corresponding changes in rates of self-renewal and differentiation. However, ISC-intrinsic responses that regulate epithelial replacement, independent of damage to differentiated cells, cannot be ruled out. In this review, we focus mostly on findings from the adult midgut of the Dipteran, *D. melanogaster*, because this is the model in which the vast majority of research on regeneration has been performed (Miguel-Aliaga et al. 2018; Colombani and Andersen 2020). Whereas the fly midgut is endodermal, the foregut ( pharynx, crop) and hindgut are ectodermal, and comprise significantly different cell types. Interestingly, however, the ectoderm-derived hindgut can also regenerate, both during larval development and in the adult (Fox and Spradling 2009; Cohen et al. 2018, 2021). Furthermore, the adult *Drosophila* renal (Malpighian) tubules, which are comprised of a hybrid of endodermal, mesodermal, and ectodermal cells (Takashima et al. 2013b), also contain endodermal midgut-like stem cells in their proximal, endodermal section (the ureters). These "renal stem cells" are capable of mediating regeneration (Singh et al. 2007; Li et al. 2015; Wang and Spradling 2020) using mechanisms similar to those elucidated in the midgut, as described below.

## DEVELOPMENT OF THE *Drosophila* DIGESTIVE TRACT

During embryonic development, the GATA family member serpent (srp) and HNF/Fork Head (Fkh) transcription factors direct endoderm specification and subsequent morphogenesis of the digestive tract (Stainier 2005; Takashima et al. 2013a), whereas homeobox genes regulate the anteroposterior patterning of the endoderm (Beck 2002). Endodermal cells then undergo further specification by activating multiple signaling pathways (e.g., Wnt/Wingless [Wg], Decapentaplegic [Dpp], etc.) (Immerglück et al. 1990; Reuter and Scott 1990). From embryonic stage 11, the *Drosophila* midgut epithelium consists of two distinct cell populations: (1) undifferentiated adult midgut progenitors (AMPs), which persist through larval development and generate the adult midgut epithelium during metamorphosis (Hartenstein et al. 1992); and (2) differentiating midgut epithelial cells, which become functional enterocytes (ECs) or enteroendocrine (EE) cells of the larval midgut. During larval development, the midgut grows substantially due to dramatic cell size increases, and corresponding increases in EC ploidy. In the meantime, the diploid AMPs undergo extensive proliferation to form distinctive cell clusters. In contrast to the situation in Lepidoptera (and presumably many other insects), *Drosophila*'s AMPs are not known to produce differentiated progeny cells (e.g., ECs) during the larval molts, but maintain their undifferentiated progenitor identity until metamorphosis in the pupa (Jiang and Edgar 2009; Mathur et al. 2010; Micchelli et al. 2011; Takashima et al. 2011). Epidermal growth factor receptor (EGFR) and ecdysone receptor (EcR) signaling are pivotal for AMP proliferation (Jiang and Edgar 2009; Micchelli et al. 2011), and Dpp signaling is important for maintaining AMPs in an undifferentiated state in a transient niche (Mathur et al. 2010). The visceral muscle (VM)-derived EGFR ligand vein is

required for early AMP proliferation, while AMP-derived EGFR ligands, Spitz and Keren, promote AMP mitosis during late larval stages. To our knowledge, no evidence of bona fide regeneration in the larval midgut of *Drosophila* has been reported, although AMPs in the larval organ can increase their rates of division in response to stimulation by the commensal microbiota (Jones et al. 2013; Reedy et al. 2019). Reports from other insects, in contrast, show that new ECs can be generated from the AMPs with each molt (Baldwin and Hakim 1991; Franzetti et al. 2016), suggestive of regenerative capability that has been lost in *Drosophila*.

During early pupal development, the fly's larval midgut contracts and shortens to bring the AMP cell clusters together, forming a new epithelium. Following this, as part of metamorphosis, Notch signaling drives most AMPs to differentiate into ECs (Takashima et al. 2011), and the older larval ECs are displaced into the intestinal lumen and die. Although most AMPs differentiate during metamorphosis, a small number remain undifferentiated, divide to expand their numbers, and are retained as the ISCs of the adult midgut (Jiang and Edgar 2011). In addition, two opposing migrations of progenitor cells have been observed during early metamorphosis: (1) During early pupal development, AMPs from a hybrid midgut/hindgut transition zone form the posterior compartment of the adult midgut (Sawyer et al. 2017); and (2) AMPs migrate posteriorly to form the adult ureters at the base of the Malpighian (renal) tubules. A subset of these cells then moves from the ureters to the more distal region of the Malpighian tubules to establish renal stem cell populations during later pupal stages (Takashima et al. 2013b).

## CELL TYPES AND COMPARTMENTALIZATION OF THE ADULT *Drosophila* MIDGUT

The adult *Drosophila* midgut comprises a monolayer cell epithelium, without the crypt/villus structure observed in mammals, wrapped by two layers of mesoderm-derived visceral muscle (VM). Crypt-like epithelial structures have been observed in the midguts of other insects, however, for instance in beetles (Oishi et al. 2019). Circular muscles ensheath the midgut epithelium, and multinucleate longitudinal muscles make up an outer muscle layer (Aghajanian et al. 2016). The VM is penetrated by a web of tracheal tubes, which brings oxygen to the midgut (Li et al. 2013b; Perochon et al. 2021; Tamamouna et al. 2021), and the midgut also has a number of neural connections (Miguel-Aliaga et al. 2018; Hadjieconomou et al. 2020). Inside the midgut lumen, apical to the epithelial cells, a chitinous membrane called the peritrophic matrix (PM), separates the epithelium from ingested food, and serves as a barrier against the gut bacteria (Kuraishi et al. 2011). Mucin proteins, which make up the mucosal apical coating of vertebrate intestines, are also expressed in the *Drosophila* gut, although there is not an obvious mucosal barrier. Within the epithelium, ISCs reside basally, next to a basement membrane that lies between the epithelium and the circular VM cells (Fig. 1; Micchelli and Perrimon 2006; Ohlstein and Spradling 2006). This overall structure, and the cell types present, are strikingly similar to that found in the mammalian colon and small intestine (Colombani and Andersen 2020; Beumer and Clevers 2021). For instance, human intestinal epithelium comprises stem cells that generate absorptive ECs and secretory EE cells, layered on a stromal surface that is primarily mesodermal VM. However, in contrast to insects, the human gut has more specialized epithelial cell types (e.g., Paneth, goblet, tuft, and transit-amplifying absorptive cells), and a much more complex stromal cell population that includes fibroblastic, endothelial, neural, and lymphoid cell types that are largely absent in insects.

ISCs in adult *Drosophila* can proliferate throughout the life of the animal, and are the only self-renewing cell type yet detected in the fly midgut. At division, ISCs renew themselves and also generate postmitotic committed progenitors called enteroblasts (EBs), which can differentiate directly without division into ECs (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006), and pre-EEs, which differentiate into EE cells, typically after dividing once. The adult *Drosophila* midgut maintains about 600 ISCs and an equal number of EBs that can

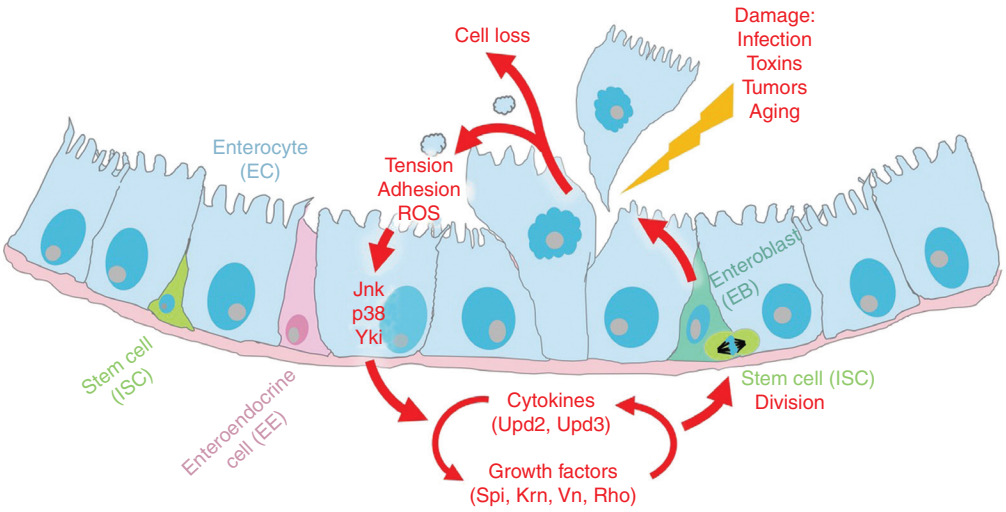


**Figure 1.** General model for regeneration in the *Drosophila* midgut epithelium. Cells are color coded by cell type. (ISC) Intestinal stem cell, (ROS) reactive oxygen species.

be identified by their small size, basal location, and the expression of the snail-type zinc-finger transcription factor escargot (*esg*), a specific marker of diploid progenitor cells (both ISCs and EBs) in the midgut (Fig. 2; Micchelli and Perrimon 2006; Korzelius et al. 2014). ECs are large, polyploid absorptive cells that comprise the bulk of the intestinal epithelium. The POU/homeodomain transcription factor (Pdm1/nubbin), and nonmuscle myosin IA (MyoIA), expressed in the apical brush borders of the midgut epithelium, specifically mark differentiated ECs (Morgan et al. 1994; Jiang et al. 2009; Lee et al. 2009). EEs are small postmitotic secretory cells that express neuroendocrine markers such as Prospero (pros) and hormones (e.g., allatostatin, tachykinin), which drive peristalsis by controlling the contraction of VM (Yoon and Stay 1995; Siviter et al. 2000; Micchelli and Perrimon 2006; Ohlstein and Spradling 2006), and most likely have many other functions (Amcheslavsky et al. 2014; Miguel-Aliaga et al. 2018; Colombani and Andersen 2020).

Although these features are common throughout the midgut (Fig. 1), regional anatomic, cellular, and molecular heterogeneities have led researchers to subdivide it into 10–14 regions (Murakami et al. 1994; Buchon et al. 2013; Marianes and Spradling 2013). Each region is characterized by different cell compositions, physical properties (e.g., luminal pH), histological features, stem cell proliferative rates, and gene expression profiles (Murakami et al. 1994; Strand and Micchelli 2011, 2013; Buchon et al. 2013; Marianes and Spradling 2013; Dutta et al. 2015). For instance, morphologically distinct ECs are found in the different regions, one region (R3) has distinct "copper" and "interstitial" cell types (Strand and Micchelli 2011; Marianes and Spradling 2013), and the proportions of the many (>12) different types of EE cells vary by gut region, with some types being region-specific (Beehler-Evans and Micchelli 2015; Hung et al. 2020). ISCs of all regions are able to regenerate all cell types of their particular region (Buchon and Osman 2015), but ISCs in the posterior midgut differ in some respects from those in the anterior. For example, ISC proliferation rates are relatively greater in the posterior regions, both during normal tissue maintenance and episodes of damage-induced regeneration. Similarly, regional effects in ISC-tumor models have been reported, such that the posterior midgut is generally more tumor-prone (Martorell et al. 2014; Patel et al. 2015; Sawyer et al. 2017). Several transcription factors (e.g., Pdp1, Stat92E, GATAe, Sna, Ptx1, etc.) have unique region-specific expression patterns and regula-

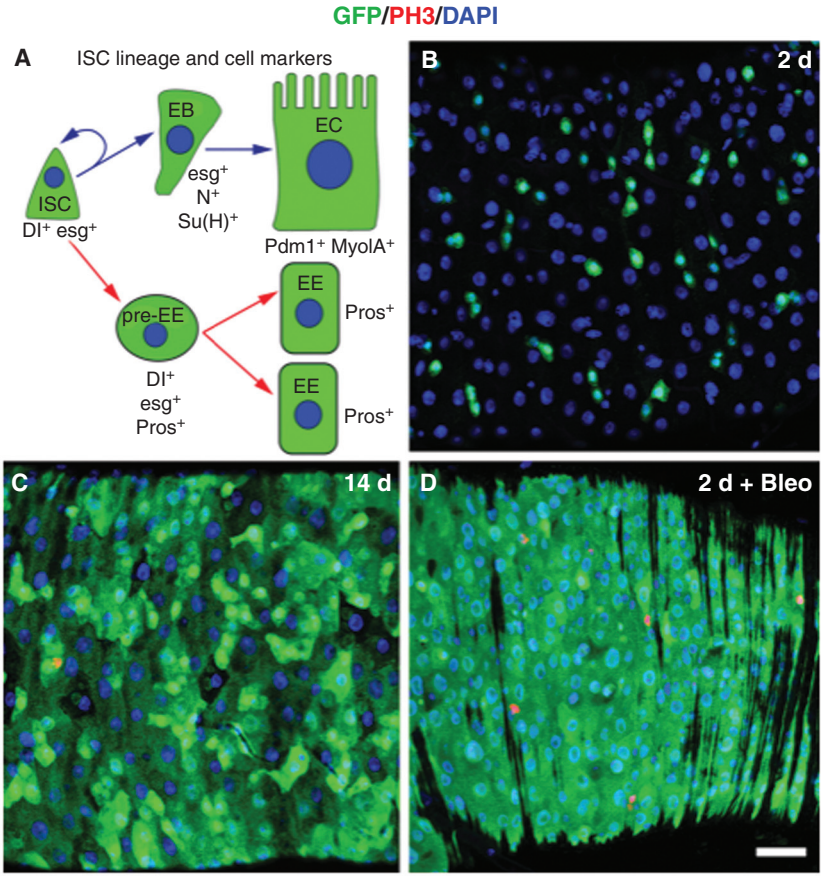


**Figure 2.** Renewal of the *Drosophila* midgut epithelium under normal and stress conditions. (*A*) Lineage relationships of intestinal stem cell (ISC) progeny, and markers for each cell type. ISC markers: Delta (Dl), escargot (esg); enteroblast (EB) markers: escargot (esg), Notch (N), Suppressor-of-Hairless (Su(H)); enterocyte (EC) markers: Pdm1, myosin IA (MyoIA); pre-enteroendocrine (EE) markers: Delta (Dl), escargot (esg), Prospero (Pros); EE marker: Prospero (Pros). (*B–D*) Examples of gut epithelial renewal using the conditional "*esg$^{ts}$-FlipOut*" lineage-tracing system (genotype: *esg-Gal4 tub-Gal80$^{ts}$ act>CD2>Gal4 UAS-GFP*) (Jiang et al. 2009). In this system, ISCs are labeled with the expression of green fluorescent protein (GFP) following a temperature shift, and then all newborn ISC progeny inherit GFP expression, marking newly generated epithelium. (*B*) Lineage tracing in a healthy adult female midgut for 2 d. Only ISCs and EBs are labeled with GFP, indicating very little epithelial replacement. (*C*) A healthy adult female was lineage traced for 14 d. Nearly complete epithelial replacement is observed. (*D*) An adult female was lineage traced for 2 d and then fed 500 µM bleomycin for 18 h to damage the gut. Nearly complete epithelial replacement is evident, and mitotic ISCs (red) are detected. Midguts were dissected and stained with anti-GFP (GFP, green), anti-pH3 antibodies (mitosis, red), and DAPI (DNA, blue). Scale bar, 30 µm.

tory roles (Marianes and Spradling 2013; Dutta et al. 2015) that may explain these differences. Thus far, however, how gut regionalization is established and maintained, and how it affects digestion and physiology, is not so well understood.

## CELLULAR DYNAMICS OF GUT EPITHELIAL MAINTENANCE AND REGENERATION

Many studies have addressed gut epithelial turnover in the *Drosophila* midgut. These find that rates of cell replacement in the *Drosophila* mid-

gut are extremely variable, and can be modulated by many extrinsic and intrinsic factors (e.g., Fig. 2). Regeneration of the midgut epithelium can thus be understood as working along a continuum from extremely slow to very fast, with slow rates used for basal tissue maintenance and fast rates used for tissue repair after acute injury. Published information pertains nearly exclusively to ISC-dependent epithelial regeneration, and evidence for regeneration of *Drosophila*'s VM is lacking. The gut's tracheal network, however, is highly dynamic, and can expand during regeneration of the gut epithelium (Perochon et al. 2021; Tamamouna et al. 2021). Lineage analyses in young, healthy guts show that ISCs can be relatively quiescent for long periods, dividing less than once every 2–3 d, and indicate that ECs are replaced at a rate of about once per 2–3 wk in females, and somewhat slower in males, which consume considerably less food (Fig. 2; Jiang et al. 2009; de Navascués et al. 2012; Hudry et al. 2016). ISC mitoses and gut renewal can be dramatically accelerated, however, by treatments that stress or damage the intestinal epithelium (Fig. 2D). These include ingested detergents (DSS, SDS), enteric bacterial infection (e.g., by *Pseudomonas entomophila*, *Pseudomonas aeruginosa*, *Erwinia carotovora carotovora*), commensal bacteria (*Lactobacillus plantarum*), DNA-damaging agents (bleomycin), oxidative stress (paraquat, $H_2O_2$), physical cutting, stretching by food particles, or the genetic activation of apoptosis, autophagic cell death, or stress signaling genes (Biteau et al. 2008; Amcheslavsky et al. 2009; Apidianakis et al. 2009; Buchon et al. 2009a,b; Jiang et al. 2009). In response to such stresses, damaged and dying ECs are typically shed (extruded) from the intestinal epithelium, and ISC mitotic rates rapidly increase by 10- to 100-fold (Biteau et al. 2008; Amcheslavsky et al. 2009; Apidianakis et al. 2009; Buchon et al. 2009b; Chatterjee and Ip 2009; Cronin et al. 2009; Jiang et al. 2009). Genetically induced tumors also promote turnover of the surrounding epithelium (Cordero et al. 2012a; Patel et al. 2015). During regenerative episodes resulting from acute epithelial damage, ISCs can divide several times per day, and are capable of reconstituting a completely new epithelium in 2–3 d (Jiang et al. 2009). Following the mitotic activation of ISCs, newborn EBs and pre-EEs quickly differentiate into ECs or EEs to replenish lost and damaged cells, thereby regenerating the midgut epithelium. In response to regenerative signals, newborn, postmitotic EBs and ECs can also grow faster and become larger than normal (Xiang et al. 2017). During this process, endoreplicating ECs can reach DNA ploidies of 16–32C rather than the 8C typical of mature ECs in healthy guts. As with many polyploid cell types, EC cell size is generally proportional to nuclear DNA content. Like new cell production, this relative increase in the size of ECs produced following damage may enable faster reconstitution of tight epithelial barrier function. Several genes have been identified that are required for the proper cessation of ISC proliferation as an episode of regeneration comes to a close (Takemura and Nakato 2017; Takemura et al. 2021). The signaling mechanisms that mediate the various regenerative responses have been extensively characterized, as summarized below (Table 1).

In addition to damage- or cell-loss-dependent regeneration, *Drosophila* research has shown that (1) changes in commensal bacteria can accelerate "basal" rates of ISC division and epithelial replacement (Reedy et al. 2019; Ferguson et al. 2021); (2) dietary manipulations can alter gut cell turnover rate (McLeod et al. 2010; Choi et al. 2011; Akagi et al. 2018); and (3) changes in the microbiota with aging can accelerate gut epithelial turnover (Biteau et al. 2008; Buchon et al. 2009a; Guo et al. 2014). It is also interesting to note that forcing ISCs to divide, by inducing cell cycle or growth control genes, is sufficient to drive the shedding of older ECs as they are displaced by newborn ECs (Jin et al. 2017; Loudhaief et al. 2017). This may be due to space limitations on the VM, to which ECs must adhere for viability. Conversely, arresting ISC divisions can prolong the life of preexisting ECs (Jin et al. 2017). These observations suggest that, in addition to EC loss through damage, intrinsic rates of ISC division have a role in driving gut epithelial turnover. Pertinent to this, the genetically determined sex of ISCs affects their proliferative capacity (Hudry et al. 2016). Systemic signaling from mating-dependent hormonal

**Table 1.** Signaling mechanisms and regenerative responses

| Signaling pathway | Functions at homeostasis | Functions after injury | Sending cell types | Receiving cell types | References |
|---|---|---|---|---|---|
| Notch/Delta | Enterocyte (EC) differentiation | EC differentiation | Intestinal stem cell (ISC) | Enteroblast (EB) | Micchelli and Perrimon 2006; Ohlstein and Spradling 2006, 2007; Bardin et al. 2010 |
| Wnt/Wingless | ISC proliferation and maintenance | ISC proliferation | Epithelial cells at the foregut/midgut and midgut/hindgut boundaries, EB, visceral muscle (VM) | ISC, EC | Thuringer and Bienz 1993; Lin et al. 2008; Takashima et al. 2008; Fox and Spradling 2009; Lee et al. 2009; Singh et al. 2011; Cordero et al. 2012b; Dutta et al. 2015; Suijkerbuijk et al. 2016; Tian et al. 2016, 2017, 2018, 2019; Perea et al. 2017; Sawyer et al. 2017; Ngo et al. 2020 |
| Unpaired/Jak/Stat | None (Upd2,3), EC/enteroendocrine (EE) differentiation (Stat) | ISC proliferation, EC differentiation (Stat), activation of epidermal growth factor (EGF) signaling | EB, EC, hindgut | ISC | Buchon et al. 2009a,b; Jiang et al. 2009; Beebe et al. 2010; Lin et al. 2010; Liu et al. 2010; Patel et al. 2015, 2019; Tian et al. 2015, 2016; Houtz et al. 2017; Sawyer et al. 2017; Cohen et al. 2021 |
| Epidermal growth factor receptor (EGFR) | Larval adult midgut progenitor (AMP) proliferation, ISC survival, and proliferation | ISC proliferation, EB, and EC growth | ISC/EB (Spi), EC (Krn), VM (Vn) | ISC, EB | Buchon et al. 2010; Ren et al. 2010; Biteau and Jasper 2011; Jiang et al. 2011; Jin et al. 2015; Xiang et al. 2017 |
| JNK | ISC differentiation, ISC symmetric division | ISC proliferation, ISC pool size, EC apoptosis | ISC/EB (Jun amino-terminal kinase [JNK] ligand Eiger) | EC, ISC, EB | Biteau et al. 2008; Apidianakis et al. 2009; Buchon et al. 2009a; Jiang et al. 2009; Patel et al. 2015; Houtz et al. 2017; Hu and Jasper 2019; Mundorf et al. 2019; Rodriguez-Fernandez et al. 2020 |
| p38 | Needs to be determined | ISC proliferation | EC | EC | Houtz et al. 2017; Patel et al. 2019 |

*Continued*

**Table 1.** *Continued*

| Signaling pathway | Functions at homeostasis | Functions after injury | Sending cell types | Receiving cell types | References |
|---|---|---|---|---|---|
| Hippo/Yki | ISC self-renewal | ISC proliferation | EC | ISC, EB | Karpowicz et al. 2010; Ren et al. 2010; Shaw et al. 2010; Staley and Irvine 2010; Poernbacher et al. 2012; Jin et al. 2013; Huang et al. 2014; Li et al. 2014, 2018; Patel et al. 2015; Suijkerbuijk et al. 2016; Zhang et al. 2019; Hao et al. 2020 |
| PVF | ISC proliferation, EC differentiation | None | ISC/EB (PVF2) | ISC (Pvr) | Choi et al. 2008; Bond and Foley 2012 |
| Dpp | Copper cell and EC differentiation | Restrict ISC proliferation | VM | EC | Guo et al. 2013; Li et al. 2013a, 2016; Tian and Jiang 2014; Ayyaz et al. 2015; Zhou et al. 2015; Ma et al. 2019; Takemura et al. 2021 |
| Duox/Nox/ROS | Needs to be determined | Larval AMP proliferation, ISC proliferation | EC | EC, ISC, EB | Buchon et al. 2009a; Lee 2009; Lee et al. 2015, 2018; Patel et al. 2019; Reedy et al. 2019 |
| $Ca^{2+}$ | ISC self-renewal | ISC proliferation | ESC/EB | ISC, EB | Deng et al. 2015; Xu et al. 2017 |
| Insulin-like peptides/ PI3K | ISC proliferation, ISC pool size, EC growth | Needs to be determined | VM | ISC, EB | Choi et al. 2011; O'Brien et al. 2011; Amcheslavsky et al. 2014 |
| Ecdysone | ISC proliferation, ISC pool size | None | Ovary | ISC, EB | Ahmed et al. 2020; Zipper et al. 2020 |

(ecdysone, juvenile hormone) signaling also affects ISC proliferation (Reiff et al. 2015; Ahmed et al. 2020; Zipper et al. 2020), in both cases favoring higher rates of ISC division and epithelial replacement in females.

## REGULATION OF ISC POOL SIZES

Many researchers have noted that the numbers of *esg*$^+$ progenitor cells (ISCs and EBs) can increase transiently during episodes of damage-induced regeneration, and some authors have inferred that this indicates expansion of the stem cell pool. However, Jin et al. found that ISC numbers remain stable through cycles of infection-stimulated regeneration and, using ISC depletion experiments, concluded that infection-induced regeneration was unable to increase stem cell numbers (Jin et al. 2017; see also Biteau et al. 2008; Hu and Jasper 2019; Rodriguez-Fernandez et al. 2020). In an extensive lineage analysis study, de Navascués et al. (2012) reported that, as in the mammalian intestine, *Drosophila* ISCs typically exist in a state of neutral drift, wherein symmetric, duplicative ISC divisions occur at a significant rate (~10%), but are exactly balanced by an equal number of symmetric divisions that produce two postmitotic EBs, and thereby extinguish an ISC (Lopez-Garcia et al. 2010; de Navascués et al. 2012). But while this clonal drift may be neutral at homeostasis, the symmetry of ISC divisions can be biased to increase the stem cell pool under certain conditions. During cycles of fasting and refeeding, the fly gut can shrink and regrow, with numbers of total and stem cells decreasing and then rebounding accordingly (McLeod et al. 2010; O'Brien et al. 2011; Lucchetta and Ohlstein 2017). The fasting-dependent decrease in ISCs was attributed to cell death, whereas the feeding-dependent increase in ISCs was attributed to higher rates of symmetric, duplicative ISC divisions (McLeod et al. 2010; O'Brien et al. 2011), or by an unusual process termed "amitosis" in which polyploid ECs reduce their ploidy to become diploid cells (Lucchetta and Ohlstein 2017). Feeding-dependent gut expansion was found to be dependent on an insulin-dependent peptide, dILP3, produced by the VM in response to signals from EE cells (O'Brien et al. 2011; Amcheslavsky et al. 2014). Duplicative ISC divisions have been attributed to altered orientation of the ISC mitotic spindle during refeeding, such that duplicating ISCs divide more often in the plane of the VM, whereas asymmetric divisions that produce an ISC and an EB occur more orthogonal to the VM (Ohlstein and Spradling 2007; Goulas et al. 2012; Hu and Jasper 2019). In addition, Hu and Jasper found ISC symmetric divisions could be promoted by oxidative stress, which triggered a JNK-dependent reorientation of the mitotic spindle. Interestingly, this oxidative stress/JNK-dependent effect occurs naturally during aging of the *Drosophila* gut, in response to microbiota imbalances (dysbiosis), and gives rise to gut dysplasia (Biteau et al. 2008; Hu and Jasper 2019; Rodriguez-Fernandez et al. 2020). Finally, mating-dependent ecdysone signaling can drive an expansion of ISC numbers in females, and with it an increase in overall gut size that facilitates nutritional influx and reproduction (Reiff et al. 2015; Ahmed et al. 2020; Zipper et al. 2020). These effects may be triggered by sex peptide (SP), a male factor that is delivered to the female in the seminal fluids during mating, and which is also necessary and sufficient to drive mating-dependent increases in the overall female gut size (White et al. 2021). Thus, under some specific conditions, the ISC pool can be plastic and contribute to adaptive growth.

## NOTCH SIGNALING, ENTEROCYTE, AND ENTEROENDOCRINE CELL DIFFERENTIATION

Binding of the Notch (N) receptor by ligands such as Delta (Dl) leads to the cleavage of Notch, generating an "activated" fragment that regulates target gene transcription in combination with Suppressor-of-Hairless (Su(H)), an HES family transcription factor, and cell-type-specific cofactors. Early work on the *Drosophila* midgut revealed a central role for Dl/N signaling in directing the differentiation of ISC progeny (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006, 2007; Bardin et al. 2010). As in the mammalian intestine (van Es et al. 2005), blocking Notch signaling in *Drosophila*'s ISCs suppresses EC differentiation and causes excessive EE

differentiation. Loss of Notch also generates an excess of ISC/EB-like cells that proliferate exponentially to form large clones, sometimes referred to as "stem cell tumors" (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006; Patel et al. 2015). Conversely, forced activation of Notch signaling induces rapid EC differentiation, extinguishing the stem cell population (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006, 2007). ISCs specifically express the Notch ligand, Dl, which is a specific ISC marker. A Notch activity reporter gene containing binding sites for Su(H) (Furriols and Bray 2001) is specifically expressed in Dl-negative EBs adjacent to Dl-positive ISCs. Thus, high-level Dl/Notch signaling from ISCs to their EB daughters directs EBs to differentiate into ECs. Attaining a sufficient threshold of Notch signaling in the EB appears to take several hours (Martin et al. 2018), and is potentiated by a strong E-cadherin-based cell junctional complex between ISCs and their EB daughters (Maeda et al. 2008). Moreover, the repression of Notch target genes in ISCs by H/Su (H)/Gro/CtBP complexes is necessary to maintain stem cell identity (Bardin et al. 2010). Enhancer of split complex (E(spl)-C) genes are an essential target of this repression and promote differentiation when derepressed by Dl/N signaling. Stem cell progeny that receive relatively little Dl are thought to differentiate as EEs (Ohlstein and Spradling 2007; Guo and Ohlstein 2015) but might also be retained as undifferentiated progenitors that can differentiate later, on demand, or possibly revert to ISC behavior.

In addition to *Delta*, gene expression mapping has identified a number of other genes specific to ISCs (Dutta et al. 2015; Hung et al. 2020). Among these, the *escargot* (*esg*) transcription factor is noteworthy as an ISC/EB-specific factor that promotes stemness, diploidy, and mesenchymal cell behaviors (Korzelius et al. 2014; Loza-Coll et al. 2014; Antonello et al. 2015). Loss of *esg* promotes the rapid differentiation of ISCs and EBs, into ECs and EEs, and is required to repress Pdm1, a transcription factor that promotes EC differentiation and along with it the establishment of polarized columnar epithelial cell morphology (Korzelius et al. 2014; Chen et al. 2018a).

Recent studies have expanded our understanding of EE specification. Lineage-tracing indicates that EEs arise from precommitted Prospero-positive ($Pros^+$) ISCs rather than from EBs that are positive for Notch signaling activity (Biteau and Jasper 2014; Guo and Ohlstein 2015; Zeng and Hou 2015). $Pros^+$ pre-EEs can divide, although typically only once before terminal differentiation (Chen et al. 2018b). EE specification requires the phyllopod-mediated repression of Tramtrack 69 (Ttk69), a transcriptional repressor, which promotes Scute-mediated activation of Pros (Amcheslavsky et al. 2014; Li et al. 2017; Chen et al. 2018b; Yin and Xi 2018). This process may be limited by Slit, an EE-derived ligand for the Roundabout 2 (Robo2) receptor (Biteau and Jasper 2014; Nagy et al. 2017). EE differentiation can also be triggered by calcium signaling in response to the activation of the stretch-activated ion channel, Piezo (He et al. 2018). Further studies are needed to clarify the exact signaling events that govern EE versus EC specification, and which determine how EEs are further differentiated to secrete different endocrine hormones (Beehler-Evans and Micchelli 2015; Hung et al. 2020).

## WNT SIGNALING IN MIDGUT REGENERATION

Although Wnt signaling is critical for mammalian ISC proliferation and maintenance (van der Flier and Clevers 2009) and is mutationally activated in a majority of human intestinal cancers (Barker et al. 2009; Raskov et al. 2020), manipulation of Wnt/Wingless (Wg) signaling in the *Drosophila* adult midgut has relatively mild effects, suggesting a divergence of function. Ectopic expression of the *Drosophila* Wnt ligand, Wg, or a constitutively active form of the downstream effector, β-catenin/armadillo, promotes ISC proliferation (Lin et al. 2008; Lee et al. 2009). Similarly, deletion of negative Wnt pathway components such as Shaggy (*sgg*), Axin (*Axn*), or adenomatous polyposis coli (*Apc1*, *Apc2*) also promotes ISC proliferation (Lin et al. 2008; Lee et al. 2009). Although several groups have used *Drosophila* $Apc^{-/-}$ (*Apc1*, *Apc2* double mutant) intestinal tumor models to investigate tumor

progression (Suijkerbuijk et al. 2016; Tian et al. 2018; Ngo et al. 2020), these studies came to different conclusions regarding the role of Wnt signaling in ISC self-renewal and differentiation. Lin et al. (2008) found that Wg signaling promotes ISC division, that prolonged activation of Wg signaling caused ISC expansion, and that ISCs defective in Wg pathway components (*frizzled*, *fz2*, *disheveled*, *armadillo*) were poorly maintained. Genetic epistasis tests led these authors to propose a hierarchical model wherein Wg and Notch signaling regulate ISC self-renewal and EB differentiation. However, another study (Lee et al. 2009) disputed this model and concluded that loss of *Apc* did not affect cell specification in the ISC lineage, although it did promote cell proliferation and disrupt epithelial structure. Another mystery is the source of Wnt ligands in the fly midgut. Under normal conditions, Wg is expressed in epithelial cells at the foregut/midgut and midgut/hindgut boundaries (Takashima et al. 2008; Fox and Spradling 2009; Singh et al. 2011; Sawyer et al. 2017), and also in a small band of VM cells (Thuringer and Bienz 1993; Lin et al. 2008). While Wg at the midgut boundaries likely regulates the development of foregut and hindgut during metamorphosis (Takashima et al. 2008; Singh et al. 2011; Tian et al. 2016, 2019), its function in the band of VM cells is uncertain. Additionally, Cordero et al. reported that EBs are a source of Wg, which signals to the ISCs to induce their proliferation in response to damage (Cordero et al. 2012b). In this case, EB-derived Wg was required for damage-induced ISC proliferation but dispensable for normal self-renewal and gut maintenance without stress. A follow-up study reported that the Ret tyrosine kinase receptor is specifically expressed in progenitors to sustain ISC proliferation through the posttranscriptional up-regulation of Wg (Perea et al. 2017). In addition, Tian et al. reported an important role for downstream Wg signaling components in ECs, where they restrain the production of Upd cytokines and, indirectly, ISC proliferation (Tian et al. 2016). Interestingly, RNA sequencing experiments show that not only Wg (Wnt1 in mammals), but also Wnt2, Wnt4, Wnt6, and Wnt10 are strongly induced in different midgut cell types during episodes of infection-induced regeneration (Dutta et al. 2015). However, functional tests of these ligands have not yet been reported. Given the widespread use of Wnt signaling in regeneration, the functions of these Wnt ligands in the fly gut should be an interesting topic to explore.

## DAMAGE-DEPENDENT CYTOKINE/JAK/STAT SIGNALING

The Janus tyrosine kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway has emerged as a major regulator of *Drosophila* midgut regeneration (Buchon et al. 2009a; Jiang et al. 2009; Beebe et al. 2010; Lin et al. 2010; Liu et al. 2010). In JAK/STAT signaling, extracellular ligands (cytokines) bind a transmembrane receptor associated with JAKs via its intracellular domain. This activates the JAKs, which *trans*-phosphorylate tyrosines within both the receptors and JAKs. Phosphorylated receptor/JAK complexes act as docking sites for cytosolic STAT molecules, which are also tyrosine phosphorylated. STATs activated in this manner dimerize and translocate to the nucleus to bind DNA and regulate target gene transcription. Core JAK/STAT signaling components in *Drosophila* include three leptin/IL6-like cytokines called Unpaired (Upd), Upd2, and Upd3, a receptor called domeless (Dome), a JAK called hopscotch (Hop), and a single Stat, Stat92E. Accessory regulators include three suppressor of cytokine signaling (SOCS) genes, the PIAS-like Stat-binding protein (ZIMP) and others. In the midgut, stress or damage to ECs stimulates regeneration by triggering a rapid, powerful JAK/STAT signaling response in which Upd2 and Upd3 are transcriptionally induced in ECs and EBs, and trigger STAT signaling in ISCs, promoting their division (Buchon et al. 2009a, b; Jiang et al. 2009). STAT signaling also promotes and is required for EC and EE differentiation (Jiang et al. 2009; Beebe et al. 2010), but it is not clear whether the Upd ligands are needed for this function. Upds can also activate JAK/STAT signaling in the VM, which responds by producing another ISC mitogen, the EGFR ligand Vein (Vn) (Jiang et al. 2011). Diverse types of midgut

stress and damage trigger the production of Upds by ECs and EBs. These include bacterial pathogens, chemical toxins, induced apoptosis, the activation of JNK- or $p38^{MAPK}$-mediated stress signaling (Buchon et al. 2009a; Jiang et al. 2009; Patel et al. 2019), or the loss of Wg signaling (Tian et al. 2016). EBs can also be a source of Upd2 upon stress (Patel et al. 2015; Tian et al. 2015). Although it is not entirely clear how Upd2 and Upd3 are induced by gut epithelial stress, an analysis of the *upd3* promotor implicated the transcription factors Sd, Mad, and D-Fos as direct regulators, downstream of stress-dependent Hippo, Dpp, and JNK/$p38^{MAPK}$ signaling, respectively (Houtz et al. 2017). Paralleling the midgut, regeneration of the fly hindgut also requires Upd3, which can be induced by apoptotic injury within the developing hindgut (Sawyer et al. 2017; Cohen et al. 2021). Notwithstanding the central role of Upd/Jak/Stat signaling in intestinal regeneration, genetic tests show that Upd2 and Upd3 are not essential for ISC proliferation (Jiang et al. 2009; Beebe et al. 2010). Moreover, these cytokines are not required for development of the gut or other organs, although they are used extensively during stress responses and regeneration. Thus, this pathway appears to be relatively dedicated to damage-dependent regeneration.

## REGENERATIVE GROWTH DIRECTED BY EGFR SIGNALING

The EGFR/Ras/mitogen-activated protein kinase (MAPK) signaling pathway is an essential driver of both ISC proliferation during adult gut regeneration, and for AMP cell proliferation in *Drosophila* larvae and pupae. EGFR signaling plays an equally important role in promoting stem and transit-amplifying cell proliferation in vertebrate intestines, where it is essential for epithelial regeneration and commonly hyperactivated in intestinal cancers (Dekker et al. 2019; Jardé et al. 2020; Beumer and Clevers 2021). In *Drosophila*, three secreted ligands (Vein, Spitz, and Keren) activate Ras/Raf/Mapk signaling through the EGFR, which is expressed in ISCs and EBs and required for their growth and/or division. Loss of EGFR or many other components in this highly conserved signaling system arrests ISC division and compromises ISC survival (Buchon et al. 2010; Ren et al. 2010; Biteau and Jasper 2011; Jiang et al. 2011). Gut epithelial stress, damage, or infection up-regulate all three EGFR ligands in the adult intestine. Spitz (Spi) is expressed mostly in progenitor cells (Buchon et al. 2010), whereas Keren (Krn) is expressed mainly in ECs and Vein (Vn) is induced in VM (Jiang et al. 2011). Several Rhomboids (Rho), proteases that cleave and activate Spi and Krn, are concurrently induced in ECs (Jiang et al. 2011; Liang et al. 2017). These factors activate the EGFR/Ras/MAPK signaling cascade in ISCs where they act as potent mitogens, driving rapid ISC growth and division to facilitate midgut renewal (Jiang et al. 2011). During normal gut epithelial renewal, and perhaps also in instances of stress, the induction of Rho by the loss of E-cadherin and release of β-catenin/armadillo in apoptotic ECs is a driving factor for Spi and Krn secretion, and for EGFR activation in ISCs (Liang et al. 2017). Unlike JAK/STAT signaling, EGFR/Ras/MAPK signaling is not required for EE or EC differentiation (Jiang et al. 2011), although it might accelerate these processes. To address how EGFR signaling promotes ISC proliferation, Jin et al. used a combination of DamID-seq and RNA-seq to investigate the genome-wide regulatory targets of this pathway. They found that MAPK-dependent inactivation of the conserved transcriptional repressor, Capicua (Cic), promotes the transcription of a large set of proliferation genes, including cell-cycle regulators and the ETS domain transcription factors Ets21C and Pointed (pnt) (Jin et al. 2015), which were also sufficient to promote ISC divisions. Beyond this mitogenic role, EGFR/Ras/MAPK signaling also promotes damage-induced growth and polyploidization of EBs and newborn ECs (Xiang et al. 2017), and is required for proper delamination of damaged ECs from the gut epithelium (Buchon et al. 2010). The postmitotic growth of differentiating ECs, which increase their DNA content to 8-32C and their mass proportionally, is an important aspect of gut epithelial regeneration in *Drosophila*, and is probably equally important in a wide range of invertebrates.

Cross talk between EGFR/Ras/MAPK and Upd/JAK/STAT signaling has also been investigated in *Drosophila*. Interestingly, the two pathways are each capable of activating the production of the others' ligands (Buchon et al. 2010; Jiang et al. 2011), reminiscent of inflammatory responses in mammals. In a signaling relay, Upd2 and Upd3 produced by stressed ECs triggers STAT activation in the VM, which responds by producing the Vn ligand, to activate EGFR in ISCs (Jiang et al. 2011). However, gut epithelial stress can activate EGFR/MAPK signaling even if JAK/STAT signaling is suppressed, implying that the induction of EGFR ligands and Rhomboids by midgut stress, and their impact on ISC proliferation, can be mediated independently of and in parallel with Upd/JAK/STAT signaling (Liang et al. 2017).

## STRESS SENSING BY JNK AND P38$^{MAPK}$

Jun amino-terminal kinase (JNK) and p38$^{MAPK}$ are MAPK family members that initiate cellular responses to stress in many animal cells. In *Drosophila*, both of these MAPKs, and their regulators, play important roles in gut regeneration during aging and in response to acute stress. During aging, intestinal microbiota can change substantially, both in species composition and overall load (Buchon et al. 2009a; Guo et al. 2014). Microbiotic imbalances (termed "dysbiosis") activate dual oxidase (DUOX) signaling in the epithelial cells to produce reactive oxygen species (ROS) (Kim and Lee 2014), which in turn up-regulate signaling via JNK and p38$^{MAPK}$ to promote ISC proliferation (Jasper 2020). Upon infection by pathogens that directly damage the gut epithelium, such as *Pseudomonas,* JNK signaling is activated mostly in ECs (Apidianakis et al. 2009; Jiang et al. 2009). However, infection by *Erwinia*, a gram-negative bacterium that induces an oxidative burst, or feeding the ROS-producing agent paraquat, activates JNK signaling in both mature ECs and progenitor cells (Biteau et al. 2008; Buchon et al. 2009a). JNK activation then promotes Upd2 and Upd3 expression, thereby stimulating ISC proliferation and facilitating regeneration (Biteau et al. 2008; Buchon et al. 2009a; Jiang et al. 2009; Patel et al. 2015; Houtz et al. 2017). JNK activation in damaged ECs can also kill these cells, potentiating midgut turnover (Apidianakis et al. 2009; Jiang et al. 2009). JNK activation in ISCs likely has somewhat different functions, such as activating stress-responsive genes as protection from oxidative damage (Buchon et al. 2009a), stimulating proliferation (Biteau et al. 2008; Buchon et al. 2009a), and altering ISC differentiation by inducing Dl expression and/or promoting ISC symmetric divisions (Biteau et al. 2008; Hu and Jasper 2019; Rodriguez-Fernandez et al. 2020). Most effects of JNK signaling are thought to be executed through the Jun/Fos transcription factor complex (AP-1, encoded by *jra* and *kay* in *Drosophila*), which is activated via phosphorylation by JNK (*bsk* in *Drosophila*) (Houtz et al. 2017). However, a recent study showed that the ETS-type transcription factor, Ets21C, also acts downstream of JNK to induce ISC proliferation and EC apoptosis (Mundorf et al. 2019).

Despite its apparently central role in transducing damage signaling to activate ISCs during regeneration, genetic tests show that JNK signaling is only partially required in ECs for the induction of Upd3 and ISC activation. This suggests that other independent pathways act in parallel to couple stress to regeneration (Houtz et al. 2017; Patel et al. 2019). Signaling via the p38$^{MAPK}$ is one such pathway (Patel et al. 2019). In this pathway, stress from bacterial infection, as well as mechanical or detergent damage, activate the NADPH oxidase, Nox, generating ROS, which in turn activates Ask1, a ROS-sensing kinase upstream of p38$^{MAPK}$. But like the related JNK pathway, p38$^{MAPK}$ signaling is only partially required for regenerative responses in the *Drosophila* midgut, suggesting that these two stress-detection systems might function in concert, or as accessories to yet other damage-sensing pathways.

## MECHANO-STRESS SENSING BY Hippo/Warts/Yorkie SIGNALING

The Hippo/Warts/Yorkie pathway comprises a conserved kinase cascade that regulates regeneration in several organs in *Drosophila*, mice, and presumably all higher animals (Yu et al. 2015; Moya and Halder 2019). Core components of

this pathway include the Hippo kinase (Hpo; MST1/2 in mammals), which activates Warts (Wts; LATS1/2 in mammals), which in turn phosphorylates and inactivates the transcriptional coactivator Yorkie (Yki; YAP/TAZ in mammals). Inactivation of the Hippo or Warts kinases leads to the nuclear translocation of Yki, where it binds to a transcription factor, Scalloped (Sd or TEAD1-4 in mammals), to induce downstream targets that promote cell growth, proliferation, and survival (Zhang et al. 2017). Studies in diverse *Drosophila* and mouse cell types show that Hpo and Wts can transduce mechanical inputs including stretch, loss of cell adhesion, and epithelial disruption by interacting with cytoskeletal and cell junctional factors. Accordingly, Hippo signaling has essential functions in maintaining epithelial homeostasis and regulating regeneration in the *Drosophila* midgut (Karpowicz et al. 2010; Ren et al. 2010; Shaw et al. 2010; Staley and Irvine 2010; Jin et al. 2013), and the mouse intestine (Cai et al. 2010; Serra et al. 2019; Cheung et al. 2020). Inactivation of Hpo or Wts in ECs causes ISC hyperproliferation, an effect achieved in part by the induction of multiple Upd cytokines and EGFR ligands (Karpowicz et al. 2010; Ren et al. 2010; Shaw et al. 2010; Staley and Irvine 2010; Poernbacher et al. 2012). Consistent with this, disruption of the gut epithelium by infection or tumors up-regulates Yki levels in ECs and the expression of Yki target genes (Karpowicz et al. 2010; Shaw et al. 2010; Patel et al. 2015; Suijkerbuijk et al. 2016). Loss of Yki (Ren et al. 2010; Zhang et al. 2019) or its downstream target *bantam* (Huang et al. 2014) in ISCs blocks their mitotic response, indicating a separate requirement in ISCs. However, compared to its robust function in ECs, inactivation of Wts or overexpression of Yki in ISCs induces relatively mild ISC responses, indicating that Hpo pathway components execute their most important functions in ECs (Ren et al. 2010; Shaw et al. 2010; Hao et al. 2020). A recent paper reported another mechanism, in which the *longitudinals lacking* (*Lola*) transcriptional repressor is stabilized by Wts to restrain ISC proliferation, independently of Yki-Sd activity (Hao et al. 2020).

How does Hippo signaling monitor gut epithelial integrity? Experiments from *Drosophila* show that septate junctional (SJ) proteins (e.g., Tetraspanin 2A [Tsp2A], Snakeskin [Ssk], Mesh, etc.) in ECs restrict Yki activation, thereby controlling ISC proliferation non-cell-autonomously (Xu et al. 2019; Chen et al. 2020). SJ structures between ECs are maintained by autophagy, which has an inhibitory effect on Yki via Wts, a Ref(2)P–Dachs complex (Nagai et al. 2021) and aPKC (Xu et al. 2019). Other mechanical sensors that feed into Hpo-Wts-Yki signaling include the atypical proto-cadherins Fat (*Ft*) and Dachsous (*Ds*), the ERM family member Merlin/NF2, the tumor-suppressor gene Scribble (*Scrib*), the cortical cytoskeletal protein Crumbs (*crb*), and even Actin (Yu et al. 2015; Fletcher et al. 2018). Of note, ISC tumors or overproliferation can cause long-range activation of Yki in the fly gut epithelium (Patel et al. 2015; Zhang et al. 2019), but whether this is mediated by propagation of mechano-stress or secreted molecules is unknown. In one particularly interesting study, food particle ingestion was used to promote mechano-stretching of the midgut, and this up-regulated Upd3 in EBs through Hpo-related Tao/Msn/Wts/Yki signaling pathway (Li et al. 2014, 2018). Altogether, work from *Drosophila* and mice indicate that Hpo and Msn signaling are important sensors of gut epithelial integrity that integrate with other pathways (e.g., Upd/Jak/Stat, Rho/EGFR) to mediate gut regeneration.

## OTHER REGULATORY PATHWAYS

In addition to the signaling network summarized above, many other factors affect homeostasis and regeneration in the *Drosophila* midgut. For instance, aging and oxidative stress increase the expression of PVF2, a PDGF/VEGF-like growth factor that feeds into the Pvr/Ras/Raf/Mapk pathway, and this is pro-proliferative for midgut ISCs (Choi et al. 2008; Bond and Foley 2012).

The BMP family factor, Dpp, also plays multiple, somewhat complex roles in the gut. Sustained Dpp signaling is required in the middle midgut to direct the differentiation of a specialized EC subtype called the copper cell (Guo et al. 2013; Li et al. 2013a, 2016), and Dpp signaling has been proposed to promote EC differentia-

tion in general (Tian and Jiang 2014; Zhou et al. 2015). But upon injury, Dpp signaling is up-regulated in both ECs and progenitors along the entire anteroposterior axis, in part by DPP secreted by hemocytes recruited to the damaged gut (Ayyaz et al. 2015) and in part by DPP secreted by the VM (Guo et al. 2013; Zhou et al. 2015). This damage-dependent up-regulation of Dpp signaling is necessary for regeneration (Tian et al. 2017), but eventually functions to restrict ISCs proliferation (Guo et al. 2013; Ayyaz et al. 2015; Zhou et al. 2015; Ma et al. 2019; Takemura et al. 2021). Thus, inactivation of Dpp signaling in progenitors or depletion of Dpp in the VM leads to ISC hyperproliferation and gut dysplasia, a condition likened to human juvenile polyposis (Guo et al. 2013), which is also caused by loss of BMP signaling.

The enteric commensal microbiota also contributes signals that promote gut regeneration. In larval midguts, *L. plantarum* stimulates the generation of microbicidal ROS, which activate CncC/Nrf2 signaling in ECs, thus promoting AMP proliferation nonautonomously (Reedy et al. 2019). In the adult midgut, bacterial-derived uracil activates dual oxidase (DUOX) in ECs to generate microbicidal ROS (Lee et al. 2013), which in turn promotes ISC proliferation and gut renewal (Buchon et al. 2009a; Lee 2009) possibly via $p38^{MAPK}$ (Patel et al. 2019). PLCβ/PKC/$Ca^{2+}$ signaling and Hedgehog/Cadherin 99C (Cad99C) signaling-dependent endosome formation have been found to be required for this uracil-induced DUOX activation (Lee et al. 2015, 2018).

## ISC-INTRINSIC FUNCTIONS DURING REGENERATION

How ISCs integrate the many signals described above to maintain gut homeostasis is still a matter of speculation, but recent studies show that intracellular calcium signaling may respond to a wide range of mitogenic signals to integrate stress-based inputs with ISC proliferation. Elevation of cytosolic calcium in ISCs occurs in response to various stresses, and is required and sufficient to trigger ISC proliferation (Deng et al. 2015; Xu et al. 2017). The proliferative effect of $Ca^{2+}$ signaling requires EGFR/RAS signaling, but precisely why is not yet clear. Accumulating evidence indicates that calcium fluxes are involved in many constitutive membrane-trafficking events (Hay 2007), suggesting that intracellular vesicle trafficking should be critical for ISC division and gut homeostasis. Indeed, rerouting intracellular vesicle traffic by suppressing autophagy or endocytosis can drastically up-regulate Ras/Mapk and Jak/Stat signaling and ISC proliferation, leading to gut hyperplasia, barrier breakdown, and mortality (Ren et al. 2015; Zhang et al. 2019). The function of the endocytosis-autophagy network declines in aged ISCs (Du et al. 2020), contributing to gut dysplasia, and this can be rescued by lipoic acid supplementation. Yet how vesical trafficking is regulated in ISCs upon stress-induced gut regeneration is still unknown.

*Drosophila* Myc (dMyc), an important regulator of growth-associated metabolism, integrates various stress-dependent signals (Hpo, JAK-STAT, WNT, EGFR) that promote ISC proliferation, and dMyc is crucial for sustaining ISC self-renewal and differentiation (Cordero et al. 2012b; Ren et al. 2013). Also pertinent, a recent study (Morris et al. 2020) found that during episodes of regenerative growth ISCs sustain influxes of calcium into their mitochondria that stimulate ATP production to meet the higher demands of proliferation. These studies of Myc and mitochondria emphasize an expected role of growth-associated metabolism in ISC activation during regeneration, but more research is required to determine how ISC metabolism is regulated by the signaling pathways detailed above. Another fundamental question that research in *Drosophila* can shed light on is how epithelial damage is sensed in the first place. Although a diverse array of environmental insults can trigger regenerative signaling, the proximal sensors of some of these inputs are still unidentified.

## INTERORGAN COMMUNICATION AND FLY GUT REGENERATION

In addition to organ-intrinsic responses, the fly midgut maintains active communication with other organs that influence its regenerative ca-

pabilities (Colombani and Andersen 2020). Examples of ovary-to-gut (Ahmed et al. 2020; Zipper et al. 2020), testis-to-gut (Hudry et al. 2019), trachea-to-gut (Li et al. 2013b; Perochon et al. 2021; Tamamouna et al. 2021), and hemocyte-to-gut (Ayyaz et al. 2015; Chakrabarti et al. 2016) signaling interactions have all been described. These interorgan communications each significantly alter ISC behaviors during gut regeneration, and are also used to resize the gut according to the needs of the animal.

## GUT REGENERATION IN OTHER INSECTS

### Lepidoptera (Butterflies and Moths)

The Lepidopteran larval gut has a monolayer epithelium comprised of four major cell types: ISCs, goblet cells (GCs), columnar cells or ECs, and EE cells (Castagnola and Jurat-Fuentes 2016). Circular and longitudinal muscle fibers form an extracellular matrix (ECM) and produce a basement membrane located basal to the epithelial layer, and this is interwoven with trachea that provide oxygen to the muscle and underlying epithelium (Castagnola and Jurat-Fuentes 2016). This structure is very similar to that in *Drosophila*, and it is also grossly similar to that found in vertebrates. Lepidopteran larval ISCs are capable of proliferation during both larval molting and pathogenic infections. In some species (e.g., the silk moth, *Bombyx mori*), larval ISCs undergo rounds of division and differentiation to generate new cells (mostly ECs) prior to each molt, thus increasing gut cell numbers dramatically (~200-fold) as the larval gut grows (Baldwin and Hakim 1991; Franzetti et al. 2016). Much of the research on Lepidopteran gut regeneration has been done with larval midgut primary cell cultures. This in vitro culture system is advantageous since it can maintain the proliferation and differentiation features of ISCs as observed in vivo during molting and injury-induced gut regeneration (Sadrud-Din et al. 1996; Loeb et al. 2001). Isolated Lepidopteran (*Heliothis virescens*) ISCs undergo asymmetric cell divisions during epithelial regeneration but can also undergo symmetric differentiation after treatment with peptides called midgut differentiating factors (MDFs) (Loeb 2010; Castagnola et al. 2011; Castagnola and Jurat-Fuentes 2016). Other factors (e.g., ecdysone, α-arylphorin, and the insulin-related peptide hormone bombyxin) have also been demonstrated to be required for the differentiation of cultured Lepidopteran ISCs (Blackburn et al. 2004; Goto et al. 2005; Smagghe et al. 2005). However, in contrast to *Drosophila*, little is currently known about the molecular mechanisms involved in gut-healing responses in Lepidoptera. Nevertheless, some gene products (e.g., arylphorins [Willott et al. 1989]) are critical in the regenerative response to ingested Bt toxins. Notably, α-arylphorin, a protein secreted by the larval fat body into the insect hemolymph, has proliferative effects on ISCs in vitro and can induce gut hyperplasia in vivo (Baldwin and Hakim 1991; Blackburn et al. 2004; Hakim et al. 2007).

### *Aedes* (Mosquitos)

As important vectors of human and animal diseases, several mosquito species (*Aedes albopictus*, *Culex pipiens*, *Aedes gambiae*) represent serious threats to public health. Understanding the mechanisms that regulate regenerative capacity of the mosquito intestine should therefore be advantageous for developing novel vector control strategies. Different species of mosquitoes exhibit distinct susceptibilities to gut damage (Janeh et al. 2019). Feeding *A. albopictus* with the detergent SDS or the bacterial pathogen, *Serratia marcescens*, significantly increased expression levels of Keren (an EGFR ligand) and Socs36E (a target of the JAK/STAT pathway), suggesting that EGFR and JAK/STAT signaling are involved in mosquito gut regeneration (Janeh et al. 2017), similar to the situation in *Drosophila* as described above. In *Aedes aegypti* larvae, exposure to the Bt Cry family of toxins up-regulates several processes, including vesicular trafficking, MAPK pathways, lipid metabolism, and the unfolded protein response (UPR) (Bedoya-Pérez et al. 2013; Canton et al. 2015). These clues suggest that gut regeneration in mosquitos probably involves molecular and cellular mechanisms closely related to those that have been intensively characterized in *Drosophila,* also a Dipteran.

## CONCLUSIONS AND PERSPECTIVE

As discussed, insect ISCs engage in a multitude of interactions with their neighboring cells, the nutritional and microbiotic contents of the gut, and other organs to facilitate gut epithelial maintenance, regeneration, and adaptive growth. A prevalent model supported by extensive literature is that ECs in the gut epithelium act as primary sensors of stress, and generate and deliver mitogenic signals to ISCs, which then divide to drive epithelial renewal (Fig. 1). However, this model is definitely not the entire story, and clear exceptions can already be found in the literature. Several experiments have shown that even without EC damage, ISC division promotes the shedding of ECs, and that excess ECs and EBs that are healthy can be shed through active culling (Jin et al. 2017; Loudhaief et al. 2017; Reiff et al. 2019). These and other observations are consistent with mechanisms in which environmental signals directed at ISCs, as well as ISC-intrinsic factors, promote epithelial renewal. Other modes of regeneration that do not even require ISC division have also been described. For instance, after *S. marcescens* infection, *Drosophila* ECs undergo cytoplasmic extrusion to purge damaged cellular components, shrinking the epithelium but protecting it from infection stress (Lee et al. 2016). As the infection is cleared by the immune system, ECs then initiate rapid cytoplasmic biosynthesis to recover their lost mass, reestablishing a healthy epithelium. Similarly, after infection with *P. entomophila* (which does kill ECs), newborn ECs endoreplicate their DNA more and grow larger than normal, generating additional epithelial mass independent of ISC division (Xiang et al. 2017).

The conservation of arthropod genomes and gut structures suggests that the damage-response pathways that mediate *Drosophila* gut regeneration will prove to be largely conserved among the different insect orders and into other arthropods. Information from *Drosophila* should prove useful in developing gut-based strategies to control insects that are agricultural pests and vectors of human disease. Moreover, the similarities between mechanisms of gut homeostasis in insects and humans (Colombani and Andersen 2020; Beumer and Clevers 2021) imply that many of the genes and regulatory interactions described in *Drosophila* will also be relevant to human diseases that involve deregulated stem cell growth (e.g., cancers, chronic inflammation), and to stem-cell-based regenerative therapies.

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