Intestinal Stem Cell Proliferation and Epithelial Homeostasis in the Adult *Drosophila* Midgut.

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Abstract

Adult tissue homeostasis requires a tight balance between the removal of old or damaged cells and the production of new ones. Such processes are usually driven by dedicated stem cells that reside within specific tissue locations or niches (Nystul and Spradling, 2006).

The intestinal epithelium has a remarkable regenerative capacity, which has made it a prime paradigm for the study of stem cell-driven tissue self-renewal. The discovery of the presence of stem cells in the adult midgut of the fruit fly *Drosophila melanogaster* has significantly impacted our understanding of the role of stem cells in intestinal homeostasis. Here we will review the current knowledge of the main mechanisms involved in the regulation of tissue homeostasis in the adult *Drosophila* midgut, with a focus on the role of stem cells in this process. We will also discuss processes involving acute or chronic disruption of normal intestinal homeostasis such as damage-induced regeneration and ageing.

Introduction

The gastrointestinal tract, referred to as the gut, is a hollow muscular tube lined with a highly specialized epithelium. This organ occupies a large portion of the body cavity and it is in charge of multiple biological roles, which are vital to maintain organismal fitness (Figure 1). Functions of the gut include nutrient absorption, activation of the immune response against pathogens and regulation of multiple metabolic and endocrine functions. Furthermore, due to its remarkable self-renewing capacity, the gut epithelium has long been a prime paradigm for the study of stem cell function during adult tissue homeostasis.

The fruit fly *Drosophila melanogaster* is one of the longest-established genetically tractable model organisms. Its short life cycle and amenable genetics has made it an unbeatable model system for the study of key developmental processes (Nusslein-Volhard and Wieschaus, 1980). Furthermore, since the groundbreaking discovery of the presence of stem cells in the adult *Drosophila* midgut (Micchelli and Perrimon, 2006) (Ohlstein and Spradling, 2006) the fly gut has represented an invaluable research system in fields such as stem cell biology, host-pathogen interactions, metabolism and ageing among others. A comprehensive overview of the multiple physiological functions of the *Drosophila* gut can be found in a recent review by B. Lemaitre and I. Miguel-Aliaga (Lemaitre and Miguel-Aliaga, 2013). Here, we will focus our efforts in summarizing the current knowledge on the role and regulation of intestinal stem cells during homeostasis of the adult *Drosophila* midgut and its impact on animal fitness.

Origin and specification of intestinal stem cells

Drosophila intestinal stem cells (ISCs) derive from adult midgut precursors (AMPs).

AMPs are cells of endodermal origin, which are specified during the early embryonic stages and form part of the embryonic midgut (Micchelli, 2012). The embryonic midgut is retained throughout larval development. Larval AMPs undergo a series of cell divisions, which are regulated through cell autonomous activation of the EGFR/MAPK signalling by EGF ligands emanating from the visceral muscle and AMPs themselves (Jiang and Edgar, 2009). Additionally, a transient Dpp/BMP niche provided by peripheral cells (PC) enwrapping AMPs is required to allow AMP proliferation while preventing their differentiation (Mathur et al., 2010). As the animal enters metamorphosis, the larval midgut degenerates except for clusters of AMPs, which merge throughout the pupal stage to generate the adult midgut epithelium (Mathur et al., 2010) (Nakagoshi, 2005) (Takashima and Hartenstein, 2012) (Micchelli, 2012) (Jiang and Edgar, 2009). It is still unclear how a single AMP per cluster is selected to become an adult ISC. Detailed morphological analysis of the developing midgut reports the presence of a transient pupal midgut, which generates from AMPs at the start of metamorphosis. The pupal midgut intercalates between the larval and adult midgut and later degenerates to form the 'yellow body' towards the end of metamorphosis (Takashima et al., 2011).

Structure of the adult *Drosophila* midgut

The adult *Drosophila* gut has a tubular structure and is surrounded by visceral muscle, nerves and trachea. This muscular tube is lined with a pseudostratified monolayered epithelium and it is divided into three compartments defined by their embryonic origin: the foregut, midgut and hindgut (Figure 1B). The foregut and the hindgut are of ectodermal origin, while the midgut originates from the endoderm. A protective cuticle covers the apical surface of the foregut and hindgut whereas a chitin-rich layer, the peritrophic membrane, lines the midgut epithelium (Lehane, 1997). The foregut is composed of the pharynx, the oesophagus and the crop. The midgut extends from the

cardia until the junction with the hindgut, where the Malpighian tubules connect with the gut (Figure 1B). Detailed histological and molecular analysis demonstrates a complex degree of compartmentalization in the adult fly midgut (Buchon et al., 2013b) (Marianes and Spradling, 2013) (Li et al., 2013a). Further details on gut compartmentalization, the perithropic membrane and Malpighian tubules can be found in different articles of this issue.

The adult midgut epithelium is composed of 4 different cell types: intestinal stem cells (ISCs), undiferentiated progenitor cells called enteroblasts (EBs) and specialized absorptive enterocytes (ECs) and secretory enteroendocrine cells (EEs) (Figure 1D). ISCs are randomly scattered along the basal membrane of the intestinal tube and, following division, they are proposed to give rise to EBs, which differentiate into either EEs or ECs (Ohlstein and Spradling, 2006) (Micchelli and Perrimon, 2006). ISCs can divide both symmetrically and asymmetrically. A combined approach of mathematical modeing and genetic experiments suggest that, as in the mouse intestinal epithelium (Snippert et al., 2010), ISCs in the adult *Drosophila* midgut can devide symetrically and stochastically give rise to either two stem cells or two differentiated daughter cells (de Navascues et al., 2012). On the other hand, integrin-dependent adhesion to the basal membrane— leading to apical localization of the Par complex— and assymetric localisation of Sara endosomes contribute to Notch signalling bias and assymetric division of ICSs to produce EBs (Goulas et al., 2012) (Montagne and Gonzalez-Gaitan, 2014). Recent work also suggests that ISCs could directly differentiate into EEs whithout involving EBs (Biteau and Jasper, 2014). It is unclear what might influence the intestinal epithelium to chose between these different modes of ISC division and lineage production. Assymetric ISC dvision may be a predominant feature of the homeostatic self-renewing epithelium while the presence of stressors or changing eviromental conditions, requiring a more robust production of stem cells or specialized daughter cells, may favour symmetric divisions. This hypotheis is

supported by elegant lineage tracing experiments performed in the midgut of newly eclosed feeding animals (O'Brien et al., 2011).

Basal homeostatic self-renewal of the adult *Drosophila* midgut

As in the case of the mammalian intestine, the adult *Drosophila* midgut is constantly self-renewed by its resident stem cells (ISCs) (Ohlstein and Spradling, 2006) (Micchelli and Perrimon, 2006). Normal self-renewal of the intestinal epithelium requires a tight regulation of the activity of multiple conserved signalling pathways (Figure 2A).

Basal levels of activation of EGFR/Ras/MAPK and Wg signalling are required to maintain homeostatic self-renewal of the intestinal epithelium. EGF-like and Wg/Wnt ligands secreted from the visceral muscle, ECs and stem/progenitor cells (ISCs/EBs) result in pathway activation within ISCs (Biteau and Jasper, 2011) (Jiang et al., 2011) (Buchon et al., 2010) (Cordero et al., 2012b). Knocking down signal transduction from the EGFR/Ras/MAPK or Wg pathways within stem/progenitor cells leads to progressive cell loss and subsequent thinning of the intestinal epithelium (Biteau and Jasper, 2011) (Jiang et al., 2011) (Buchon et al., 2010) (Cordero et al., 2012b). Conversely, hyperactivation of either pathway leads to ISC hyperproliferation (Lin et al., 2008) (Lee et al., 2009) (Biteau and Jasper, 2011) (Jiang et al., 2011) (Buchon et al., 2010) (Cordero et al., 2012b) (Cordero et al., 2012a). Multiple sources of the BMP ligands Dpp and Gbb, maintain intestinal homeostasis by constraining ISC proliferation (Tian and Jiang, 2014) (Li et al., 2013b) (Zhou et al., 2014). Recently, Hedgehog (Hh) signalling has emerged as a positive regulator of basal ISC proliferation in the midgut. In that context, Debra-dependent degradation of the transcription factor Ci controls levels of Hh pathway activation and tissue homeostasis (Li et al., 2014).

Notch and JAK/Stat signalling regulate differentiation of ISCs into the EE and EC cell lineage. Low levels of Notch activity in progenitor cells (EBs) is believed to be required

for their differentiation into EEs while high levels of the pathway are necessary for ECs specification (Ohlstein and Spradling, 2006) (Micchelli and Perrimon, 2006) (Bardin et al., 2010) (Perdigoto et al., 2011) (Perdigoto and Bardin, 2013). Preventing JAK/Stat signalling through knock down of *Drosophila* Hop/JAK, the cytokine receptor Domeless or the transcription factor Stat leads to an overrepresentation of stem/progenitor cells in the adult midgut (Jiang et al., 2009) (Beebe et al., 2010). Autocrine activation of PVF2/PVR signalling also regulates homeostatic ISC proliferation and differentiation (Bond and Foley, 2012). Additionally, recent reports show that the Snail homolog Escargot (Esg), which is expressed in stem/progenitor cells (Micchelli and Perrimon, 2006), is essential for the maintenance of ISCs in the adult midgut. Loss of *esg* induces premature differentiation of progenitor cells into ECs and EEs, while ectopic *esg* expression forces cells into a stem cell fate (Korzelius et al., 2014) (Loza-Coll et al., 2014).

Homeostatic self-renewal in the fly midgut also fluctuates in response to metabolic and environmental cues. Damage-induced ISC proliferation in midgut has been shown to follow a circadian pattern and the transcription factor *period,* a core component of the circadian clock is a critical mediator of intestinal regeneration (Karpowicz et al., 2013). The intestine of newly eclosed animals undergoes a growing phase and an increase in stem cell numbers as the animal starts feeding, which is mediated by a local insulin-like source secreted from the visceral muscle (O'Brien et al., 2011). Additionally, it has been shown that tissues directly associated with the gut can also respond to the presence or absence of nutrients. Gut-associated tracheae undergo drastic remodeling depending on nutrient availability (Linneweber et al., 2014). Whether this phenomenon is associated with nutrient-dependent adult ISC homeostasis remains unknown. Interestingly, recent work suggests a novel role for EEs — classically known for their endocrine function— as local regulators of ISC homeostasis and lipid metabolism in the adult midgut (Biteau and

Jasper, 2014) (Amcheslavsky et al., 2014) (Scopelliti et al., 2014) (Song et al., 2014). Therefore, exciting new links between ISC homeostasis and organismal physiology are likely to emerge from future research on the fly midgut.

Intestinal stem cell homeostasis during tissue regeneration

ISCs confer a powerful regenerative capacity to the intestinal epithelium (Bach et al., 2000). Pioneering studies have demostrated that the adult posterior *Drosophila* midgut is equally able to mount a very rapid and robust regenerative response to multiple agents disruptive to epithelial integrity (Amcheslavsky et al., 2009) (Buchon et al., 2009a) (Jiang et al., 2009). Conserved signalling pathways such as JNK, JAK/Stat, Hippo, EGFR, Wg, Hh and Dpp/BMP signalling have been shown to mediate damage or stress-induced intestinal regeneration in *Drosophila* (Figure 2B) (Apidianakis et al., 2009) (Jiang et al., 2009) (Buchon et al., 2009a) (Cronin et al., 2009) (Biteau and Jasper, 2011) (Jiang et al., 2011) (Shaw et al., 2010) (Staley and Irvine, 2010) (Ren et al., 2010) (Karpowicz et al., 2010) (Cordero et al., 2012b) (Li et al., 2013b) (Guo et al., 2013) (Li et al., 2013a) (Tian and Jiang, 2014) (Zhou et al., 2014) (Tian et al., 2015).

Perhaps the most physiologicaly relevant aspects of the intestinal regenerative response are the ones associated with the presence of microbes (Buchon et al., 2013a). Bacteria inhabited the earth for at least 2.5 billion years, therefore upcoming species had to coevolve with these prokaryotic organisms, leaving a noticeable mark on their physiology (Brocks et al., 1999). The surface of the intestinal epithelium is constantly exposed to microorganisms and it functions as the first line of defence against microbial pathogens while also regulating the homeostatic response to commensal microbes (Hooper and Gordon, 2001). The adult *Drosophila* midgut has proven to be a powerful model system to investigate host-microbial interactions as well as to study various cellular and molecular aspects of the behaviour of intestinal stem cells upon microbial presence

(Lemaitre and Hoffmann, 2007) (Buchon et al., 2013a) (Lee et al., 2013). On the one hand the intestine activates a local innate immune response directed to fight infection and eliminate pathogens. This is mediated by the production of antimicrobial peptides (AMPs), which are classically controlled by the Toll and IMD signalling pathways (Lemaitre and Hoffmann, 2007). However, the immune response of the midgut seems to be mostly in charge of the IMD pathway (Buchon et al., 2009b) (Tzou et al., 2000) (Ryu et al., 2006). Production of reactive oxygen species (ROS), mainly through the NADPH oxidase Duox, acts as an immune defence mechanism, which works in parallel to IMD to fight midgut infection by microbes (Ryu et al., 2006) (Ha et al., 2005) (Bae et al., 2010). Another essential element of the response to either commensal or pathogenic microbes in the gut is mediated at the level of the ISCs, which turn on a rapid and robust proliferative response that is essential to replenish the damaged epithelium (Figure 3A, B) (Buchon et al., 2009a) (Jiang et al., 2009) (Chatterjee and Ip, 2009). Flies incapable of gut epithelial renewal succumb to infection, demonstrating the importance of damage-induced ISC proliferation to overall organismal viability (Buchon et al., 2009a) (Osman et al., 2012) (Jiang et al., 2009). Multiple conserved pathways are involved in the modulation of ISC proliferation and epithelial regeneration of the adult *Drosophila* midgut in response to microbes and other damaging agents.

One key feedback mechanism activated in response to acute damage or stress within the intestinal epithelium is mediated by a crosstalk between the JAK/Stat and EGFR signalling pathways. In response to damage or stress to the intestinal epithelium the cytokines and JAK/Stat signalling ligands Upd2 and Upd3 are secreted by the ECs. These ligands induce JAK/Stat signalling in stem/progenitor cells as well as the secretion of EGF-type of ligands from multiple sources, which in turn activate EGFR/Ras/MAPK signalling in ISCs. Reciprocally, EGF ligands can induce the production of Upds. Together these event form part of a positive feedback loop, which drives the ISC proliferative response required

for regeneration of the damaged intestinal epithelium (Buchon et al., 2010) (Jiang et al., 2009) (Jiang et al., 2011) (Biteau and Jasper, 2011) (Osman et al., 2012) (Zhou et al., 2013). Another hallmark of the regenerative response to damage in the adult *Drosophila* midgut is the activation of Wg signalling. Wg is produced by progenitor cells in response to damage and this is required to drive ISC proliferation and tissue regeneration through activation of the Myc proto-oncogene (Cordero et al., 2012b). Hyperactivation of Wg signalling also induces the production of EGFs and Upds and thus contributes to EGFR-JAK/Stat signalling feedback loop (Cordero et al., 2012a). Interestingly, progenitor derived Wg is exclusively required for ISC proliferation during regeneration or ageing while it is redundant for basal tissue homeostasis (Cordero et al., 2012b). Similarly, damage-induced Hh activation in EBs is specifically required for regenerative but not homeostatic ISC proliferation (Tian et al., 2015). Perhaps, these scenarios indicate the presence of different thresholds of pathway activity required to drive ISC proliferation in the different contexts. One can envision that a local, strong signal may be required when a robust proliferative response is needed to regenerate the tissue upon injury/stress while minimal pathway activity suffices to maintain basal tissue homeostasis. Further work remains to be done to test this hypothesis.

JNK and Hippo pathway are recognised as two of the upstream sensors of tissue damage in the midgut, which lead to the production of Upds, EGF and Wg to ultimately induce ISC proliferation (Apidianakis et al., 2009) (Jiang et al., 2009) (Shaw et al., 2010) (Staley and Irvine, 2010) (Ren et al., 2010) (Karpowicz et al., 2010) (Cordero et al., 2012b). Finally, activation of DPP/BMP signalling is required for the return to homeostasis after repair of the damage epithelium has been achieved (Zhou et al., 2014) (Guo et al., 2013).

Ageing and Intestinal Stem Cell Homeostasis

The tight control of signalling networks, which dictates ISC homeostasis in the adult *Drosophila* midgut is commonly disrupted upon organismal ageing (Biteau et al., 2011) (Ayyaz and Jasper, 2013). Phenotypes such as ISCs hyperproliferation, cell lineage mis-differentiation and defective epithelial barrier and absorption functions characterize the ageing *Drosophila* midgut (Biteau et al., 2008) (Rera et al., 2012) (Choi et al., 2008) (Figure 3C).

Hyperactivation of the JNK-dependent stress response has been shown to be a main factor inducing age related hyperplasia in the *Drosophila* intestine, while exacerbated Notch signalling partly contributes to mis-differentiation (Biteau et al., 2008). Deregulation of additional pathways previously linked to the stress and damage response, such as components of the ROS and Wg/Myc signalling have also been associated with loss of homeostasis in the ageing *Drosophila* midgut (Wang et al., 2014) (Cordero et al., 2012b). Elevated levels of ROS contribute to age-dependent over proliferation of ISCs with high levels ROS signalling increasing organismal sensitivity to oxidative stress and thereby lowering lifespan (Sykiotis and Bohmann, 2010). JNK signalling has been shown to counteract the effects of ROS and antagonize Insulin/IGF signalling (IIS) leading to extension of lifespan (Wang et al., 2003) (Wang et al., 2005). Interestingly, balanced levels of JNK or IIS in ISCs contribute favourably to intestinal homeostasis and also increase animal lifespan (Biteau et al., 2010). A similar outcome has been observed upon reduction of Myc-dependent ISC proliferation in hyperplastic fly midguts (Cordero et al., 2012a). PVF2 expression in stem/progenitor cells is induced in response to oxidative stress and ageing and mediates age-related ISC proliferation and dysplasia in the midgut (Choi et al., 2008).

Signalling pathways responding to metabolic factors and components of the *Drosophila* immune pathways are also altered with age in the intestine. It has recently

been discovered that a negative regulator of the IMD/relish pathway, PGRP-SC2, plays a crucial role in preventing hyper-proliferation of ISCs in the ageing gut. PGRP-SC2 is reduced in ageing midguts leading to increased activity of Relish/NFκB, which results in ISC hyper-proliferation and disruption of host and commensal bacterial interactions in the intestine (Guo et al., 2014). A related subsequent report presents evidence for a role of systemic IMD activation in the fat body as a key mediator of homeostatic disruption in the ageing fly midgut (Chen et al., 2014). These studies emphasise the complexity of the mechanisms regulating intestinal homeostasis and the importance of achieving the right levels of ISC proliferation throughout the lifespan of the insect.

Conclusions

In the last 10 years we have witnessed an explosion in the number of studies using the *Drosophila* gut as a model system. The great plasticity and wide range of vital biological functions associated with the intestinal epithelium make it a prime paradigm for the study of questions related to developmental and stem cell biology as well as physiology. The long- and short-range connections between the gut and other tissues also establish it as an ideal system to study inter-organ communication. All of these coupled with the amazing range of genetic tools, which have always characterized *Drosophila,* guarantees our delight from seeing field-changing research using the gut of this tiny but powerful model organism.

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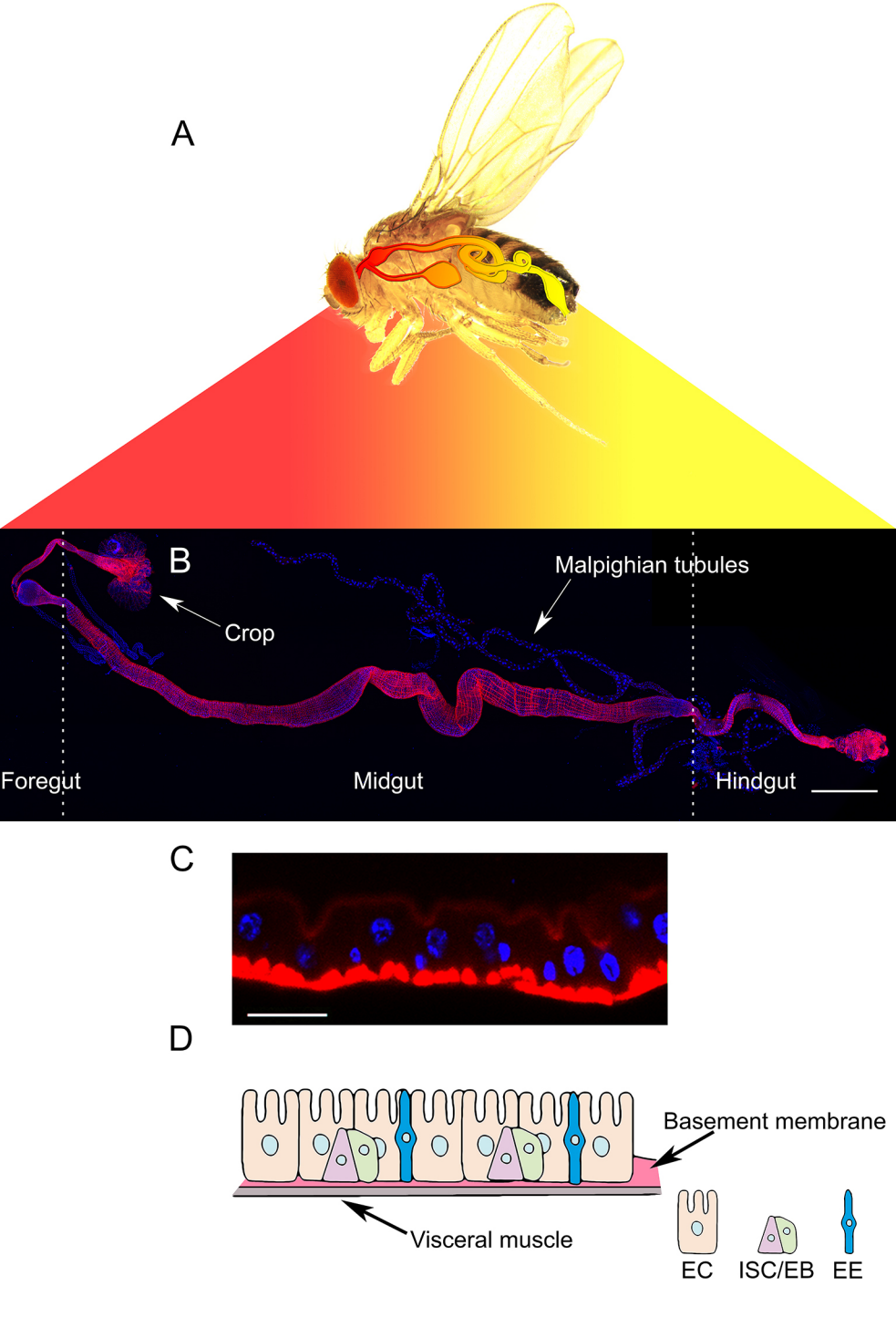
Figure Legends

Figure 1. The adult *Drosophila* gut. (A) Schematic overview of the digestive tract within the *Drosophila* body cavity. (B) Tiled confocal projection from an adult *Drosophila* gut stained with DAPI (blue) and Phalloidin (red). Dotted lines indicate boundaries between the different gut sections. Scale bar=500 μm. (C) Microscopic and (D) schematic representation of a section of the adult posterior midgut epithelium. (C) DAPI (blue) and Phalloidin (red); Scale bar=10 μm. (D) The different cell types within the tissue are indicated. EC: Enterocyte; ISC/EB: intestinal stem cell/enteroblast; EE: enteroendocrine cell.

Figure 2. Conserved pathways controlling *Drosophila* ISC proliferation. Spatial organization of signaling pathways involved in the regulation of ISC proliferation during homeostatic self-renewal (A) and upon stress/damage (B) of the midgut epithelium. For simplification purposes we have omitted the role of environmental and metabolic factors. EC: Enterocyte; ISC/EB: intestinal stem cell/enteroblast; EE: enteroendocrine cell; BM: basal membrane; VM: visceral muscle; Tr: trachea.

Figure 3. Stem/progenitor cell homeostasis in the adult *Drosophila* midgut. (A-C) Confocal projections of homeostatic (A), regenerating (B) and ageing (C) adult posterior midguts stained with DAPI (blue) and expressing GFP under the stem/progenitor driver *escargot-gal4* (*esg>gfp*; green). Scale bar=20 μm**.**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Foregut
Midgut
Hindgut
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Visceral muscle
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ISC/EB
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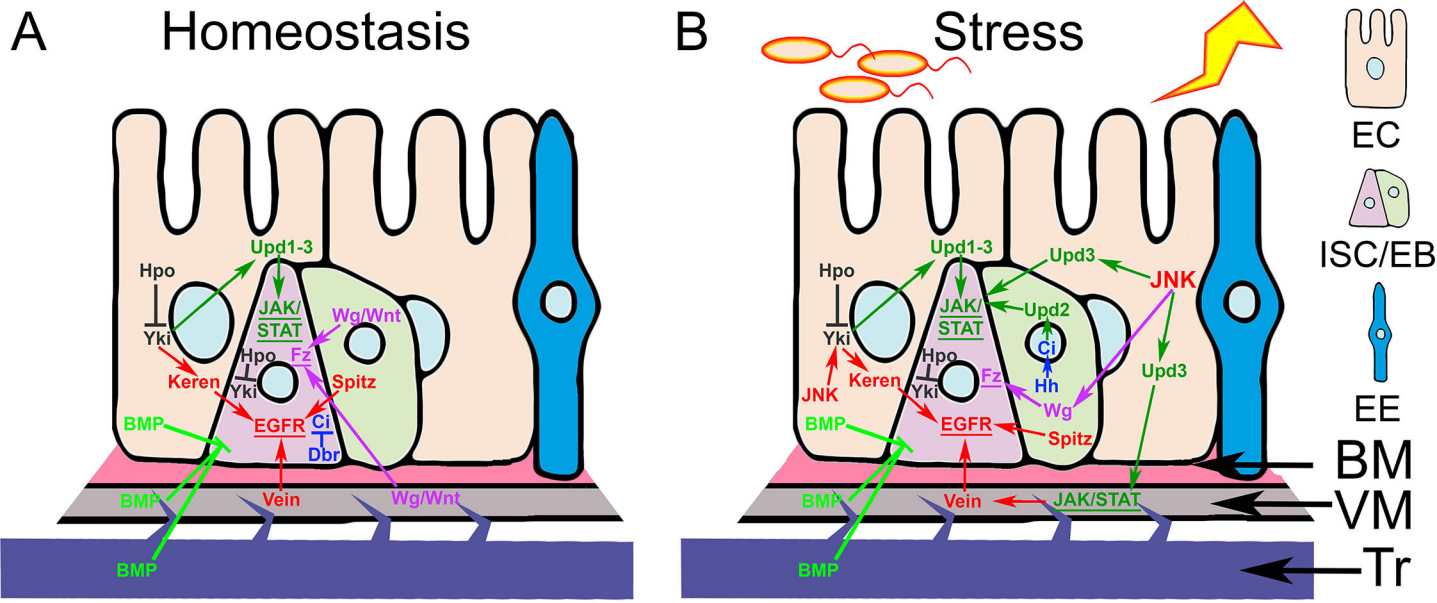
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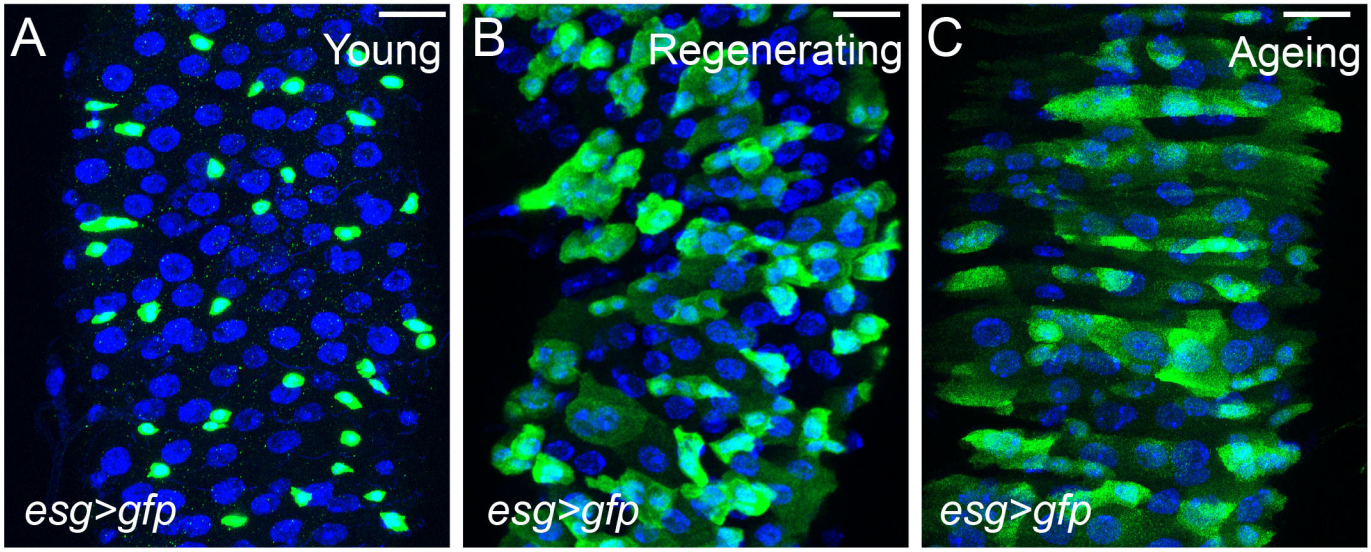
A
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esg>gfp
B
Regenerating
esg>gfp
C
Ageing
esg>gfp