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PRIMER

Model systems for regeneration: *Drosophila*

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ABSTRACT

Drosophila melanogaster has historically been a workhorse model organism for studying developmental biology. In addition, *Drosophila* is an excellent model for studying how damaged tissues and organs can regenerate. Recently, new precision approaches that enable both highly targeted injury and genetic manipulation have accelerated progress in this field. Here, we highlight these techniques and review examples of recently discovered mechanisms that regulate regeneration in *Drosophila* larval and adult tissues. We also discuss how, by applying these powerful approaches, studies of *Drosophila* can continue to guide the future of regeneration research.

KEY WORDS: *Drosophila*, Germline, Imaginal disc, Intestine, Regeneration

Introduction

In the early 1900s, Thomas Hunt Morgan turned his research focus to examine the genetic basis of heredity in the organism *Drosophila*, moving away from previous efforts that included studying the biology of regeneration in the flatworm *Planaria*. Over 100 years later, it is clear that the strength of genetic approaches developed by Morgan and his scientific descendants has circled back to impact our understanding of regeneration. Importantly, these approaches have revealed that specific developing and adult tissues in *Drosophila* are capable of regenerating damaged tissues. Moreover, given the extensive genetic resources available in this well-established model system, *Drosophila* has contributed a great deal to the study of regenerative mechanisms. Here, we provide an overview of current approaches used for regeneration research in *Drosophila*, highlighting the use of new methodology that should position the field for exciting breakthroughs. Focusing on techniques developed in the last 10 years, we discuss both recent advances and current unanswered questions in the field.

Regeneration in juvenile and adult arthropods

Drosophila is one of many arthropods that have served as model organisms for regeneration research. This research includes studies using insect models such as larvae of the flour beetle *Tribolium castaneum*, nymphs of the cricket *Gryllus bimaculatus* and cockroaches such as *Blattella germanica* and *Periplaneta americana*, as well as crustaceans such as the fiddler crab *Uca pugilator* and the beach hopper *Parhyale hawaiiensis* (for reviews, see Khan et al., 2016; Das, 2015). The regenerative capacity of the appendages or appendage primordia of these animals is constrained by the ability of each to grow. For example, holometabolous insects (see Glossary, Box 1) such as *Drosophila* cannot regenerate appendages after metamorphosis. Similarly, hemimetabolous insects (see Glossary, Box 1), which develop as nymphs, are only able to regenerate legs as long as they continue to molt. By contrast, crustaceans that grow and molt throughout adulthood retain their ability to replace appendages (for reviews, see Khan et al., 2016; Das, 2015). Indeed, the presence and maintenance of regenerative capacity across all animal species may be linked to the continual growth of the organ or appendage (Hariharan et al., 2015). Recent studies in these various arthropod species have begun to elucidate the molecular mechanisms underlying tissue injury responses such as wound closure, regeneration and re-patterning (for reviews, see Khan et al., 2016; Das, 2015), but the wealth of genetic tools and understanding of development in *Drosophila* give it an experimental advantage over many of these other model systems.

The *Drosophila* regeneration toolkit

There are many advantages to studying regeneration in *Drosophila*. Given their rapid life cycle, which proceeds from egg to adult in ∼10 days at 25°C, and short adult lifespan, which lasts ∼40 days at 25°C, regeneration experiments, including genetic screens, can be carried out quickly. In addition, the genetic strengths of *Drosophila* have facilitated in-depth mechanistic studies of regeneration, and emerging tools are rapidly propelling *Drosophila* regeneration research forward. As we highlight below, much of this work has focused on the imaginal discs, which are the larval primordia of adult structures. However, a number of more recent studies have also examined regeneration in stem cell-containing tissues in the adult, such as the midgut and germline, as well as in other tissues, such as muscle and the brain. *Drosophila* have also been used to study other responses that can occur after injury, such as wound healing and changes in tissue fate called transdetermination. However, the discussion here will focus on tissue restoration by cellular regeneration, and we direct readers to excellent reviews on wound healing (Razzell et al., 2011; Tsai et al., 2018; Zulueta-Coarasa and Fernandez-Gonzalez, 2017) and transdetermination (Beira and

Model systems for regeneration

This article is part of a series entitled 'Model systems for regeneration'. This series of articles aims to highlight key model systems and species that are currently being used to study tissue and organ regeneration. Each article provides background information about the phylogenetic position of the species, its life-cycle and habitat, the different organs and tissues that regenerate, and the experimental tools and techniques that are available for studying these organisms in a regenerative context. Importantly, these articles also give examples of how the study of these models has increased our understanding of regenerative mechanisms more broadly, and how some of the open questions in the field of regeneration may be answered using these organisms. To see the full collection as it grows, please visit: https://dev.biologists.org/collection/regeneration_models.

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Development (2020) 147, dev173781. doi:10.1242/dev.173781

Box 1. Glossary of terms

Blastema. Undifferentiated cells that proliferate to contribute to tissue regeneration.
Ecdysone. An insect steroid hormone that systemically regulates growth and development.
Endocycle. A variant cell cycle involving repeated DNA synthesis phases followed by intervening gap phases but no cell division. Cells undergoing endocycles increase in DNA content (ploidy).
Enteroblast. A transient daughter cell of a midgut intestinal stem cell that differentiates into an enterocyte.
Enterocyte. Differentiated absorptive cell in the midgut.
Enteroendocrine cell. Differentiated, endocrine hormone-expressing cell in the midgut.
Hemimetabolous insects. Insects that develop as nymphs, which progress through a series of molts before emerging from the final molt as an adult.
Holometabolous insects. Insects that develop as larvae before entering pupariation and metamorphosis to form the adult.
Imaginal rings. Adult progenitor cells that are a contiguous part of an organ, such as the hindgut or salivary gland.
Prothoracic gland. Endocrine gland that regulates development by secreting ecdysteroids.
Satellite cells. Skeletal muscle progenitor cells.
Optic lobe. A brain region involved in vision processing.

Paro, 2016; Worley et al., 2012). In addition, imaginal discs have been used to study the cellular responses that occur after the generation of 'undead' cells that initiate but do not complete apoptosis, for example by irradiating cells but then overexpressing the apoptosis inhibitors DIAP1 or p35. These cells remain in the tissue and emit pro-growth signals (Huh et al., 2004; Kondo et al., 2006; Pérez-Garijo et al., 2004, 2009; Ryoo et al., 2004; Wells et al., 2006). Key findings using these methods, and the distinctions between the tissue's response to undead cells and to removal of cells, have been discussed elsewhere (Martín et al., 2009; Mollereau et al., 2013).

Inducing injury

Numerous approaches can be used to induce injury in specific *Drosophila* tissues. The earliest *Drosophila* regeneration experiments involved the isolation and surgical fragmentation of imaginal discs, followed by their culture in the abdomen of an adult (e.g. Bryant, 1971; Hadorn et al., 1968; Schubiger, 1971). Subsequent studies induced death in scattered cells, by using X-ray irradiation (Fukunaga and Kondo, 1985; Haynie and Bryant, 1977) or mitotic recombination to generate clones of cells homozygous for a temperature-sensitive cell-lethal mutation (Addison et al., 1995; Brook et al., 1993). These elegant *in vivo* culture and irradiation studies contributed substantially to our understanding of the imaginal disc response to damage, and we direct readers to excellent reviews of this foundational work (Hariharan and Serras, 2017; Worley et al., 2012). Importantly, fragmentation, irradiation, clones of cell-lethal mutations, and 'undead cells' are still being used to ask specific questions (e.g. Diaz-Garcia et al., 2016; Fogarty et al., 2016; Gerhold et al., 2011; Verghese and Su, 2017; Yoo et al., 2016). In the adult, injury in the midgut epithelium can be accomplished through oral ingestion of agents that induce cell loss, such as the polysaccharide dextran sodium sulfate (DSS), the DNA-damaging agent bleomycin, and the oxygen radical-inducing agent paraquat. Pathogenic Gram-negative bacteria, such as *Pseudomonas entomophila*, *Serratia marcescens* or *Erwinia carotovora*, or acute starvation, can also be used to trigger midgut injury.

Using targeted genetics to induce precise injuries

Recently, increasingly intricate genetic approaches have enabled *Drosophila* researchers to injure specific tissues or remove large, contiguous portions of a tissue through targeted cell ablation (Bergantiños et al., 2010; Fox and Spradling, 2009; Jiang et al., 2009; Smith-Bolton et al., 2009). Techniques to induce cell ablation and regeneration in imaginal discs and other tissues have largely employed binary expression systems that provide spatial control over the portion of the disc to be removed, with added temporal control provided by regulators of the binary expression system. For example, tissue-specific promoters that drive the Gal4/UAS system (Brand and Perrimon, 1993) have been used to express pro-apoptotic genes, such as *eiger* or *reaper*, to ablate spatially restricted portions of the wing disc (Bergantiños et al., 2010; Repiso et al., 2013; Smith-Bolton et al., 2009), or midgut cells (Jiang et al., 2009). In addition, using the temporal and regional gene expression targeting (TARGET) system, the temperature-sensitive (ts) repressor Gal80^{ts} (McGuire et al., 2004) has been used to restrict cellular ablation to the appropriate time window during development (Smith-Bolton et al., 2009) (Fig. 1A). Such ablation methods have enabled large-scale regeneration experiments and screens for genes implicated in regeneration (Brock et al., 2017; Khan et al., 2017; Worley et al., 2018).

Although these approaches have identified numerous regulators of *Drosophila* regeneration (discussed in detail below), one challenge has been marrying the injury techniques with the vast *Drosophila* genetic toolkit. If a genetic system is used to injure a tissue, it may not be possible to use that same system to query the function of a gene. For example, if the widely used Gal4/UAS system is used to express apoptotic genes to induce injury, then theoretically this same system cannot be used to knock down or misexpress a candidate regeneration gene. One exception is the wing disc ablation system, in which 5-10% of targeted cells survive and contribute to the regenerating tissue (Smith-Bolton et al., 2009). Surprisingly, RNAi knockdown in these cells has been effective and has produced phenotypes (Skinner et al., 2015), likely owing to the 'shadow RNAi' effect that exists in wing discs (Bosch et al., 2016), whereby the RNAi-induced knockdown persists in progeny of the original cells despite cessation of expression of the RNAi transgene (Fig. 1A).

For more precise control over genetic perturbations, several next-generation systems have been developed to facilitate independent expression of transgenes in the wing disc. For example, the use of a LexA/Gal4 hybrid (LHG) transcription factor enables control of *reaper* expression through a LexA operator, but the LHG transcription factor is still regulated by Gal80^{ts}. The LHG approach achieves spatial separation of the expression of *reaper* for cell ablation and, via a Gal4-induced transgene, genetic manipulation of a regeneration factor (Santabárbara-Ruiz et al., 2015) (Fig. 1B). In addition, Gal4 has been combined with the binary Q system to control injury and transgene expression separately. In one such Q+Gal4 system, ablation using a temperature-sensitive diphtheria toxin A (Bellen et al., 1992), expressed under the control of QF/QUAS (Potter et al., 2010), enables the use of Gal4 for expression of desired transgenes (Kashio et al., 2016) (Fig. 1C). In another similar approach, the Q system drives the expression of a receptor for a toxin that is fed to the animal to accomplish temperature-independent cell ablation, while Gal4 drives transgene expression (Obata et al., 2015) (Fig. 1C). The LHG and Q+Gal4 systems require the two transcription factors to be expressed in domains that are at least partially non-overlapping. A separate system, named the dual expression method for induced site-specific eradication (DEMISE), instead requires just one binary expression

Development (2020) 147, dev173781. doi:10.1242/dev.173781

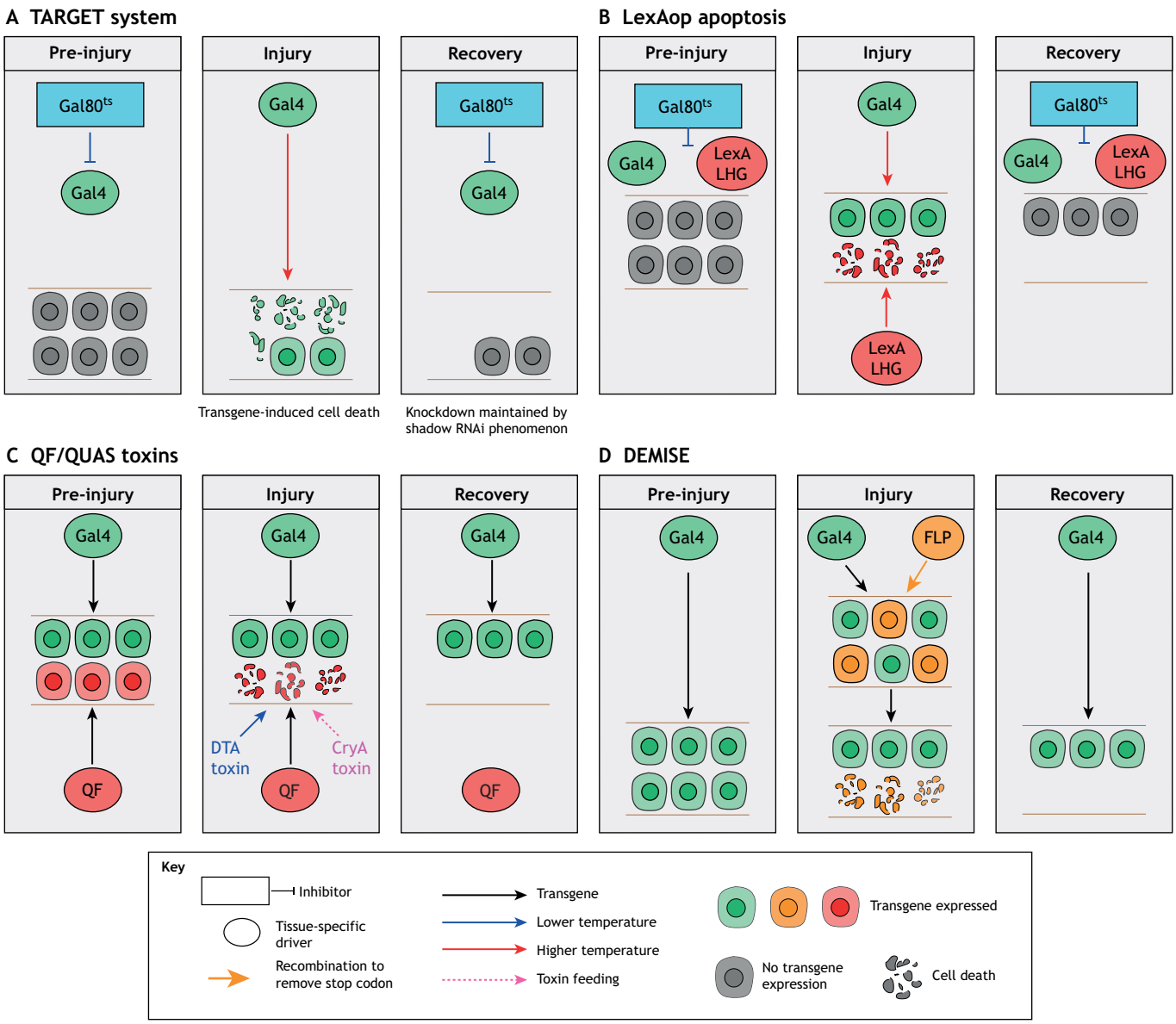

Fig. 1. Experimental tools for identifying molecular regulators of regeneration. Many transgene systems in *Drosophila* employ a transgene 'driver' that directs transgene expression, such as an overexpression construct or a hairpin encoding a double-stranded RNA for gene knockdown, under the control of a tissue-specific promoter. The driver can also be inhibited until the desired time of expression. Transgene expression systems can be manipulated in numerous ways, including via temperature change, mosaic cassette flipping, or by feeding. These manipulations turn on the transgenes of interest and/or cause cell death. Combinations of various approaches have been employed in precision injury systems, as shown in each panel and described in detail in the text. For each system, a pre-injury, injury, and recovery state is shown, using the manipulations outlined in the figure key. For simplicity, all cells are drawn to the same scale. (A) Through use of the TARGET system, one can achieve temporally controlled cell ablation via the temperature-sensitive repressor Gal80^{ts} (used to control Gal4-mediated expression of both a cell death-inducing transgene and an RNAi transgene of interest), and through the shadow RNAi effect can also achieve persistent gene knockdown. (B,C) LexA (LexAop-Apoptosis) or the Q-system (QF/QUAS toxins) can be combined with the Gal4 system to manipulate tissue injury and transgene expression separately. Note that for QF/QUAS toxins, as described in the text, the system can be adapted to be activated by either temperature (to control activation of a temperature-sensitive DTA toxin) or feeding (to deliver CryA toxin). (D) The DEMISE system relies on Gal4 to drive both cell death and transgene expression, but death is limited to those cells that also express FLP.

system. This approach enables a fraction of cells to escape Gal4-mediated injury, as FLP-FRT recombination excises a stop cassette in front of *reaper* in only a subset of the Gal4^{+} cells. The remaining Gal4^{+} cells survive the injury and thus can be genetically manipulated (Cohen et al., 2018) (Fig. 1D). This approach also avoids raising animals at 18°C, which is the permissive temperature for Gal80^{ts} and roughly doubles the time spent in development. DEMISE animals can be raised at 25°C, and injury is induced using a 37°C pulse to excise the stop cassette and activate ablation.

Approaches that employ temporal control of tissue ablation using optogenetic methods to activate gene expression are also under development (Makhijani et al., 2017). These new and emerging tools will enable large-scale screens in the background of a precision injury setting. Given the large collections of transgenic *Drosophila* RNAi (Dietzl et al., 2007; Perkins et al., 2015) and CRISPR (Ewen-Campen et al., 2017; Li-Kroeger et al., 2018; Meltzer et al., 2019) lines, the field now has an expanded toolkit that can be used to investigate regeneration mechanisms further.

Development (2020) 147, dev173781. doi:10.1242/dev.173781

Drosophila regenerative responses

Organ regeneration during development

Cell fates in *Drosophila* imaginal disc epithelia are specified during the third larval instar stage, but cells do not fully differentiate until metamorphosis (Held, 2002). With the exception of specialized regions, proliferation then continues until shortly after pupariation (Buttitta et al., 2007; Graves and Schubiger, 1982; Milán et al., 1996; Schubiger and Palka, 1987). As mentioned above, the imaginal discs can regenerate through most of the third larval instar stage, but this capacity to regenerate ceases with metamorphosis and the end of appendage growth.

Early experiments involving surgical damage and *in vivo* culture showed the formation of a zone of proliferation around the damaged site called the regeneration blastema (see Glossary, Box 1) (Abbott et al., 1981; Dale and Bownes, 1980; Karpen and Schubiger, 1981; O'Brochta and Bryant, 1987), which is similar to the regeneration blastema or zone of de-differentiated, proliferating cells observed in amputated vertebrate appendages (Tanaka, 2016). The use of transgenic tools for tissue ablation has enabled further observation of changes in proliferation and in patterning gene expression in the regenerating tissue, and changes in the resulting adult appendage. If sufficient contiguous tissue is ablated, a blastema forms (Smith-Bolton et al., 2009; Bergantiños et al., 2010), and suppression of proliferation elsewhere occurs through a mechanism in which nitric oxide synthase activity in the prothoracic gland (see Glossary, Box 1) reduces the systemic levels of ecdysone (see Glossary, Box 1) needed for disc growth (Jaszczak et al., 2015). This proliferation of cells near the wound replaces the lost tissue, and there is no evidence for a rare population of progenitor cells (Smith-Bolton et al., 2009) (Fig. 2A,B).

Tissue damage in the wing disc, via ablation or mechanical damage, also causes localized loss of expression of patterning and cell fate genes, such as the transcriptional activator *vestigial*, and markers for wing veins and interveins (Díaz-García and Baonza, 2013; Smith-Bolton et al., 2009), indicating that blastema cells lose cell fate specification, reminiscent of de-differentiated blastema cells in vertebrates. These de-specified cells and their progeny can adopt new cell identities, such as vein or intervein cells, to replace lost cell types (Repiso et al., 2013), even crossing the anterior-posterior compartment boundary under extreme conditions (Herrera and Morata, 2014) (Fig. 2C). However, adult wings resulting from damaged discs that have fully regenerated often have subtle patterning mistakes, such as anterior bristles and veins in the posterior of the wing, demonstrating that patterning can go awry during regeneration (Schuster and Smith-Bolton, 2015). In addition, adult wings resulting from damaged discs that have not fully regenerated are grossly mis-patterned (Smith-Bolton et al., 2009), indicating that insufficient regrowth inhibits proper repatterning.

Several of the conserved cell-cell signaling molecules required for regeneration in imaginal discs, such as WNT/Wingless, Jun N-terminal kinase (JNK), Jak/STAT, and calcium, were identified while studying surgically damaged discs (Bosch et al., 2005, 2008; Katsuyama et al., 2015; Restrepo and Basler, 2016; Schubiger et al., 2010), and have also been shown to drive regeneration after disc ablation (Bergantiños et al., 2010; La Fortezza et al., 2016; Smith-Bolton et al., 2009). Tissue ablation experiments enabled the identification of additional key signaling components such as reactive oxygen species (ROS) (Khan et al., 2017; Santabárbara-Ruiz et al., 2015), kinases such as p38 MAP kinase (Santabárbara-Ruiz et al., 2015), Akt1 and Ask1 (Santabárbara-Ruiz et al., 2019), chromatin modifiers such as Trithorax (Skinner et al., 2015) and Taranis (Schuster and Smith-Bolton, 2015), and transcriptional regulators such as Myc (Smith-Bolton et al., 2009), Yki (Grusche et al., 2011; Sun and Irvine, 2011), Nrf2 (also known as Cnc) (Brock et al., 2017) and Chinmo (Khan et al., 2017; Narbonne-Reveau and Maurange, 2019).

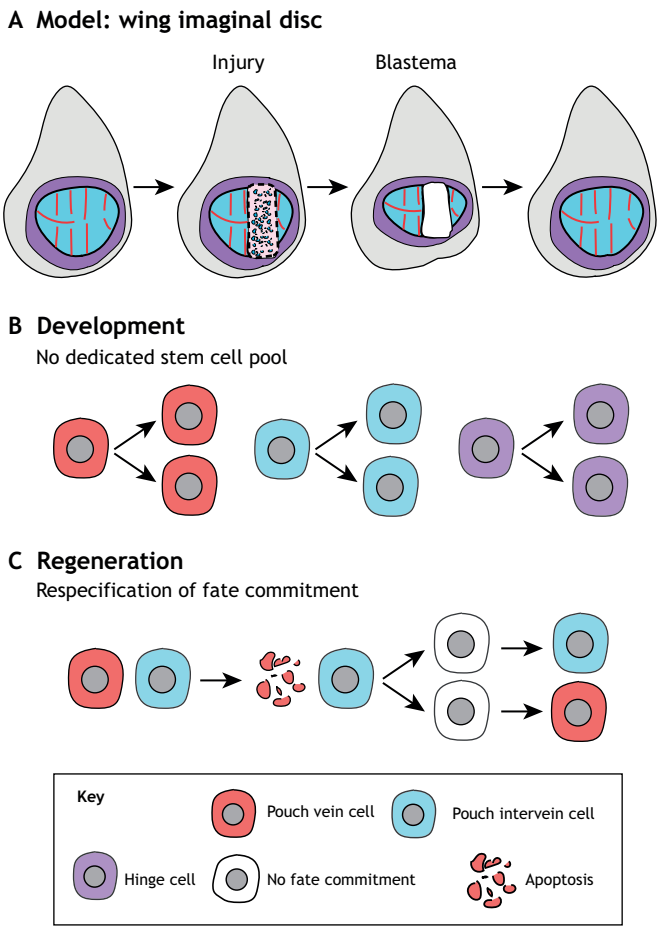

Fig. 2. Regenerative responses in larval *Drosophila* imaginal discs. Regeneration in the wing imaginal disc. (A) A tissue-level view of regeneration. Injury leads to the formation of a regeneration blastema (white), followed by full regeneration through cell proliferation. (B) During normal development, the wing imaginal disc does not appear to have stem cells or a lineage hierarchy aside from compartment restrictions. (C) During regeneration, wing cells lose commitment to particular cell fates, such as pro-vein or intervein fates, and can contribute to regeneration of multiple cell fates.

Many tissues, including tadpole tails, skeletal muscle and mammalian fingertips (Simkin et al., 2015; Slack et al., 2004; Sousa-Victor et al., 2018), lose or decrease their capacity to regenerate as they age or mature. Imaginal disc ablation experiments have confirmed that, similarly, there is a window of competence for regeneration in *Drosophila* that ends at the late third instar stage (Halme et al., 2010; Smith-Bolton et al., 2009), and that damage at the early-mid third instar stage leads to an extension of that larval phase (Smith-Bolton et al., 2009). This delay in pupariation is due to a damage- and growth-induced checkpoint that involves suppression of ecdysone production by signaling through retinoids (Halme et al., 2010) and the secreted peptide Ilp8 (Colombani et al., 2012; Garelli et al., 2012; Katsuyama et al., 2015). The surge in ecdysone prior to pupariation induces chromatin changes at damage-responsive enhancers, disabling the regenerative response (Harris et al., 2016), and these changes in chromatin are delayed by Ilp8 signaling. Thus, although the form of an imaginal disc is distinct from vertebrate

Development (2020) 147, dev173781. doi:10.1242/dev.173781

appendages and organs, many aspects of regeneration are similar, including formation of a blastema, activation of key signaling pathways (Gauron et al., 2013; Langiewicz et al., 2018; Moya and Halder, 2016; Stoick-Cooper et al., 2007), and constraints caused by maturation and cessation of developmental growth.

Regeneration in adults

A number of adult *Drosophila* tissues that undergo homeostatic renewal contain resident stem cells capable of driving organ-specific regeneration. In recent years, the mechanisms underlying this regenerative growth following tissue injury have been studied, focusing primarily on three actively renewing tissues: the midgut and the male and female germlines (Fig. 3A).

The midgut

Although it consists of regionally distinct domains (Buchon et al., 2013; Driver and Ohlstein, 2014; Dutta et al., 2015; Marianes and Spradling, 2013; Sawyer et al., 2017; Strand and Micchelli, 2011), the *Drosophila* midgut contains repeated units of multipotent intestinal stem cells (ISCs) throughout its length. During normal homeostatic conditions, ISCs divide asymmetrically to self-renew and produce daughter cells that ultimately differentiate into two cell types: enteroendocrine cells (EEs; see Glossary, Box 1) and enterocytes (ECs; see Glossary, Box 1) (Guo and Ohlstein, 2015; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006). ECs are derived from transient precursors known as enteroblasts (EBs; see Glossary, Box 1), which undergo multiple rounds of endocycles (see Glossary, Box 1) to increase in ploidy and cell size (Edgar et al., 2014; Ohlstein and Spradling, 2006), whereas EEs remain diploid and are derived from the division of endocrine progenitor cells (Guo and Ohlstein, 2015; Zeng and Hou, 2015). The differentiated cells are lost and are replaced by the continued division of ISCs, much like in the mammalian small intestine and colon (Fig. 3B).

The midgut can recover from various forms of injury to regenerate lost cells. In many respects, regeneration of the midgut epithelium represents an accelerated version of several processes that occur during homeostasis (Fig. 3C). Namely, diverse injury stimuli increase death and elimination of differentiated cells (Apidianakis et al., 2009; Buchon et al., 2009a, 2010; O'Brien et al., 2011), ISC division rates (Amcheslavsky et al., 2009; Apidianakis et al., 2009; Buchon et al., 2009a; Jiang et al., 2009, 2011; O'Brien et al., 2011; Staley and Irvine, 2010), EB differentiation into ECs (Amcheslavsky et al., 2009; Chatterjee and Ip, 2009; Staley and Irvine, 2010; Zhai et al., 2017), and EC endocycles (Xiang et al., 2017).

The molecular circuitry underlying both homeostatic and regenerative midgut renewal is complex, and we are unable to comprehensively review it here; we instead direct the reader to extensive reviews on this topic (Jiang et al., 2016; Schwartz and

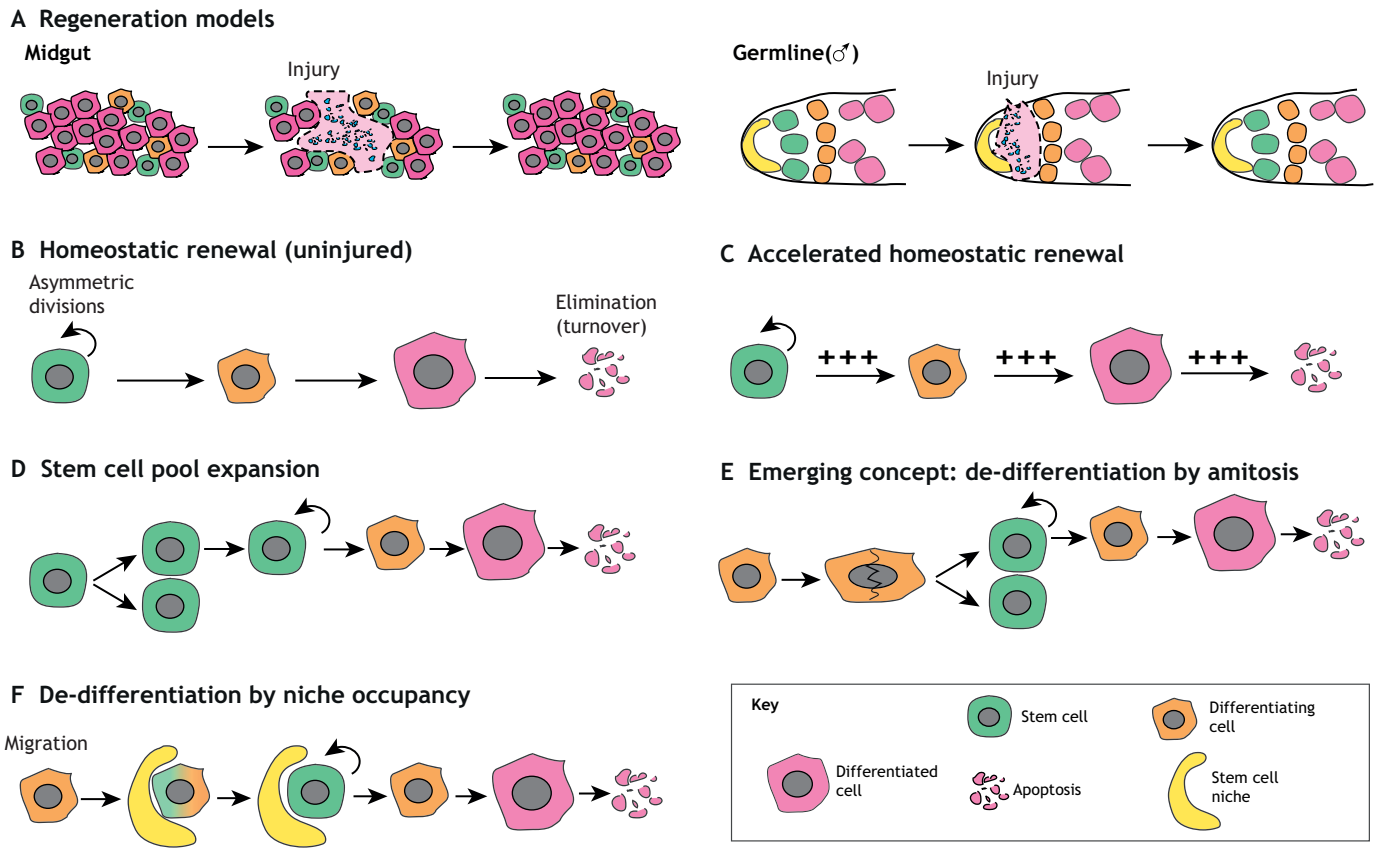

Fig. 3. Regenerative responses in adult *Drosophila* tissues. (A) Two key regeneration models – the midgut and the germline – and their responses are shown. Note that only the male is shown for the germline. Both tissues can recover from acute cell loss in specific contexts (see text for details). (B) Homeostatic renewal in the midgut epithelium. (C-F) Distinct regeneration concepts revealed in these tissues are shown. In the midgut, regeneration mechanisms include: (C) accelerated homeostatic renewal and asymmetric division of intestinal stem cells; (D) symmetric division of ISCs to expand the stem cell pool; and (E) amitosis of differentiating enterocytes. Note that amitosis is a newly proposed mechanism of regeneration observed in a specific midgut region under severe starvation conditions. (F) In the germline, regeneration is accomplished following acute depletion of germline stem cells through the de-differentiation of early-stage germ cells back into stem cells through niche occupancy. The key indicates distinct cell states that participate in adult tissue regeneration. For simplicity, all cells are drawn to the same scale.

Rhiner, 2018; Sun and Irvine, 2014). However, it is important to call attention to the overall conservation of the molecular circuitry that controls *Drosophila* midgut regeneration and regenerative responses in vertebrate tissues, such as the zebrafish fin, mouse intestine and mouse liver. For example, the Hippo signaling pathway is a crucial regeneration regulator in both the *Drosophila* midgut and all of these vertebrate tissues (Brandão et al., 2019; Karpowicz et al., 2010; Mateus et al., 2015; Moya and Halder, 2016; Ren et al., 2010; Shaw et al., 2010; Staley and Irvine, 2010). Importantly, and as we highlight below, there are a few differences between the molecular regulation of midgut regeneration versus midgut homeostasis.

Upon infection with *Erwinia carotovora*, an immune defense response triggers a regeneration program in the midgut that involves upregulation of Unpaired cytokines, which signal from injured ECs through Jak/STAT in ISCs to increase ISC proliferation. Indeed, in diverse midgut injury models, Jak/STAT signaling increases the rate of ISC division (Buchon et al., 2009a,b, 2010; Cronin et al., 2009; Houtz et al., 2017; Jiang et al., 2011). By contrast, under homeostatic conditions, Jak/STAT does not substantially regulate ISC division rates, although it does influence differentiation in the ISC lineage and can therefore impact ISC numbers (Beebe et al., 2010; Jiang et al., 2009). *E. carotovora* infection also enhances ISC/EB cell-cell contact, which is mediated by the transcription factor Sox21a. This increased contact facilitates ISC-to-EB differentiation, mediated by Delta/Notch signaling (Guo and Ohlstein, 2015; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Zhai et al., 2017). During homeostasis, EC endocycles are regulated by dietary input from insulin/Target of Rapamycin (TOR) signaling; however, *Pseudomonas entomophila* infection triggers an endocycle program mediated instead by Egfr/MAPK signaling (Xiang et al., 2017). These studies highlight examples by which multiple steps of the homeostatic midgut renewal program can be regulated to regenerate damaged or lost midgut tissue.

In addition to involving accelerated homeostatic renewal, midgut regeneration can involve cellular mechanisms not found during homeostasis. For example, following injury induced by bleomycin, ISCs divide symmetrically to expand the ISC pool in order to facilitate rapid regeneration (Tian et al., 2017) (Fig. 3D). Symmetric divisions to expand the ISC pool also occur if a new adult is starved and then re-fed (O'Brien et al., 2011). In this context, the ISCs then switch to asymmetric divisions to populate the adult gut epithelium with differentiated cells. However, because the midgut is still finishing development at the time of starvation, it is unclear whether this mechanism also contributes to mature adult midgut regeneration or recovery from ISC pool depletion. Injury stimulus may impact the mode of regeneration, as symmetric ISC divisions are not reported to increase following *P. entomophila* infection (Jin et al., 2017). Related to this idea, ISC spindle orientation was recently tied to division outcome (symmetric versus asymmetric), and was shown to be differentially regulated by varying stress stimuli (Hu and Jasper, 2019). Importantly, the injured mouse gut is also capable of ISC-mediated regeneration (Barriga et al., 2017; Metcalfe et al., 2014; Yan et al., 2012).

Interestingly, ISCs may not be the only drivers of adult midgut regeneration. In a recent study, differentiating ECs of segment R4b-R4c of the adult midgut were reported to be capable of amitosis, a cell fission mechanism previously described in polyploid ciliates (Orias, 1991) that does not require entry into mitosis. Amitosis in this gut region appears to be triggered by injury via severe starvation. Through this mechanism, differentiating ECs that are beyond the EB state split into two cells without a mitotic spindle, albeit occasionally with unequal chromosome numbers, and generate new ISCs that regenerate the local midgut (Fig. 3E) (Lucchetta and Ohlstein, 2017). This new work highlights the continued potential of *Drosophila* to reveal unexpected regeneration mechanisms.

The germline

The male and female germlines are maintained by proliferation of germline stem cells. These stem cells can be depleted by mis-expression of differentiation factors in the stem cells or by starvation (Brawley and Matunis, 2004; Herrera and Bach, 2018; Kai and Spradling, 2004). Following these acute stresses, immediate stem cell daughters can de-differentiate back into stem cells to repopulate the stem cell compartment (Fig. 3F). These reverted stem cells then regenerate the germline, which would otherwise be depleted over time (Brawley and Matunis, 2004; Kai and Spradling, 2004). Remarkably, this process involves the fragmentation of interconnected groups of germline cyst cells that then re-acquire stem cell characteristics (Brawley and Matunis, 2004; Kai and Spradling, 2004; Sheng et al., 2009). Such de-differentiation of spermatogonial progenitors can also contribute to regeneration of the male germline in mice (Barroca et al., 2009; Nakagawa et al., 2010). As a further parallel with mammals, de-differentiation of intestinal enterocytes is reported to lead to regeneration of stem cells in intestinal crypts, in situations in which stem cell activity in crypts is ablated (Tetteh et al., 2016). Going forward, this de-differentiation process may be exploited to identify the molecular determinants that control regenerative plasticity.

Restoring tissue mass without cellular proliferation

Central to most regenerative responses is the ability of an injured tissue to replace lost tissue mass through cell proliferation. However, it is increasingly clear that many tissues replace lost tissue mass without cell proliferation. Numerous *Drosophila* tissues, including the *Drosophila* hindgut pylorus, adult abdominal epidermis and adult follicular epithelium (Cohen et al., 2018; Losick et al., 2013, 2016; Tamori and Deng, 2013), as well as the mammalian liver, bladder and kidney (Lazzeri et al., 2018; Miyaoka et al., 2012; Wang et al., 2018), restore tissue mass by enlargement of the remaining cells, often through an increase in cellular ploidy. As an example, the adult *Drosophila* pylorus undergoes such a compensatory hypertrophic response, which can be converted to a regenerative proliferation response by knockdown of the cell-cycle regulator *fizzy-related*, a negative regulator of mitotic cyclin accumulation. However, inducing proliferation in the adult pylorus experiencing chronic injury leads to gut leakage (Cohen et al., 2018), highlighting that there may be trade-offs to remaining in a pro-proliferative regeneration state. The injured *Drosophila* abdominal epidermis undergoes a similar endocycle response, but also relies on cell fusion to replace lost tissue mass (Losick et al., 2013, 2016). Going forward, study of these non-proliferative injury models as companion models to proliferation-driven regeneration can reveal distinct regulation and function of diverse organ injury responses. We refer the reader to a recent review on this emerging topic (Gjelsvik et al., 2019).

Recent advances and emerging questions in *Drosophila* regeneration

The role and regulation of ROS during regeneration

One important question in the regeneration field concerns how the initial sensors of tissue damage are both constrained to limit damage and sustained to activate the cascade of regeneration signaling. In many animals, tissue damage leads to release of ROS, which serve as a damage signal and perpetuate the damage response (van der Vliet and

Janssen-Heininger, 2014). As we highlight below, genetic tissue ablation techniques in *Drosophila* have enabled identification of the regulators and downstream effectors of damage-induced ROS.

Damaged imaginal discs release ROS (Santabárbara-Ruiz et al., 2015), which in the eye disc signal to immune cells (Fogarty et al., 2016), and in the wing disc stimulate regeneration in the surrounding epithelium (Santabárbara-Ruiz et al., 2015). A crucial effector of ROS in damaged wing discs is the kinase Ask1, which is activated under oxidative stress and can activate JNK and p38 MAPKs (Santabárbara-Ruiz et al., 2015; Santabárbara-Ruiz et al., 2019). Importantly, ROS levels and the activation of their downstream effectors must be constrained, as high ROS levels paradoxically dampen JNK signaling and impair regeneration (Brock et al., 2017). ROS levels are constrained by activation of the transcription factor Nrf2, which regulates expression of anti-oxidant genes (Brock et al., 2017). In addition, Ask1 activation is constrained by phosphorylation by Akt1 (Santabárbara-Ruiz et al., 2019). Although ROS and their downstream effectors must be tightly controlled, they must also be sustained long enough for regeneration to complete. In many species, such as zebrafish and *Xenopus*, ROS are produced at a damage site for at least 24 h and for up to several days, although the mechanism underlying this sustained ROS production has been unclear (Gauron et al., 2013; Love et al., 2013). However, the recent transcriptional profiling of ablated *Drosophila* wing discs has identified a positive-feedback loop in which JNK signaling activates expression of the gene *moladietz* (*mol*), which encodes the dual oxidase (Duox) maturation factor NIP, which plays a role in ROS production (Khan et al., 2017). Thus, expression of *mol* ensures ROS production, which activates JNK signaling, thereby sustaining expression of *mol* (Khan et al., 2017).

ROS are also implicated in regulating regeneration in the midgut, and there are several similarities between ROS regulation in regenerating imaginal discs and adult midgut epithelia. Many midgut-damaging agents, such as paraquat and bleomycin, or targeted cell ablation, induce ROS in the midgut. Upon midgut infection with *E. carotovora*, the immune defense response is triggered by an oxidative burst generated by Duox activity, and blocking ROS production or detection prevents midgut regeneration (Buchon et al., 2009b). As in the imaginal disc, Nrf2 is required for midgut regeneration after paraquat ingestion (Hochmuth et al., 2011). Furthermore, Ask1 is required for midgut regeneration in response to multiple injury sources, and in these contexts both Ask1 and ROS activate p38 signaling (Patel et al., 2019). Additional ROS-responsive regeneration mechanisms in the midgut include activation of the influx channel TRPA1 and the endoplasmic reticulum cation channel RyR, both of which regulate cellular calcium signaling, to transmit a Ras/MAP Kinase signal to ISCs to increase division rates (Deng et al., 2015; Xu et al., 2017). Thus, in both regenerating imaginal discs and midguts, much of the molecular circuitry connecting ROS production to regeneration signaling, and the regulators that constrain these signals, have been identified.

Patterning during regeneration

Regenerating tissue must adopt appropriate cell fates to produce a functional replacement tissue. How cell fates are impacted by tissue damage and how the correct cells are specified in the correct positions are still largely open questions. However, genetic screens for mutations that cause consistent mis-patterning of regenerating tissue, but do not affect normal imaginal disc development, can reveal key mechanisms regulating cell fate (Schuster and Smith-Bolton, 2015). For example, JNK signaling, which is essential for wound closure and regenerative growth (Bergantiños et al., 2010; Bosch et al., 2005), can disrupt expression of the posterior selector gene *engrailed*, leading to posterior-to-anterior cell fate transformations in regenerating wing discs (Schuster and Smith-Bolton, 2015). Normally, these JNK-induced mistakes are minimized by the putative chromatin modifier Taranis (Tara), which is required for posterior fate after damage but not during normal development (Schuster and Smith-Bolton, 2015). Therefore, patterning during regeneration is not identical to patterning during development, owing to the presence of damage response signals, such as JNK, and the need for protective factors, such as Tara.

In the adult midgut, aging can cause mis-patterning during both normal homeostasis and regeneration. Aging elevates both JNK and Jak/STAT signaling in specific midgut compartments, which can disrupt midgut homeostasis through inappropriate expression of differentiation signals such as Delta/Notch. Overexpressing these signals in younger flies can mimic the effect of this age-dependent signaling disruption on regeneration, causing metaplasia during regeneration after paraquat-induced injury (Biteau et al., 2008; Li et al., 2016). Therefore, *Drosophila* is a valuable model for demonstrating how factors such as aging and the wound response can disrupt cell fate during regeneration, and can highlight differences between regeneration and development.

Regulating regeneration gene expression

The identification of numerous regeneration genes has raised the question of how those genes are regulated after tissue damage. The wing imaginal disc is competent for regeneration during a specific window of development that ends before pupariation, when expression of regeneration genes as well as activity of key signals and transcription factors are no longer strongly upregulated upon damage (Harris et al., 2016; Smith-Bolton et al., 2009). This phenomenon was explained by the identification of bipartite damage-response enhancers, which contain an activator region that induces gene expression upon disc damage, and a repressor region that mediates silencing of the enhancer via chromatin changes when ecdysone levels peak before pupariation (Harris et al., 2016). Tissue ablation methods have also been coupled with genomic approaches such as transcriptional profiling and chromatin profiling in an attempt to understand the mechanisms that drive regeneration (Khan et al., 2017; Vizcaya-Molina et al., 2018). Indeed, ATAC-seq profiling of regenerating wing discs at multiple time points has identified many regions with increased chromatin accessibility, suggesting changes in enhancer activity across the genome (Vizcaya-Molina et al., 2018). Such damage-responsive enhancer elements have also been identified in acoel worms as well as in zebrafish and mouse hearts (Gehrke et al., 2019; Kang et al., 2016; Wang et al., 2019). Future studies of these genomic loci will no doubt yield a trove of information about the mechanisms that control regeneration, and comparative analyses may reveal commonalities in how regeneration is regulated across species.

Turning regeneration off

Once enough tissue has been generated to compensate for cells lost to injury, a tissue must return to a steady state. Failure to do so risks acquiring a hyperplastic or cancerous phenotype. As discussed above, when imaginal discs are damaged, expression of Ilp8 delays metamorphosis to buy time for regenerative growth. However, metamorphosis eventually occurs, and with it silencing of the damage-responsive genes, preventing runaway regrowth (Colombani et al., 2012; Garelli et al., 2012; Katsuyama et al., 2015; Harris et al., 2016). By contrast, in the *Drosophila* midgut, hyperactivation of many pro-regeneration factors can cause hyperplasia (Biteau and Jasper, 2011; Johansson et al., 2019), and little is known about the

mechanisms that actively turn off the regeneration program. In the regenerating midgut, two distinct modes of BMP/Decapentaplegic (Dpp) signaling differentiate between the pro- and anti-regenerative states (Ayyaz et al., 2015; Tracy Cai et al., 2019). After bacterial challenge, the BMP-family receptor Punt binds to its co-receptor Saxophone and drives pro-regeneration gene expression, mediated by the transcription factor Smad on X (Smox). Later in regeneration, the Punt co-receptor Thickveins is more abundant, and this receptor complex activates different genes via Mothers against Dpp (Mad), returning the midgut to homeostasis. Future work in *Drosophila* regeneration models may benefit from focusing on negative regulation of pro-regeneration factors, such as injury-mediated activation of Jak/STAT or JNK signals. Additionally, negative-feedback signaling from newly created cells, such as from progeny to stem cells may turn out to be a common mechanism of terminating the regenerative state (Ge and Fuchs, 2018). Identifying mechanisms that end regeneration may impact our understanding of not only regenerative growth control but also cancer prevention mechanisms.

Expanding regeneration studies to other organs

Although most *Drosophila* regeneration studies have focused on imaginal discs, the midgut and the germline, additional tissue regeneration models continue to be identified. Imaginal rings (see Glossary, Box 1), for example, are constituent components of larval organs (such as the hindgut, foregut and salivary gland) that have recently been shown to regenerate. These structures persist throughout metamorphosis and contribute heavily to the replacement of larval organs with adult organs. An example is the hindgut, where the imaginal ring is housed in a structure at the midgut-hindgut junction known as the pylorus. A recent study revealed that pro-apoptotic ablation of ∼90% of all pyloric cells leads to complete regeneration of pylorus-derived tissue of the adult hindgut, through increased rounds of cell division of remaining imaginal ring cells (Cohen et al., 2018). Regeneration can also be examined in the context of muscle. Mechanical injury to adult flight muscle, for instance, triggers proliferation of cells that retain muscle progenitor properties and express a specific isoform of the transcription factor Zfh1. These cells can produce daughter cells that both renew skeletal muscle and regenerate damaged muscle fibers (Boukhatmi and Bray, 2018; Chaturvedi et al., 2017). Thus, *Drosophila* contain cells resembling the regenerative muscle stem cells (termed ‘satellite cells’; see Glossary, Box 1) found in mammalian skeletal muscle. Similarly, mechanical injury to the adult *Drosophila* brain initiates proliferation in the medulla cortex of the optic lobes (see Glossary, Box 1), likely initiated by putative progenitor cells that express the transcription factor Deadpan (Fernández-Hernández et al., 2013; Moreno et al., 2015). We also note that the brain, muscle, hindgut pylorus and adult abdomen all represent examples of cells that respond to injury from a quiescent state (Chaturvedi et al., 2017; Fernández-Hernández et al., 2013; Fox and Spradling, 2009; Losick et al., 2013). Mechanisms of injury response in these tissues may therefore differ from injury responses in the already cycling midgut and imaginal discs, and therefore may reveal injury response mechanisms that are distinct to exiting quiescence. The study of these new models of regeneration, along with the new genetic precision injury methods, will no doubt unearth new regeneration paradigms.

Conclusions

Given the impact of *Drosophila* on regeneration research in the last decade, this stalwart model of development still has much to contribute to our understanding of how injured organs are rebuilt. Moving forward, new precision injury tools in both established and new tissue regeneration models can lead the way. Other promising approaches, such as live imaging of intact adult midgut tissue (Martin et al., 2018), high-resolution chromatin mapping in imaginal discs (Uyehara et al., 2017; Vizcaya-Molina et al., 2018) and single-cell sequencing (Deng et al., 2019) will also help uncover new regeneration biology. Given the genetic prowess and the tools now available in this model, *Drosophila* will be a major player in the regeneration field for decades to come.

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Competing interests

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