Hormone Signaling Synchronizes Regeneration with Development Progression

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Dissertation Abstract

In multicellular organisms, hormones are a critical part of growth coordination between tissues and the whole organism. Systemic hormone signaling acts as a timer that ensures the developmental progression of all tissues is coordinated. This coordination is lost when tissues are injured, and the damaged tissues need to be repaired and catch up with the development of undamaged tissues. How do the organisms reestablish the coordination? In *Drosophila* imaginal disc regeneration, growth coordination is reestablished by decreasing systemic levels of the steroid hormone ecdysone, a key coordinator of their developmental progression. At the end of larval development, an increase in systemic levels of ecdysone initiates pupation and causes a loss of regenerative capacity. Regenerating imaginal discs release the relaxin hormone Dilp8, limiting ecdysone synthesis and extending the regenerative period, allowing the damaged tissue time to repair and catch up.

However, during my thesis research, I observed that *dilp8*$^{-}$ mutants can still regenerate their wing discs despite the lack of regenerative checkpoint delay following damage. I established that ecdysone coordinates regeneration with developmental progression, even as its levels are limited during regeneration. Here, I describe how regenerating tissues produce a biphasic response to ecdysone levels: lower concentrations promote local and systemic regenerative signaling, whereas higher concentrations suppress regeneration via the expression of *broad* splice isoforms. This dual role for ecdysone explains how regeneration is completed in *dilp8*$^{-}$ mutants: their higher ecdysone levels increase the regenerative activity, allowing regeneration

completion in a shorter timeframe. However, completion of regeneration within a limited time comes at the cost of pupal viability.

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I am honored to finally have all my work compiled into a story that I hope will have a lasting impact on the scientific community. This work would not have been possible without the network of family, friends, colleagues, and even acquaintances I have met along the way. I am grateful for every single bit of mentorship, support, love, faith, and advice I have received.

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List of Abbreviations

20E: 20 Hydroxy-ecdysone/ Ecdysone

AED: After Egg Deposition

A/P: Anterior/Posterior

BDSC: Bloomington *Drosophila* Stock Center

Br: all Broad transcription factors

BrZ(1-4): Broad splice isoforms (1-4)

Bx: Beadex

CW: Critical Weight

D/V: Dorsal/Ventral

DH: Dorsal Hinge

Dilp8: *Drosophila* Insulin Like Protein 8

DRE: Damage Responsive Enhancer

Drpr: Draper

EcR: Ecdysone Receptor

EcR^{DN}: Ecdysone Receptor Dominant Negative mutant

Egr: Eiger

GFP: Green Fluorescent Protein

Hid: Head Involution Defect

HRE: Hormone Responsive Element

IF: Immuno-Fluorescence

JH: Juvenile Hormone

L1: 1^{st} larval instar

L2: 2nd larval instar

L3: 3rd larval instar

L/R: Left/Right

P/D: Proximal/Distal

Pc: Polycomb

PcG: Polycomb Group

PG: Prothoracic gland

PRC(1-2): PcG Repressive Complex (1-2)

RHG: Rpr, Hid and Grim

Rn: Rotund

Rpr: Reaper

RRP: Regeneration Restriction Point

TNF- α: Tumor Necrosis Factor-α

trx: trithorax

trxG: Trithorax Group

Vg: Vestigial

VH: Ventral Hinge

WB: Western Blot

Wg: Wingless

CHAPTER 1: Introduction

1.1 Development and developmental transitions

A fully functioning adult body has its tissues and organs in the correct place and orientation and the right size relative to the body size. A failure to coordinate tissue development often results in conditions such as heterotaxy (a.k.a. Ivermark syndrome), where the internal organs in the chest and abdomen are abnormally arranged or underdeveloped (Adrian et al., 2015). The severity of this syndrome varies depending on the number of tissues affected and the extent of malformation. For example, malformation or misplacement of the heart may lead to limited functioning capacity or poor connection to blood supply (Adrian et al., 2015; Wolla et al., 2013). Affected individuals have mild health problems associated with the affected organs or problems that can be fatal in infancy, even with treatment. To ensure that the body functions correctly, the body and the individual tissues must be patterned. In this section, I will discuss how tissues establish and maintain organization during development.

1.1.A Patterning

Patterning refers to the collective sequence of events that determine the spatial organization of cells, tissues, and organs. Cells acquire different identities from their spatial positions, making patterning fundamental to cell differentiation, morphogenesis, tissue formation, and function in the adult body (Beddington & Robertson, 1999; Holló, 2017; Manuel, 2009). Patterning is based on the establishment of the principal axes during embryonic development; anterior-posterior (A/P), which positions mouth (A) and anus (P), dorsal-ventral (D/V), which separates body front (V) from the back (D), left-right (L/R) axis, which creates the mirror-like symmetry of extremities and left-right asymmetry

of the organs, and finally proximal-distal (P/D) which determines limb orientation relative to the body (Beddington & Robertson, 1999; Takahashi et al., 2001).

In the fruit fly, *Drosophila melanogaster*, the body axes are determined soon after fertilization by sequestering the mostly opposing maternal coordinate/determination factors in the cytoplasm of the fertilized egg and the subsequent multinucleated syncytial blastoderm (Fig.1-1A, (Pankratz et al., 1990; Roth et al., 1989; Schüpbach, 1987)). Following cell cleavage, daughter cells acquire the positional information according to the contents of their cytoplasm, which regulates their expression output. The output is often the cell adhesion molecules and morphogens. Morphogens are signaling proteins, such as WNTs, BMPs, and Hedgehog that diffuse in the extracellular environment(Christian, 2012). The gradient of morphogen concentration over the distance of several cells provides spatial information by inducing concentration-dependent differential gene expression in the target cells (Christian, 2012; Wartlick et al., 2011). The morphogen gradients and cell adhesions (mechanical forces) help retain the patterns and axes as the embryo moves into the following developmental stages (Heller & Fuchs, 2015; Takahashi et al., 2018).

Cell positional identity is retained as the embryo undergoes gastrulation and the body pattern gains a new layer of complexity with the formation of repeated morphological units or segments (Davis & Patel, 1999). *Drosophila* is the best-characterized model animal system for the genetic study of segmentation and subsequent segment-specific tissue formation. The entire length of the early *Drosophila* embryo is simultaneously subdivided into 12 segments that contain the future adult body plan, head (H, segmented again later in development), thorax (T1-3), and abdomen (A1-8) (Fig.1-1A, (Davis & Patel, 1999; Martinez-Arias &

Lawrence, 1985)). Established morphogen gradients induce a spatially restricted pattern of a vital gene cluster known as the Hox genes (E. B. Lewis, 1978; McGinnis et al., 1984; Struhl, 1982). The clusters of Hox genes (A–D) are activated in a region-specific manner and specify cell lineages during the differentiation process (formation of endoderm, mesoderm, and ectoderm) and body elongation along the A/P axis. The segments create smaller, manageable centers of pattern coordination.

Figure 1-1: Examples of *Drosophila* patterning during development

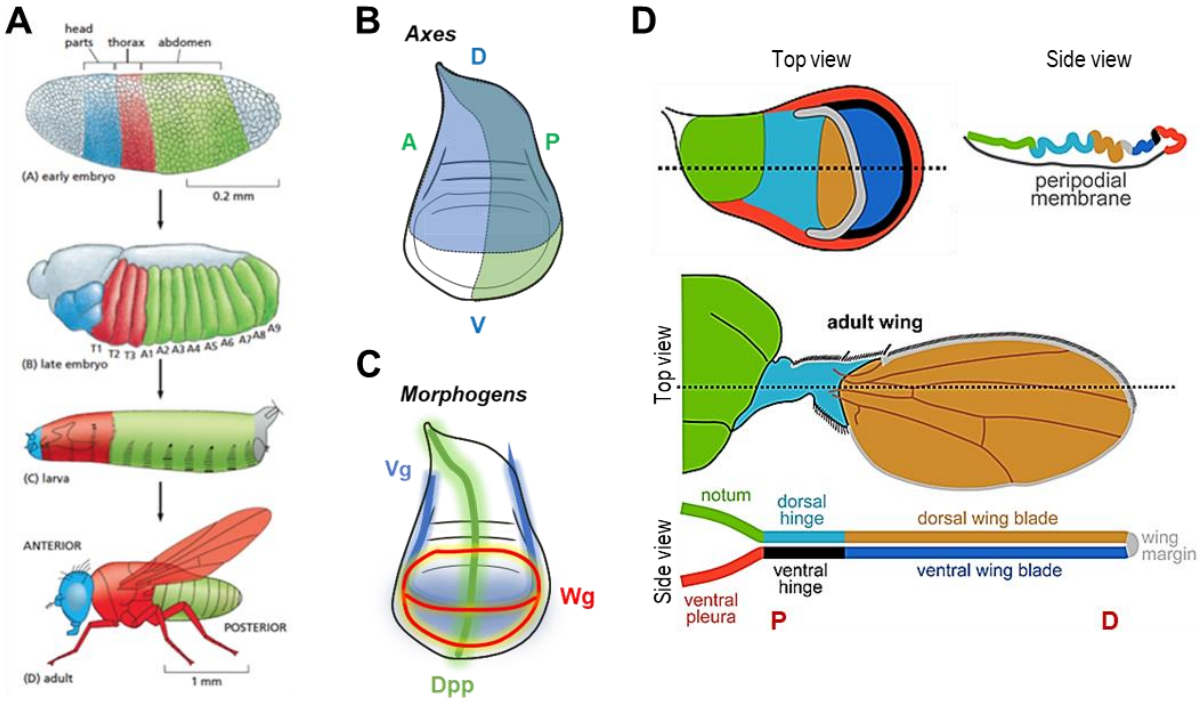

Figure 1 1: Examples of *Drosophila* patterning during development – (**A**) Hox regulated body segmentation in *Drosophila.* The diagram shows a schematic of the segment formation from early embryo development to adulthood. The segments are maintained throughout the *Drosophila* development by Hox genes. (**B-C**) Wing disc patterning development. (B) shows the principal axes of symmetry, dorsal/ventral and anterior/posterior, (C) shows some morphogen expression regions that determine the wing disc development pattern. (**D**) Representation of wing disc to adult wing transformation during metamorphosis. The diagram shows the parts of the wing disc

that become the wing blade, hinge, and part of the thorax as the wing extends from proximal to distal.

Drosophila studies show that the information used to pattern the body axis is used to pattern the primordia for appendages and organs. Although the tissue axes remain the same, the symmetry might change. For example, the overall symmetry in most animals is bilateral, but tissues like eyes and vasculature show radial symmetry, indicating tissue-autonomous patterning. Our lab studies the patterning and development of the *Drosophila* wing imaginal disc (adult wing precursor), which is found in the first thoracic segment (T1). The wing disc has well-defined patterning genes, whose expression patterns begin to be defined in the early third instar larva and organize according to the D/V, A/P, and P/D axes (Fig.1-1B). During the majority of the wing disc growth, the *wingless* gene (*wg*; ortholog of vertebrate WNTs) is at the top of the patterning hierarchy (Fig.1-1C, (Sharma & Chopra, 1976)). As the name suggests, the expression of Wg is necessary for wing development and defines wing fate early in larval development. Wg expressing cells mark the hinge, which becomes the joint connecting the wing to the thorax, the D/V boundary, which becomes the margin of the wing, and a band in the notum that becomes the dorsal part of the thorax (Fig.1-1C-E, (Zecca et al., 1996)). Although we know many vital wing-disc development genes, we still do not know much about the patterning process, especially after damage (*see Section 1.3.C*).

Patterning essentially has two parts in each process: 1) specification – the flexible spatially-determined differential gene expression, and 2) determination – the transition to an irreversible commitment to a specific lineage (Scott F Gilbert, 2000; Wieschaus, 2016).

Determination processes ensure that the lineage-specific genomic changes are stable and cell-intrinsic.

1.1.B Stabilizing patterns and developmental transitions

Development progression is like building blocks, where an increasingly complex pattern is built on a pre-established pattern. Once each stage of growth and development is completed, the animal undergoes a developmental transition that cements the previous stage before building the next stage. Developmental transitions are points in developmental progression where tissues or whole organisms undergo irreversible changes of state (Xiao et al., 2017). An example is the end of *Drosophila* larval development; imaginal tissues undergo differentiation and lose their self-renewal capabilities while the larva also begins pupating to undergo metamorphosis (Berreur et al., 1979; Smith-Bolton et al., 2009). Developmental transitions are vital since they often act as a juncture for development coordination; all tissues must be on track before progressing to the next stage. Any perturbations in tissue development often delay developmental transitions or activate mechanisms that repair and restore tissue patterning and development before the next transition (*see Section 1.3*).

Patterning and developmental transitions are completed when stable, heritable gene silencing or activation changes are made by Polycomb Group (PcG) and Trithorax Group (trxG). PcG and trxG are evolutionally conserved multiprotein complexes first identified in *Drosophila* that set epigenetic marks that allow cells to have genetic memory after a transient internal or external stimulus (Capdevila et al., 1986; Gellon et al., 1997; Kassis et al., 2017; Maurange & Paro, 2002; Schuettengruber et al., 2017). When bound

to the nucleosomes of active genes, trxG proteins keep these genes active, whereas PcG proteins, which bind to condensed nucleosomes, keep the genes in a repressed state. PcG and trxG work in opposition and usually interact with chromatin at different times except for some bivalent chromatin sites often found on pluripotent stem cells (Bernstein et al., 2006).

The first identified PcG subunit was *esc* (*extra sex combs*), whose recessive mutation caused the formation of sex combs (usually on T1 legs only) on T2 and T3 legs (Slifer, 1942). However, PcG gains its name from the dominant mutant with a similar phenotype *Polycomb* (*Pc*) that was identified several years later (P. H. Lewis, 1947). Over the years, several dominant and recessive mutations with similar phenotypes have helped identify the subunits of PcG (Alonso et al., 2007) and that the esc phenotype is due to ectopic expression of the Hox gene, *Sex comb reduced* (*Scr*) (Struhl, 1982). PcG suppresses loci through a three-step process by PcG Repressive Complexes (PRC): 1) recruitment of PRC2 by transcription factors, non-coding RNA or Pho (PcG subunit) that binds PcG response elements (PREs), to the (euchromatin) site of suppression (Blackledge et al., 2015; L. J. Brown et al., 1998; Van Kruijsbergen et al., 2015), 2) tri-methylation of lysine27 on histone3 (H3K27me3) by PRC2, and finally, 3) the methylation recruits PRC1 which mono-ubiquitinates histone2A at Lysine119 (H2AK118Ub) causing the gene suppression (heterochromatin) (Dorafshan et al., 2017; Kahn et al., 2016).

The trxG was identified much later than PcG. The *trithorax* (*trx*) mutant phenotype mimics previously identified loss of function phenotypes for Hox genes. Heterozygous double mutants of *trx* and *Pc* demonstrated that *trx* mutations are dominant suppressors of *Pc* mutant (Capdevila & García-Bellido, 1981). Since these initial studies, several other

trxG subunits have been identified (Florence & McGinnis, 1998; Gellon et al., 1997). TrxG activates gene transcription by inducing trimethylation of lysine 4 and 36 of histone3 (H3K4me3 and H3K36me3) and interacting with acetyltransferases that acetylate H3K27 preventing suppression by PcG (Bannister & Kouzarides, 2011; Schuettengruber et al., 2017).

Developmental transitions often involve changes in all the tissues in the body. To ensure the timing of these transitions and patterning changes is synchronous, a coordinating stimulus is needed. Hormones are the systemic signals that instruct the simultaneous development of all organs and direct tissue-specific developmental programs. In the next section, I will discuss hormones and their role in the coordinated development progression of all tissues.

1.2 Hormones: Multi-tissue coordination of developmental progression

The range of most of the morphogens that coordinate tissue patterning is insufficient to coordinate patterning and developmental progression between tissues. Instead, long-range signaling is required to keep coordinating development progression and growth rate for more distant tissues, and regulated production of circulating hormones performs this function.

1.2.A Introduction to hormones

Hormones are the small, secreted chemical (molecule or peptide) messengers that coordinate activities in distant cells or tissues. Unlike morphogens that function mainly by

signaling to adjacent cells (paracrine) and themselves (autocrine), hormones are primarily endocrine. For this reason, the term endocrine signaling is used synonymously with hormone signaling. Most hormones are often produced by specialized glands and reach all tissues via the bloodstream or hemolymph. At present, virtually every process in complex organisms is regulated by one or more hormones. The first hormone identified and isolated was insulin, a long-range signaling molecule produced by the pancreas and released into the bloodstream (Scott & Fisher, 1935; Vecchio et al., 2018). The glands that synthesize hormones act as signaling centers where signals from different tissues are received, and hormone production is enhanced or suppressed (Ou et al., 2016). For example, in *Drosophila,* the prothoracic gland (PG) is a signaling center (Selcho et al., 2017). Hormone and, at times, morphogen signals from peripheral tissues regulate the PG's biosynthesis and release of the steroid hormone ecdysone, which regulates developmental progression (*see Section 1.2.B*).

a) Classification of hormones

Hormones are often classified according to 1) structure (protein, steroid, amine, etc.) and 2) solubility (lipid or water-soluble) (Hiller-Sturmhöfel & Bartke, 1998). Below, I will discuss endocrine hormones according to their diverse structures, which determine their function, solubility, and eventually mechanism of action.

- *Amine or catecholamine hormones* are relatively small molecules that are derived from amino acids tyrosine and tryptophan. These molecules include dopamine, epinephrine (adrenaline), and norepinephrine (noradrenaline). Catecholamines produced in the CNS are also neurotransmitters but synthesized and secreted by

adrenal glands (Bhathena, 2006). They act through surface receptors to initiate intracellular second messengers for various physiological responses to stress.

- *Peptide hormones* include insulin, glucagon, parathyroid, and all hormones of the hypothalamus and pituitary. Peptide hormones usually are 3-200 amino acids long. However, they are expressed as longer proteins (pro-hormones) and then packaged into secretory vesicles, where they are proteolytically cleaved to form the secreted active peptide (3-200 amino acids) (Floyd et al., 1999; Given et al., 1985).

  In addition to the known peptide hormones, recent studies in *Drosophila* have found that some morphogens can function similarly to peptide hormones. *Decapentaplegic* (*dpp,* BMP2/4 ortholog), expressed by A/P boundary cells of developing wing discs, likely diffuses through the epithelium and reaches the PG via the hemolymph to inhibit ecdysone biosynthesis (Dacrema, 2020; Setiawan et al., 2018). Dpp signaling seems to communicate the developmental progression of imaginal discs to the PG for inter-organ growth coordination.
- *Retinoid Hormones* are potent hormones that regulate growth, survival, and differentiation through nuclear retinoid receptors (RXR) (Mangelsdorf & Evans, 1995). Retinol is synthesized from vitamin A in the liver and converted to retinoic acid in the target cells. All tissues express at least one form of the retinoid receptor.
- *Steroid hormones* are endocrine hormones synthesized from cholesterols and phytosterols in animals that cannot synthesize sterols (Beato & Klug, 2000). Steroid hormones include adrenocortical, sex, and vitamin D (vit.D) hormones. The synthesis of steroid hormones is a multistep process that usually involves the modification of the sterol molecule by adding keto (name ends with "-one") and hydroxyl (ends with "-ol")

groups and/or removing the hydroxycarbon side chain. In the case of vit.D hormones, the cholesterol is photolyzed to form the vit.D (cholecalciferol) molecule that is then modified by adding hydroxyl groups (Nair & Maseeh, 2012). The active forms of steroid hormones, usually the final product, act through nuclear receptors (*see b) mechanisms of action*) to activate the expression of specific genes.

- *Thyroid hormones* are a class of hormones found in vertebrates similar to peptide, amine, and steroid hormones. Thyroid hormones T_4 (thyroxine) and T_3 (triiodothyronine) are synthesized from precursor protein thyroglobulin (Rousset et al., 2000). About 20 tyrosines in thyroglobulin are enzymatically iodinated in the thyroid gland. These iodotyrosines are condensed in pairs to form the precursors to T_4. T_4 is proteolytically released when needed, or a T_3 is released following the condensation of a single diiodotyrosine with a monoiodotyrosine. Both T_4 and T_3 are active hormone forms and signal through nuclear receptors (Bianco et al., 2002; Rousset et al., 2000).

There are other classes of hormones (small signaling molecules), but their targets are often not as long-range as other hormones described above:

- *Fatty acid hormones,* also known as eicosanoids, are derived from 20-carbon polyunsaturated fatty acid arachidonate (20:4). They include prostaglandins, thromboxanes, and leukotrienes (Bhathena, 2006). Unlike other hormones, eicosanoids are not premade and stored. Instead, they are made as needed from enzymatically (phospholipase A_2) phospholipids released from the membrane. The enzymes that make eicosanoids and their specific membrane receptors are found in every cell.

- *Nitric Oxide (NO)* can be considered the final class of hormones. NO is a highly soluble free radical synthesized in many cells by NO synthase, which catalyzes the addition of molecular oxygen to the guanidino nitrogen of L-arginine (Chachlaki et al., 2017). NO is a neurotransmitter that activates the secondary messenger cGMP in its target cells.

b) Mechanisms of action

Although there are diverse hormone types, there are only two primary mechanisms of action dependent on the hormone's solubility. The solubility of the hormones determines whether they can travel through the cell's lipid bi-layer or require an extracellular binding domain.

Water-soluble or polar hormones, such as peptide hormones, bind to membrane-bound receptors. The receptors may not be exclusive to one ligand and may initiate different signaling cascades depending on the ligand. The receptors can initiate signaling transduction pathways by activating secondary messengers, phosphorylation cascade, or G-proteins. An excellent example of this is *Drosophila* insulin-like peptides, which are structurally related (three disulfide bonds motif) to human insulin/IGF(insulin growth factor)/relaxin, which activate the insulin receptor (InR). Depending on the ligand and the intracellular domain (Insulin Receptor Substrate), the receptor can activate the PI3K-Akt signaling and/or Ras-MAPK signaling. Through differential expression of InR(s) in the target cells, insulin-like peptides can regulate sugar metabolism, cell growth, and mitogenesis.

Most lipid-soluble or non-polar hormones are steroid hormones. Lipid-soluble hormones enter the cell by diffusing through the lipid-bilayer or a transporter when increased activation is needed (Beato & Klug, 2000; Okamoto et al., 2018). Once in the cell, hormones bind to nuclear receptors (NR) in the cytoplasm, nucleus, or both depending on receptor type. Hormone binding triggers conformation changes in the NR to bind regulatory loci known as Hormone Responsive Elements (HREs) to activate or suppress gene expression (Beato & Klug, 2000). NRs are ligand-regulated transcription factors that control various biological processes, including cell proliferation, development, metabolism, and reproduction (Mangelsdorf et al., 1995). Although the primary role of NR is transcription, some, like estrogen receptors in endothelial cells, act in the cytoplasm for more rapid activation of signaling (Wu et al., 2011).

Although lipid-soluble hormones can diffuse through the membrane, there is evidence of alternative entry methods. Okamoto et al. identified an evolutionarily conserved anion transporting polypeptide transporter, Ecdysone Importer (EcI), necessary for ecdysone-dependent signaling by ecdysone receptor. Loss of EcI *in-vivo c*auses phenotypes almost indistinguishable from 20E- or EcR-deficient animals, meaning there is a limitation in the cellular uptake of ecdysone (Okamoto et al., 2018). A transporter may be target tissue's way of amplifying 20E signaling when systemic 20E levels are limited.

1.2.B Hormones and the coordination development transitions

During normal development, hormones are produced at specific stages, and their combined actions trigger developmental transitions, organ re-arrangements, promoting

growth or cell death. The extent of hormone-signaling's influence on developmental transitions is seen in organisms that undergo metamorphosis. Metamorphosis in amphibians such as frogs is initiated by thyroid hormones that reach all organs through the bloodstream and induce growth, death, and remodeling to form functional adult-specific structures. Blocking T_3 activity during frog metamorphosis prevents innervation of the forming hindlimbs, impairing muscle growth and causing limb paralysis (B. Das et al., 2002; Marsh-Armstrong et al., 2004). T_3 signaling also induces degeneration of the paddle-like tail, the oxygen-procuring gills, and tadpole red blood cells/hemoglobin important for larval but not adult movement and respiration (Nakajima et al., 2005; Riggs, 1951). T3 induces shortening of the intestines (for carnivorous diet) and activates adult hepatic (urea-cycle) genes while repressing larval ammonia synthesis genes (Atkinson et al., 1998; Helbing et al., 1992, 1996; Sachs & Buchholz, 2019).

Hormones also communicate the state of the external environment. The progression of development is dependent on the availability of nutrients and favorability of the environment. In adverse conditions and malnutrition circumstances, the animal needs to trigger metabolic changes to maintain energy homeostasis and sustain growth and development (A. C. Oliveira et al., 2021). According to the animal's nutritional state, this metabolic flexibility is mediated by hormones that drive developmental encoded metabolic transitions and adaptation responses throughout development (Yamada et al., 2020). Adaptation responses may include slowing down the patterning and development process for energy conservation.

Several nutrient- and environment-regulated hormones control *Drosophila* development with signaling cross-talk. These hormones include neuropeptide

prothoracicotropic hormone (PTTH), Insulin and insulin-like peptides (Dilps), Juvenile Hormone (JH), and the steroid hormone ecdysone (in its active form 20-hydroxyecdysone, 20E) (De Loof et al., 2012; Rewitz et al., 2013; Shimell et al., 2018; Truman et al., 1974). Of the hormones produced, ecdysone, which is produced by the prothoracic gland (PG, one of three glands that make up the larger endocrine organ called the ring gland), and secreted into the hemolymph, is the master regulator of all developmental transitions *Drosophila* (Fig.1-2) (King et al., 1974; McBrayer et al., 2007). Ecdysone initiates and coordinates each molt; all other hormones regulate developmental transitions by modifying 20E biosynthesis or signaling outputs. 20E acts through a heterodimer complex of the receptor that binds it (ecdysone receptor, EcR, which comes in 3 isoforms EcR.A, B1, and B2) and another member of the NR superfamily, Ultraspiracle USP), the insect homolog of the vertebrate retinoid X receptor (RXR), found in all cells in the *Drosophila* body (Bender et al., 1997; Lynn M. Riddiford et al., 2003). EcR-Usp complex is structurally similar to the TR-RXR complex in amphibians (Szamborska-Gbur et al., 2014). The PG secretes ecdysone in distinct pulses that trigger different transcriptional events and distinct developmental transitions throughout development. The timing of ecdysone production and the output of ecdysone signaling in target tissues are regulated by other hormones.

During the larval molts, JH limits ecdysone signaling, allowing only molt-related transcriptional changes and no metamorphosis-related gene expression. Once JH levels decrease, ecdysone signaling promotes transcription of genes that drive pupation and metamorphosis (Jindra et al., 1996). Ectopic application of a JH analog during prepupal stages in *Drosophila* results in lethality with defects in the abdominal, nervous system,

and muscular development. JH signaling suppresses developmental changes mediated by downstream targets of ecdysone signaling, the Broad transcription factors (X. Zhou & Riddiford, 2002). Recent studies have shown that the downstream target of JH signaling, Krüppel Homolog 1 (Kr-h1), directly suppresses the transcription of Broad isoforms (Kayukawa et al., 2016). Studies in holometabola insects show that elevated JH levels reappear late (between wandering and white pre-pupa stage), preventing the premature adult differentiation of imaginal primordia that the more significant pre-pupa pulse of ecdysone would cause (Kayukawa et al., 2014; Minakuchi et al., 2009; Ureña et al., 2016). As larvae gain competence to undergo metamorphosis (Smykal et al., 2014), the JH titer drops, reducing expression of the anti-metamorphic, JH response gene Kr-h1, which lifts suppression on the pupal or adult developmental programs (Jindra et al., 2013).

Figure 1-2: Drosophila developmental transitions are guided by hormones

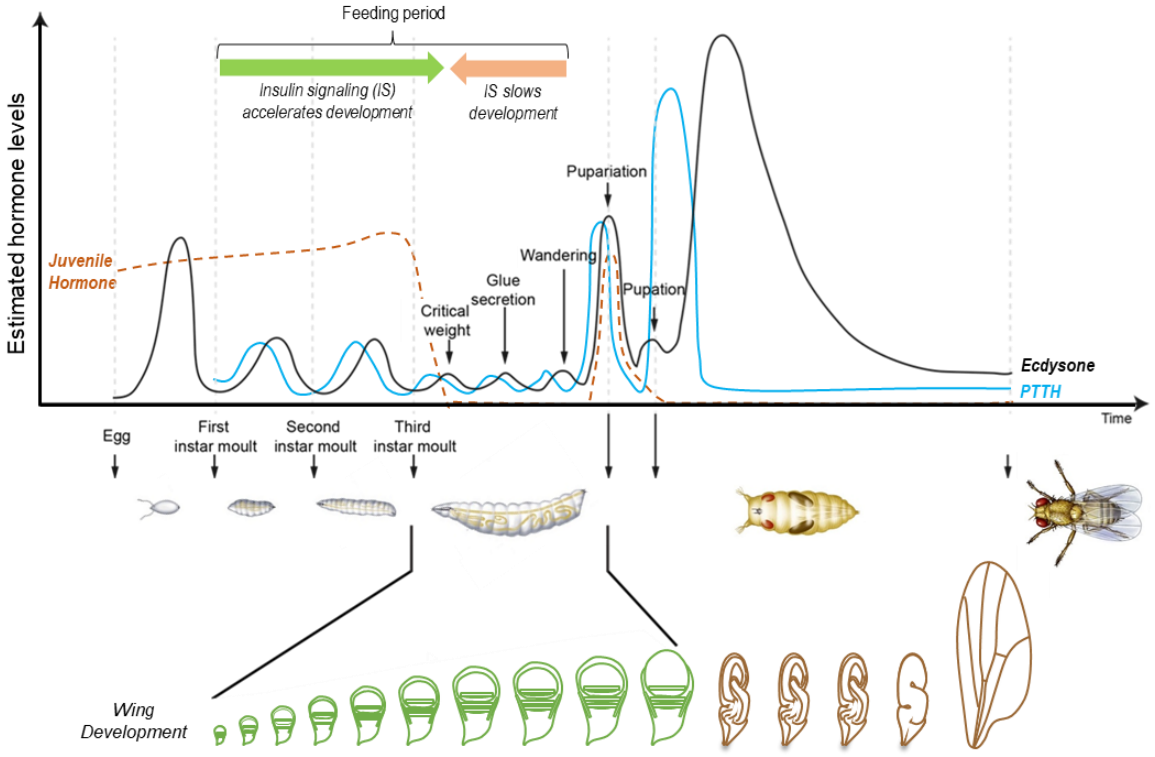

Figure 1-2: Drosophila developmental transitions are guided by hormones – The diagram outline Drosophila development and a rough estimate of levels of hormones produced in Drosophila or other holometabola insects. The hormones highlighted include ecdysone, the primary regulator of developmental transitions, juvenile hormone, and the Dilps. The Dilp levels have not been indicated, and instead, their influence on ecdysone production is indicated. Before critical weight (CW), Dilps promote ecdysone production and the switch to suppressing ecdysone production after CW. The diagram also shows the progression of wing development during L3.

JH regulates ecdysone's transcriptional activity, whereas PTTH and Dilps regulate the production of ecdysone. Nutritional and environmental factors, such as temperature shifts regulate the rate of ecdysone production, making the PG a decision-making center where external cues are converted into an actionable response. PTTH is a ~25kDa disulfide-linked neuropeptide hormone secreted by PTTH-producing neurons which sense physiological and environmental changes. For example, some blood-feeding hemipteran species, such as milkweed bugs, distention of abdominal stretch receptors by feeding or injecting air signals for PTTH release from PTTH neurons (Nijhout, 1979). PTTH neurons also receive circadian- and temperature-regulated clock input via the inhibitory neuropeptide F (NPF) produced by lateral neurons (Selcho et al., 2017); Sensory inputs such as these allow PTTH-neurons to help set the developmental clock. PTTH-neurons transmit this temporal information to the PG via the PTTH-*torso* (receptor) signaling regulating the timing of the ecdysone peaks (Di Cara & King-Jones, 2016; Selcho et al., 2017; Vafopoulou & Steel, 1996). McBayer et al. (2007) demonstrated that PTTH is necessary for the ecdysone pulses that initiate development transitions. PTTH-null

mutants have a prolonged feeding period, enhanced critical weight requirement, and developmental delay due to lower ecdysone production (McBrayer et al., 2007).

In addition to physiological and environmental cues, nutrition also regulates ecdysone-induced developmental transitions. During the larval period, animals feed and Drosophila insulin-like peptides (Dilps) produced by the brain and peripheral tissues couple nutrient uptake with systemic growth. Eight functionally diverse and differentially expressed Dilps have been described (Ikeya et al., 2002). Dilps 1–7 signal through Insulin Receptor (InR), while Dilp8 (more closely related to relaxins) binds to Lgr3 receptor (Garelli et al., 2015; Nässel et al., 2015). Of the 8 Dilps, only Dilp2, 3, 5, and 7 are expressed in the CNS insulin-producing cells (IPCs) in response to nutrition. Reducing Dilp secretion by the IPCs prolongs development time and results in much smaller adults suggesting that IPC-Dilps regulate 20E biosynthesis (Walkiewicz & Stern, 2009). Dilp signaling, through TOR signaling, regulates the cellular localization of FoxO. In the absence of Dilp signaling, FoxO remains in the nucleus and binds Usp, limiting ecdysone biosynthesis genes' expression (Koyama et al., 2014). Interestingly, Dilp signaling plays different roles in ecdysone regulation in response to nutrition pre- and post-critical weight (CW). In pre-CW larvae, once the appropriate nutrients have been acquired, the insulin signaling pathway promotes the production of ecdysone (Caldwell et al., 2005; Colombani et al., 2005; C. Mirth et al., 2005). By contrast, post-CW insulin signaling is thought to repress ecdysone production as starvation (low insulin signaling), resulting in accelerated development (C. Mirth et al., 2005; Stieper et al., 2008). However, the mechanism that causes the shift of Dilp signaling output in the PG remains unknown.

The following section will discuss how damage or development irregularities affect hormone signaling and the coordination of patterning and development progression.

1.3 Regeneration: Restoring patterns and inter-organ growth coordination following damage

At any point, the developing organism may encounter adverse conditions that cause tissue damage or even a loss of tissue sections. Following injury, the coordination of patterning is lost, morphogen gradients and cell-cell contacts are disrupted. The tissue needs to recover any lost tissue, restore cell contacts and reestablish the morphogen gradient. The process of fully restoring patterns and regrowing damaged cells, tissues, or organ systems after tissue damage is regeneration. The ability to regenerate is widespread in animals; however, the timing of regeneration and the extent of damage that can be restored varies widely between species (Brockes & Kumar, 2008; Sanchez Alvarado, 2000). In the next section (*1.3.A*), I will discuss the different ways in which different models restore tissue pattern

1.3.A Regeneration mechanisms

A single organism can utilize multiple regeneration mechanisms (*discussed below*) in different tissues or a combination of mechanisms in the same tissue. For example, the tail regenerates in zebrafish using epimorphosis while the heart employs epimorphosis followed by a compensatory proliferation. It is unclear why tissues use one regeneration

mechanism over another. There are four main types of regeneration mechanisms that restore tissue organization and function.

a. *Stem-cell mediated regeneration* – This is the regeneration mechanism often found in adult mammalian tissues. Tissues that use this regeneration method have a set of stem cells (in a niche) that allow the regrowth of specific organs or tissues that have been lost. For example, the regrowth of hair shafts from follicular stem cells in the hair bulge and the continual replacement of blood cells from hematopoietic stem cells in the bone marrow (S. F. Gilbert & Barresi, 2016; Kniss et al., 2016). A more striking example is planaria, which can regenerate after being cut to half, creating two separate planaria. Using lineage tracing, cell ablation, and several cell-transplant experiments, Wagner et al. (2011) demonstrated that no dedifferentiation occurs during planaria regeneration. Instead, the regeneration blastema (mass of cells that close the wound) and subsequent regeneration are produced by pluripotent stem cells known as clonogenic neoblasts (cNeoblasts) (Wagner et al., 2011). Loss of cNeoblasts results in a complete loss of regenerative capacity, and the planaria die following irradiation damage. However, transplanting a single cNeoblast into cNeoblast-deficient planaria restored regenerative capacity and replaced all dying cells (Wagner et al., 2011). Patterning is reestablished by Wnt signaling gradients that direct the blastema cells' anterior-posterior differentiation (De Robertis, 2010; Gurley et al., 2008). The Wnt pattern is reinforced by a gradient of head-expressed Wnt inhibitor Notum (Petersen & Reddien, 2011).

b. *Epimorphosis* – In this regeneration model, differentiated structures undergo dedifferentiation to form a relatively undifferentiated mass of cells (a blastema) that then re-differentiates to form the new structure. This regeneration model is characteristic of

regenerating amphibian limbs. Salamanders accomplish epimorphic regeneration by cell dedifferentiation to form a regeneration blastema (Brockes & Kumar, 2002). Although the cells dedifferentiate, they become lineage-restricted progenitor cells; lineage tracing shows that new muscle cells arise only from old muscle cells, similar to dermal and cartilage cells (Kragl et al., 2009). Re-establishing the body pattern requires nerves to direct regeneration and growth (Kumar, Godwin, et al., 2007). The neurons produce newt anterior gradient protein (nAG), which causes blastema cells to proliferate in culture, and is necessary to provide ectopically to permit regeneration in denervated limbs. If nAG is not administered, the denervated limbs remain stumps. (Kumar, Godwin, et al., 2007). In order to pattern the growth along the P/D axis, the receptor for nAG, Prod1, is expressed in a proximal to distal gradient (Geng et al., 2015; Kumar, Gates, et al., 2007). Other hormones and morphogens have also been found in the blastema, such as opposing retinoic acid-FGF gradients and expression of Wnts, BMPs, Hh, and Notch (Makanae et al., 2014; Singh et al., 2015).

c. *Morphallaxis* – Here, regeneration occurs through the repatterning of existing tissues and with very little new growth. After tissue damage or loss, the undamaged tissue's cells trans-differentiate to repattern a new primary axis. Freshwater Cnidarian hydra provides a remarkable example of this regeneration model. An adult hydra retains enormous regenerative capacity. When cut into 40+ pieces, each piece would regenerate new individuals with a head at its original apical end and a foot at its original basal end (Bosch, 2007). Hydra body patterns are set by having a head-activation gradient, head-inhibition gradient, foot-activation gradient, and foot-inhibition gradient. Apoptosis at the site of hydra decapitation is both necessary and sufficient to induce Wnt3 production and

head regeneration. Wnt3 was identified as a head-activator and can induce head formation even at ectopic sites (Chera et al., 2009). Wnt3 signaling remodels the remaining cells through canonical signaling (Broun et al., 2005).

d. *Compensatory regeneration* – This regeneration model only involves the proliferation of surrounding differentiated cells to replace lost tissue. The new cells do not come from stem cells or dedifferentiated cells. Each cell produces cells similar to itself, with no blastema formation. This type of regeneration is characteristic of the mammalian liver. Liver regeneration appears to have two other lines of defense. The first, mature quiescent hepatocytes are instructed to rejoin the cell cycle and proliferate until they have compensated for the missing part. However, the extent of damage determines whether the compensatory regeneration in the liver is through hypertrophy (1/3 of the tissue lost) or hyperplasia (2/3 of the tissue lost) (Miyaoka et al., 2012). The second is a population of quiescent hepatic progenitor cells activated when 80% to 90% of the liver is lost injury is too severe for hepatocytes alone. The regeneration model becomes epimorphic with the dedifferentiation of biliary epithelial cells (BECs) (Lu et al., 2015).

1.3.B Loss of regenerative capacity

Regardless of the regeneration mechanism, the regenerative ability seems to decline in two dimensions, phylogeny and complexity of body organization. So far, regeneration studies show that only animals from lower evolutionary-tree levels can regenerate at all levels of structural organization. However, organisms with higher body complexity regenerate some tissue, cells, and organelles, as well as organisms with less complexity, do, but not appendages or significant fractions of their body. Unlike

diploblastic animals like a hydra, most mammalian tissues lose regenerative capacity with the progression of development. For instance, regeneration restriction is seen in the development of the mammalian heart. Neonatal mice are capable of regrowing the ventricular apex of the heart after it is surgically removed. This regenerative capacity is lost within the first week of life (Hirose et al., 2019; Porrello et al., 2011). A similar loss of regenerative capacity is seen in digit tips (Borgens, 1982; Reginelli et al., 1995; Yun, 2015). After the loss of regenerative capacity, adult tissues form mis-patterned scar tissue following damage. Scar tissue fails to fully restore the original biochemical, structural, and functional properties of the tissue (Halder & Johnson, 2011; Porrello et al., 2011). It is unclear how developmental processes in vertebrates limit the timing of regeneration. An excellent review of the possible evolutionary reasons for the gain and loss of regenerative capacity can be found in '*Evolution of Regeneration in Animals*: *A Tangled Story*' (Elchaninov et al., 2021). The authors discuss the effect of evolution on regenerative capacity. The authors argue that adversities encountered in the environment and the cost of regeneration (extent of damage) to the organism often influence whether regenerative capacity is maintained, gained, or lost.

In organisms that lose regenerative capacity, it is clear that the loss of regenerative capacity often coincides with major developmental transitions. In arthropods, loss of regenerative capacity often coincides with the onset of pupation and metamorphosis(Goss, 1969b). Since hormones regulate developmental transitions, the correlation between hormone signaling and regenerative capacity has been long studied.

1.3.C Hormones in regeneration

In addition to morphogens that induce repatterning during regeneration, hormones also regulate regenerative activity. Almost all studied regeneration models show some relationship between hormones and regulation of regenerative activity.

Hormone signaling often suppresses regenerative activity. Hormone-induced regeneration suppression often coincides with the developmental transition. In male zebrafish, sexual maturity leads to increased systemic androgen levels and loss of pectoral fin regenerative capacity (Nachtrab et al., 2011; Yun, 2015). Androgen signaling reduces blastema proliferation and patterning by maintaining the expression of *dkk1b* and *igfbp2a* genes, which encode secreted inhibitors of Wnt and IGF signaling, respectively (Nachtrab et al., 2011). Similarly, metamorphosis (Xenopus) and maturation (mice) are induced by elevated thyroid hormone signaling and often result in loss of regenerative capacity (Hirose et al., 2019; Marshall et al., 2019). This correlation between metamorphosis/TH and regeneration was demonstrated in axolotls, where natural metamorphosis is rare. Experimental induction of metamorphosis in axolotls with TH treatment reduced the ability to regenerate and the number of cells proliferating in the limb and heart(Monaghan et al., 2014). Additionally, as postnatal development continues, there is a rise in TH production and a decrease in cell proliferation, especially in the central nervous system as it matures(S. F. Gilbert & Barresi, 2016). TH inhibits tail regeneration of tadpoles and induces tissue resorption in the tail regenerates as late metamorphic stages approach. The resorption of regenerative tissue appears to require a critical concentration of TH at a particular metamorphic stage(B. Das et al., 2002). These studies hypothesized that TH-mediated differentiation of cells and the downregulation of

developmental signaling pathways prevent cells from dedifferentiating into precursors after injury, thereby preventing regenerative capacity. This suppression of regenerative activity is similar to ecdysone's role in arthropods' suppression of regenerative activity just before metamorphosis(Goss, 1969b). The mechanism of which remains unknown.

Although ecdysone suppresses regenerative activity, a complete loss of ecdysone limits regeneration. Experiments in *Sarcophaga* leg discs demonstrate that the absence of ecdysone limits Wg expression at the wound site (Kunieda et al., 1997). Kunieda et al. established an *in-vitro* culture system for imaginal discs in which they demonstrated that discs cultured in 20E-free media failed to close wounds or express Wg. Similar observations have been made in moths (Madhavan & Schneiderman, 1969), *Sarcophaga* (Kunieda et al., 1997), fiddler crabs (S. Das & Durica, 2013; Hopkins, 1989), and newts (Goss, 1969c), where removal of the PG and subsequent loss of ecdysteroids leads to incapability to regenerate. Testosterone in deer antler regeneration also seems to play a dual role in regulating antler regrowth. Loss of testosterone limits activation of antler growth, but testosterone is also necessary for ending the regenerative response. A striking example is in the annual shedding and regrowth cycle of male deer antlers, where castration during antler regrowth leads to tumorous overgrowth of the antler (Goss, 1969a; Price & Allen, 2004). In addition, deer cannot initiate antler growth if castrated during the resting phase between antler regenerative cycles (Goss, 1969a). The dependence of antler regeneration on the sex organs indicates hormonal regulation of regeneration timing and regeneration extent (Bubenik et al., 1987; Kierdorf et al., 2004). Testosterone suppresses the activity of thyroid hormone and growth hormone (GH/IGF), which promote regenerative growth (Price & Allen, 2004; Shi & Barrell, 1994). Similar

endocrine regulation of regenerative capacity is found in amphibians, fish, and arthropods (S. Das, 2015; Goss, 1969c; Nachtrab et al., 2011). However, the mechanism of regulation is still not well understood (Goss, 1969c).

Understanding the developmental changes and hormones that limit the activation of regeneration mechanisms is one focus of our research. Another question is how developmental coordination is maintained or reestablished between the regenerating and undamaged tissues. While repairing itself, the damaged tissue is out of sync with the developmental progression of the other tissues; reestablishing the developmental program before the next developmental transition is part of the regenerative process. We are addressing these questions using *Drosophila* wing disc regeneration.

1.3.D Regeneration model: Drosophila wing disc

Drosophila melanogaster imaginal discs (adult organ precursors) have a tremendous capacity to regenerate during specific periods of their development. After the damage to an imaginal disc, *Drosophila* initiates regenerative pathways that restore imaginal disc function and size, even after losing almost 60% of the tissue (Bergantinos et al., 2010). Successful imaginal disc regeneration requires the coordination of local and systemic developmental pathways (Andersen et al., 2013; Gokhale & Shingleton, 2015; Wartlick et al., 2011).

The wing discs were chosen as their development has been well-described, and they have been a classic model for studying the genetic and molecular basis of organ regeneration. Tissue damage in the wing disc, via ablation or mechanical damage,

causes cell death, inducing Jnk signaling (Bergantinos et al., 2010). The repatterning of the wing disc begins with a marked increase in Wg expression, predominantly at the hinge region, suggesting that the wing hinge is the region of most significant regenerative activity (Harris et al., 2016; Smith-Bolton et al., 2009; Verghese & Su, 2016). Upregulation of Wnts is a common occurrence in almost all regeneration models (*see Section 1.3.A*). The increased expression of *wg* during regeneration is dependent on JNK signaling activation at a specific *wg* enhancer defined by the wgDRE (a.k.a. BRV118) transgenic enhancer reporter. Loss of the wgDRE-defined regulatory region (wg^{1} mutant) does not limit the developmental expression of *wg* but limits *wg* expression after damage (Harris et al., 2016). Recent studies have shown that *wg* expression at the hinge is necessary for regeneration (Verghese & Su, 2016). Tissues damaged using high ionizing irradiation showed an area of intact cells in the dorsal hinge labeled the 'smile' region. In collaboration with JAK-STAT signaling, Wg defines the damage-resistant cells that repopulate a significant portion of the wing pouch during regeneration (Verghese & Su, 2016). Surgical ablation experiments show that regeneration induces cell-fate commitment and patterning changes (Díaz-García & Baonza, 2013). The regenerating region showed localized loss of patterning and cell fate genes such as *vestigial* and *Iro* from the vein cells and dedifferentiated cells crossing the A/P boundary in severe cases, indicating an epimorphic regeneration model. Through these experiments, Diaz-Garcia and Baonza demonstrate that tissue patterning and tissue reorganization are independent of cell division during regeneration.

Damage to imaginal discs also regulates the timing of the late third instar ecdysone pulse through signaling by *Drosophila* insulin-like peptide 8 (Dilp8). Dilp8 is a peptide

hormone that belongs to the family of relaxin proteins, a class of hormones structurally related to insulin and involved in sexual maturation and musculoskeletal tissue repair after damage in mammals (Dehghan et al., 2014; Zeng et al., 2014). Dilp8 was identified using microarray analyses for genes differentially expressed following growth perturbations (neoplastic tumor) that induced a developmental delay (Colombani et al., 2012; Garelli et al., 2012). Dilp8, whose expression is activated by JNK signaling, is primarily expressed in regenerating tissues and acts remotely by binding to Lgr3 receptors in the brain and prothoracic gland to inhibit the production of ecdysone (Colombani et al., 2015; Garelli et al., 2015; Vallejo et al., 2015). By limiting systemic ecdysone levels, Dilp8 delays the late third instar (pupariation) ecdysone peak, extending the larval developmental period and providing regenerating tissues ample time to repair damage and reach target size (Colombani et al., 2012; Garelli et al., 2012, 2015). Thus, Dilp8-Lgr3 signaling is necessary for the proportional growth and the maintenance of body symmetry after damage as it ensures that the growth of damaged and undamaged tissues is coordinated (Boone et al., 2016; Jaszczak et al., 2015, 2016).

Like most mammalian tissues, imaginal discs lose the capacity to regenerate with the progression of development. This loss of regenerative capacity has been demonstrated in studies where imaginal discs were damaged at different points in their development. Discs damaged later in disc development (close to pupation) fail to repair and result in malformed adult tissues (Halme et al., 2010; Smith-Bolton et al., 2009). The timing of this loss of regenerative capacity coincides with the increase in systemic levels of ecdysone that initiates the larva-pupa developmental transition (Baehrecke, 1996; Lavrynenko et al., 2015). *Drosophila* wing disc regeneration, therefore, serves as an

excellent model for investigating how developmental transitions and hormone signaling regulate regenerative capacity.

1.4 Thesis overview

Our lab and others have made considerable progress in understanding the coordination of wing imaginal disc regeneration, but a lot remains unresolved. Our findings have been built on understanding the normal patterning and development of *Drosophila* and the imaginal discs. Here, I outlined the importance of patterning and developmental transitions for proper body formation. I discussed how hormone signaling coordinates developmental transitions by inducing changes in gene transcriptional activity. These changes involve silencing the genes and mechanisms that restore tissue patterns following damage (regeneration genes). Using well-known patterning and growth-coordination markers regulated during regeneration, we identified a system to quantify regenerative activity in the developing wing disc (Chapter 2).

For my dissertation work, I wanted to address how regenerative capacity is limited with developmental progression. Losing the capacity to repattern and regrow tissues often coincides with the major hormone-regulated developmental transitions. In chapter 3, I describe how the increase in hormone (ecdysone) levels at larva-pupa transition induces expression of Broad (Br) transcription factors. The sequential expression of Br splice isoforms induces the transcriptional changes that initiate pupation. The Br isoforms directly suppress the wgDRE locus, which activates Wg expression and subsequent

regenerative mechanisms. Our findings also suggest that Broad isoforms may be involved in the recruitment of epigenetic gene repressor PcG.

In chapter 4, we explored a phenomenon we observed in Dilp8 mutants. Since the discovery of Dilp8, it has been assumed that its role in regeneration was solely to provide the regeneration tissues with additional time to catch up with the development of undamaged tissues. In this chapter, we demonstrate that the tissues can still regenerate Dilp8 mutants. We determined that that hormone (ecdysone) signaling ensures regeneration is completed within the development period. Instead, the role of Dilp8 appears to be to ensure the adult viability of Drosophila following regeneration.

In these two chapters, I demonstrate that imaginal discs have a biphasic response to hormone levels. This biphasic response drives the tissue patterning and growth coordination before pre-pupa suppression of regenerative capacity. In Chapter 5, I discuss the mechanics of how the biphasic response could be achieved and the significance of Dilp8 for energy homeostasis.

CHAPTER 2: Methods and materials

2.1 Assessment of tissue regeneration

For our regeneration assessment assays, I primarily used X-irradiation to cause damage to the imaginal tissues. The regeneration outcomes and assessment methods differ slightly from those previously used for genetic damage due to eiger overexpression (Harris et al., 2016; Smith-Bolton et al., 2009).

2.1.A Adult wing phenotypes

A simple method to assess successful regeneration is the appearance of adult tissue following damage. Here I categorize the damaged wings into different categories depending on the damage location and damage combinations, Fig.2-1A. I find defects to the margin of the wing (edge of the wing) are most common following irradiation damage, and they can serve as an indicator of incomplete regenerative activity. Regeneration seems to occur from the hinge to the margin, as an incomplete repair often results in a margin with dents or missing chunks. In addition to margin defects, adult wings may show defects in the formation of veins and the orientation of wing hairs. Vein defects include misalignment and the presence of bubbles and breaks in the vein. In our experiments, hair alignment defects were marked by a general discoloration of the surrounding area. Lastly, a severe lack of regeneration may result in a reduction in wing size. I quantitatively characterized the wing size changes by measuring the wing area as depicted in Fig.2-1B.

Figure 2-1: Adult wing phenotypes and size quantification method

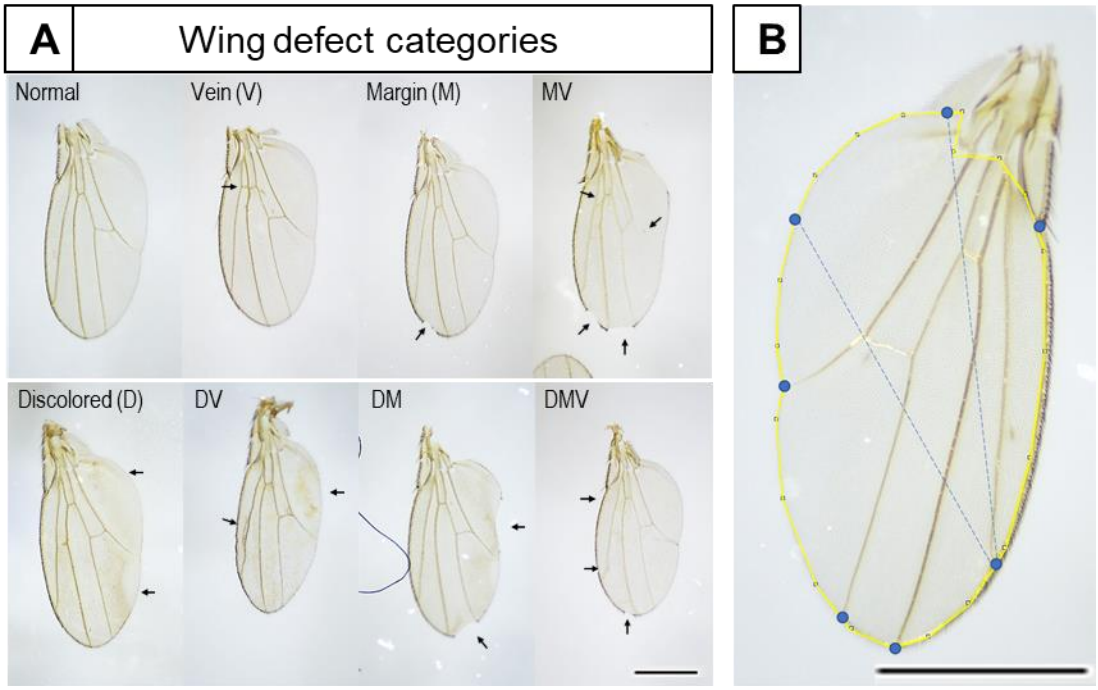

Figure 2-1: Adult wing phenotypes and size quantification method - (A) Examples of wings from irradiated and unirradiated larvae. The wings show the damage categories used to qualitatively assess wing regeneration. The data is represented as fractions of each phenotype in the whole wing population. (**B**) Representative image of wing area quantification method. The yellow line indicates the region of focus when measuring wing area. The blue dots indicate the points used when centroid calculation was used instead. Centroid points were located at margin-vein junctions and points extrapolated from wing landmarks. Centroid calculation could not be used on severely damaged wings. Scales=500cm. **Genotype** – (A-B): w^{1118}

2.1.B Pupariation time and developmental delay

Damaged imaginal discs produce *Drosophila* Insulin-like Protein 8 (Dilp8), which causes a delay in pupation timing (Colombani et al., 2012; Garelli et al., 2012). Unless saturated, the length of the delay is often equivalent to the amount of damage and regenerative activity in the organism (Hackney et al., 2012; Halme et al., 2010). We refer

to the difference in pupariation time between the experimental (damaged) and control groups as developmental delay. This difference may be quantified between undamaged and damaged tissues or between damaged tissues of different genetic backgrounds. In order to quantify the pupariation time, pupae in each vial were counted approximately every 12 hours, starting from the 104hAED timepoint and ending at least three days after the most recent pupation. For calculating purposes, the middle of the egg-laying interval (described in Section 2.2A) was considered as 0hAED. The data were pooled from multiple vials of the same genotype laid on the same day. Data from separate lays were calculated separately, and at least three lays were represented in each day's experiment. Median pupariation time was then calculated as shown in Equation 1.

Equation 1: *Median pupariation time calculation*

$$Median = T1 + ((T2 - T1) * \frac{0.5 - S1}{S2 - S1})$$

Median pupariation time was calculated by first determining the sum fraction of total pupae in a genotype at each time point. The first time point with a sum fraction of total pupae exceeding 50% indicates that the median pupariation time occurred between that point and the following point. I next calculated how long past the initial time point 50% of larvae pupated and the difference between the sum fractions. To determine how far past the first timepoint the median pupariation time was, I divided the difference from the mid-point by the difference between the sum fractions then multiplied this by the difference between time points. I added this number to the preceding time point. T2 indicates the later timepoint, T1 indicates the earlier timepoint, S2 indicates the sum fraction of pupae at T2, S1 indicates the sum fraction of pupae at T1.

2.1.C *Markers of regenerative activity*

Several genes undergo differential expression following damage, and some of these genes are essential to completing the regenerative response (Blanco et al., 2010; Katsuyama et al., 2015; Klebes et al., 2005). In our study, I use some of these essential genes as markers of regenerative activity.

***a) Drosophila Insulin-like Protein 8 (Dilp8)*:** Dilp8 is expressed following any perturbations in the development of imaginal discs. The native and induced expression of dilp8 was examined using an enhanced green fluorescent protein (eGFP) trap in the gene's first intron (Dilp8::GFP, BDSC_33079, (Garelli et al., 2012; Nagarkar-Jaiswal et al., 2015)), in which GFP is inserted into the *dilp8* locus, to assess the spatial and temporal expression of Dilp8 following damage. Using the reporter and confocal imaging, I see little to no expression of Dilp8 in undamaged wing discs (Fig.2-2A). However, following damage, I see a robust upregulation of Dilp8, especially in the wing pouch (Fig.2-2A'). Similarly, I see an increase in Dilp8 expression in the blastema following targeted genetic damage (Fig.2-2A''). I quantified changes in Dilp8 expression in the pouch using the fluorescence intensity of the GFP reporter. GFP fluorescence was quantified in the pouch region of the discs as defined by the outer edge of hinge Wg expression surrounding the wing pouch. The quantification and data analysis methods are described in section 2.2.E. In experiments where the Gal4-UAS system was used for gene over-expression or knockdown, I limited the Dilp8 quantification to the primary area of expression (Fig.2-2C). In quantification of each damage model, the expression of GFP in the notum was not quantified.

Figure 2-2: Markers of regenerative activity in the wing disc

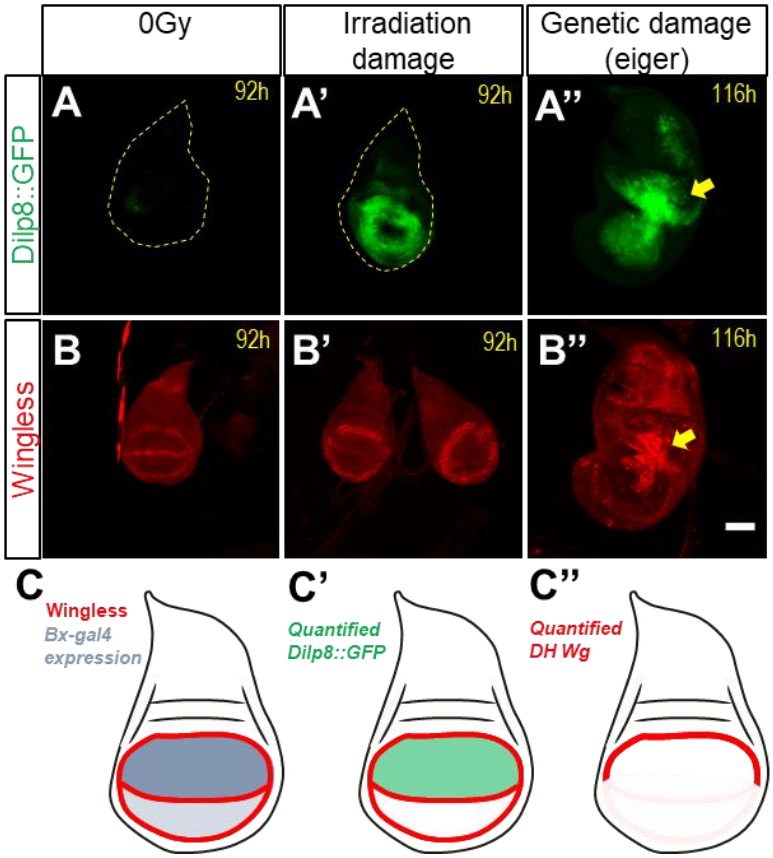

Figure 2-2: Markers of regenerative activity in the wing disc - (A) Representative images of wing imaginal discs expressing Dilp8 in undamaged (A), irradiation-damaged (A'), and eiger-damaged tissues (A''). Cells expressing Dilp8 are identified by the expression of GFP from the Dilp8::GFP transgene construct. **(B)** Representative images of wingless (Wg) expression in undamaged (B) irradiation-damaged (B') and eiger-damaged tissues (B''). Wg expression increases at the hinge and decreases at the margin following early irradiation damage (B'). Wg expression is high in the blastema (indicated by the yellow arrow, B''). **(C)** A representative figure of a wing disc showing an area of targeted gene expression using the Gal4-UAS system (C). *Bx-gal4* was most commonly used. Quantification of Dilp8 expression was focused on the area with the highest Bx expression (C'). Quantification of Wg was focused on the dorsal hinge, DH (C''). I traced the Wg expression area from the anterior margin-hinge intersection to the posterior margin-hinge intersection.

Genotypes – (A, A', B, B'): *dilp8*^{MI00727}/+, (A'', B''): Bx-Gal4/+; UAS-Eiger/+; *dilp8*^{MI00727}/+

b) ***Wingless (Wg):*** To further demonstrate changes in regenerative signaling with development in this study, I measured irradiation-induced Wingless (Wg) expression. A critical regenerative morphogen, Wg, is upregulated in the regeneration blastema following targeted damage (Smith-Bolton et al., 2009). I see that Wg is expressed in the hinge, margin, and nodum cells using IF (Fig.2-2B) or a gene reporter. In quantification of each damage model, the expression of Wg or GFP in the notum was not quantified, and blastema Wg was determined by the area of Dilp8 expression. In irradiation damaged tissues, Wg expression in the hinge region surrounding the wing pouch defines the radiation-resistant cells that contribute to regeneration(Fig.2-2B', (Verghese & Su, 2016)). In eiger damaged tissues, the blastema area shows high Wg expression (Fig.2-2B''). I quantified Wg in the dorsal hinge of the imaginal disc pouch by tracing Wg in this region from the dorsal edge of the margin (Fig.2-2C''). Margin Wg was not quantified, as is it not associated with regeneration and is instead transiently lost (Díaz-García & Baonza, 2013). The ventral hinge Wg was not quantified because tissue evagination or folding during mounting often interfered with distinguishing margin and ventral hinge Wg.

c) ***Tissue growth:*** In addition to DIlp8 and Wg, I also used tissue growth (represented by the entire wing disc area or wing pouch diameter) to supplement our analysis. Since compartmentalized gene expression often affects a specific region, overall tissue growth was not a suitable analysis parameter for such experiments. Therefore, I limited tissue growth assessment to experiments with whole disc or whole pouch effects. Pouch diameter was measured as the distance between the furthest points of the wing pouch parallel to the wing margin.

2.2 Experiment set up and data collection

The following sections describe the materials and methods used to set up the work in this dissertation. The methods were kept consistent throughout, and any deviations will be indicated in the figure legends.

2.2.A Drosophila Culture and Stocks

All experimental lines and crosses were maintained at 25^{o}C with 12-hour alternating light-dark cycles with a diet of standard (cornmeal-yeast-molasses) media (Archon Scientific B101). Since our experiments assess changes during developmental progression, it is essential to ensure that the development of the animals at each time point is equivalent. In order to achieve synchronized development, I restricted egg-laying to a designated 4-hour interval. The egg-laying (staging) was done on grape agar plates (Genesee Scientific). The middle of the staging period was considered as the 0hr of egg deposition. Twenty-four hours after egg deposition (AED), 20 1st instar (L1) larvae were transferred into vials or plates containing standard media. The larvae remained undisturbed in the media at 25^{o}C or 18^{o}C (for temperature-sensitive stocks) until experiment treatment conditions began in the third larval instar (L3).

The *Drosophila* lines used are: w^{1118} (BDSC_5905), Bx-Gal4;UAS-Dcr2; (Bilder lab stock), Dilp8::GFP/TM6B (Derived from BDSC_33079), UAS-LacZ.NZ (BDSC_3956), UAS-EcR.A^{W650A} (BDSC_9451), y^{1},br^{2Bc-2}/Binsn (BDSC_29969), y^{1},br^{npr-6}/Binsn (BDSC_36562), y^{1},br^{rbp-5}/Binsn (BDSC_30138), y^{1},br^{28},w^{1}/Binsn (BDSC_36565), FM7c^{tb} (BDSC_36337), UAS-BrZ1 (BDSC_51190), UAS-BrZ2 (BDSC_51191), UAS-BrZ3 (BDSC_51192), UAS-BrZ4 (BDSC_51193), UAS-Dcr2;UAS-BrRNAi (Derived from BDSC_27272), Bx-Gal4;UAS-Eiger; (Derived from H. Kanda's $regg^{1}$ stock),

wg[1],FRT40A;Dilp8::GFP/SM6-TM6B (Derived from BDSC_2978 and BDSC_33079), Bx-Gal4;*wg*DRE-GFP;dcr (Derived from Hariharan lab BRV118-GFP stock), UAS-mCD8-GFP,hsFlp;tub-Gal4;FRT82B,tubGal80/TM6B (from S. Siegrist), UAS-EcR.A^{W650A}; FRT82B/SM6-TM6B (Derived from BDSC_9451), Ubx-Flp;FRT40A;FRT82B (BDSC_42733), *wg*-LacZ,UAS-EcR.A^{W650A};FRT82B/CyO (Derived from BDSC_11205 and BDSC_9451). All stock genotypes used were derived from crosses of these stocks. The specific genotypes used are indicated in the figure legends.

2.2.B Irradiation damage and ecdysone feeding

The two primary larvae experimental treatments used in this dissertation were damage to larval tissues using X-irradiation and exogenous increase of systemic ecdysone by feeding larvae 20-hydroxyecdysone (20E). Both treatments were conducted on staged larvae cultured as described in 2.2.A, until developing to the desired early (80h AED) or late (104h AED) L3 developmental stage.

Tissue damage was caused by exposing larvae to 10Gy, 20Gy, or 25Gy X-irradiation generated from a 43805N X-ray system Faxitron operating at 130kV and 3.0mA. X-irradiation damages cells by causing double-stranded breaks in the DNA (Morgan & Sowa, 2005). The levels of ionizing irradiation we administer are relatively low and mainly induce apoptosis in the more irradiation-sensitive diploid cells (mainly in imaginal discs) (B. Zhang et al., 2014). The polyploid cells (almost all larval tissues) resist cell death and remain mostly unaffected (Nandakumar et al., 2020; B. Zhang et al., 2014).

20E is the active form of the ecdysone hormone used by most cells. 20E can be absorbed through the gut into the hemolymph of the larvae, there for feeding larvae, 20E

is sufficient to increase systemic levels of 20E in the larvae. 20E rich food was prepared by dissolving 20E (Sigma #H5142 – starting concentration: 20mg/ml in 95% ethanol) in standard food media at final concentrations of 0.1mg/ml, 0.3mg/ml, 0.6mg/ml and 1.0mg/ml. For 0mg/ml control vials, an equivalent volume of the 20E solvent (95% ethanol) was added to the food. Only 2ml of food was prepared per 20E experiment vial; therefore, the number of larvae transferred to these vials was limited to 6-7 larvae per vial(Halme et al., 2010)

2.2.C Tissue isolation

a) *Isolation of adult wings*: Approximately 36 hours after eclosion, adult *Drosophila* were separated according to the experimental condition and sex then stored in 70% ethanol. The wings were isolated by clipping as close to the thorax as possible. Without additional staining or tissue treatment, wings were mounted onto slides using Gary's Magic Mounting media (GMM – Balsam powder dissolved in methyl salicylate, (O'Dell, 1995)).

b) *Isolation of larvae wing discs for imaging:* To isolate the imaginal discs from larvae without damaging the tissues, I dissected the larva closer to the posterior end and gently inverted the larva body inside-out. The dissection and cleaning (removal of the fat body) of larvae bodies were done in PBS. The larvae carcass (cuticle with attached imaginal discs) was then fixed with 4% paraformaldehyde in PBS for 20 mins, followed by two 5-min washes in PBS. The tissues were then stored in PBS at 4oC until staining was done. In Section 3.2.C and 3.2.D (*broad* mutant experiments), only hemizygous male larvae were isolated. The broad gene is located on the X chromosome, and the mutations

used were recessive (DiBello et al., 1991; Kiss et al., 1988). Given our experimental cross set up, only the hemizygous males would exhibit the mutant phenotype. I identified the male larvae by their gonads located in the lower abdominal flanks (Fig.2-3). Male larvae gonads appear as circular translucent discs visible through the cuticle (Selva & Stronach, 2007).

Figure 2-3: Identification of male and female larvae using gonads

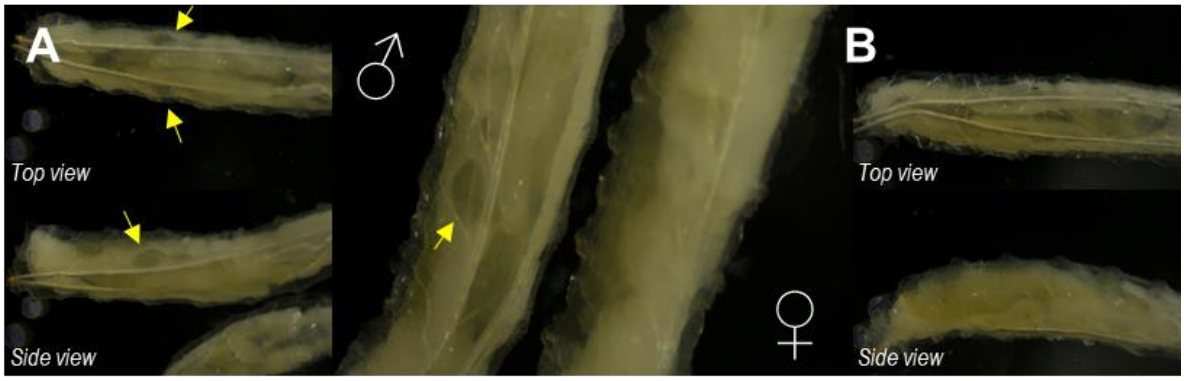

Figure 2-3: Identification of male and female larvae using gonads - The figure shows representative images of male (**A**) and female (**B**) L3 larvae at 92hAED. The middle image shows side by side male and female larvae for comparison. The male larvae have visible gonads on either side of the posterior-most third of their body. These circular structures (indicated by yellow arrows) are not found in female larvae. The gonads are often challenging to see in the early stages but become easy to see in late L3. **Genotypes** – w^{1118}

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c) *Isolation of larvae wing discs for western blot:* 40-80 wing discs (depending on tissue size at each time point) were isolated from L3 larvae. Due to tissue size limitations, isolation of wing discs started at 92hAED for undamaged larvae and 104hAED for damaged larvae. The wing discs were plucked off the inverted larvae carcass while fully immersed in chilled Schneider's Insect Medium (Sigma-Aldrich #S0146). The dissection

dish was also placed on ice to maintain the temperature of the Schneider's medium. The isolated wing discs were transferred to an Eppendorf tube, washed twice in chilled PBS by rinsing and spinning down for 15 seconds using a C1008-R Benchmark myFUGE mini centrifuge. Excess PBS was aspirated, then the tissues pellet (in approximately 50ul of PBS) was frozen in dry ice and stored at -80^{o}c.

2.2.D Immuno-Histochemistry

In order to not lose any tissues, all immunostaining was done with imaginal discs still attached to the carcass. The imaginal discs were permeabilized for immunofluorescent (IF) staining using two 10-min washes in 0.3% Triton in PBS (PBST), then incubated for 30 mins in a blocking solution – 10% goat serum (GS) and 0.1%PBST. The tissues were then incubated in primary antibody solutions overnight at 4^{0}C on a nutator. Antibody solutions were prepared in 10%GS in 0.1%PBST. The primary antibodies used most in this dissertation are mouse β-Gal (1:250; Promega#Z3781), mouse anti-Wingless (1:100; DSHB #4D4), and rabbit anti-GFP (1:1000; Torrey Pines Biolabs #TP401). The samples were washed in 0.3% PBST and blocked again before incubating in the appropriate secondary antibody solutions (1:1000; ThermoFisher Alexa488, Cy3, or Alexa633) prepared in 10% GS in 0.1% PBST for 2-4 hours at room temperature. After two 10-min 0.3% PBST washes and one 5-min PBS wash, the tissues were stored in 80% glycerol in PBS at 4^{o}C. For mounting, imaginal discs were isolated from the stained carcass and mounted on glass slides with Vectashield (Vector Laboratories).

2.2.E *Imaging, Quantification and Statistical Analysis*

Adult wings were imaged using MU530-Bi AmScope Microscope Digital Camera and software. Any additional high magnification images were taken using Leica MZ16F. The wing Confocal imaging was done using an Olympus FluoView1000 from the University of Virginia Department of Cell Biology and Zeiss LSM 700 and LSM 710 in the University of Virginia Advanced Microscopy Facility (RRID: SCR_018736). Laser power and gain settings for each set of stained samples were based on the experimental group with the highest fluorescence intensity in each channel and kept constant within the experiment. All confocal images were taken as z-stacks of 10um intervals.

Images were processed and quantified using Fiji/ImageJ. Adult wings were categorized according to damage features and quantified by measuring the wing area. The area measured is shown in Fig.2.1. Representative confocal images used in figures are composites of the image stacks using max fluorescence projection, while quantification was done using sum fluorescence composites. I quantified the fluorescence intensity using integrated density which takes into account both the area and the mean fluorescent expression within the area of interest

Prism 8 software was used for the statistical analysis. The specific tests used are listed in the figure legends. To compare between independently repeated experiments, I normalized within the experiment as indicated in figure legends.

2.2.F *Western Blot*

Proteins were extracted in 50ul SDS lysis buffer (2%SDS, 60mM Tris-Cl pH6.8, 1X protease inhibitors, 5mM NaF, 1mM Na orthovanadate, 1mM β glycerophosphate in

dH_2O), sonicated using two 5-sec pulses (microtip Branson sonifier), boiled for 10 minutes at 95^0C, and centrifuged at 15000rpm for 5 mins at RT. The supernatant was collected for BCA assay and analyzed by SDS-PAGE using Mini-Protean® TGXTM 4–15% (BioRad) and transferred to nitrocellulose membranes. For Western blot analysis, membranes were incubated with blocking solution (1% cold water fish gelatin; Sigma#G7765), primary antibodies (1:500 Broad Core, DSHB#25E9.D7 and 1:10,000 a-tubulin, Sigma#T6074), followed by appropriate LI-COR IRDye® secondary antibodies and visualized using the Li-COR Odyssey® CLx Imaging System. Quantifications were done using LI-COR Image Studio™ Software.

CHAPTER 3: Hormone signaling suppresses regeneration via Broad transcription factors

Abstract

As tissues develop, their regenerative capacity is often diminished. In *Drosophila* melanogaster, imaginal discs (larval precursors to adult tissues) lose the ability to regenerate near the end of larval development. This loss of regenerative capacity coincides with an increase in systemic levels of the steroid hormone ecdysone, a key coordinator of *Drosophila* developmental progression. Experimentally increasing systemic ecdysone levels by feeding larvae ecdysone limited regeneration that was observed in adult tissues. To determine how ecdysone impacts regenerative signaling, I looked at downstream targets of ecdysone. I find that the expression of broad (br) splice variants (Z1-Z4), early prepupal ecdysone signaling targets, coincides with regeneration restriction in wing discs. I determined that the expression of *br* splice variants is necessary for regeneration restriction. Loss of Br expression using Br^{RNAi} or variant-specific mutants allows activation of essential regeneration genes, wingless (*wg*) and dilp8, past the regeneration restriction timepoint. In addition, loss of br allows for increased and extended activation of regeneration pathways. By overexpressing the variants early in imaginal disc development, I determined that *brZ1*, *brZ2,* and *brZ4*, limit regenerative activity. Sequential expression of Br isoforms coordinates the end of regeneration with the end of larval development. Our findings provide insight into how endocrine signals regulate the regenerative competence of cells.

3.1 Introduction

In most animals, regenerative capacity is lost with developmental progression(Seifert & Voss, 2013; Yun, 2015). Often, loss of regenerative capacity coincides with changes in systemic hormone signaling. For example, loss of regenerative capacity in the heart tissues of *Xenopus laevis* and mice is preceded by a sharp increase in systemic thyroid hormone levels (Hirose et al., 2019; Marshall et al., 2019). Similarly, *Drosophila melanogaster* imaginal discs (larval precursors to adult tissues) lose regenerative ability near the end of larval development (Halme et al., 2010), coinciding with an increase in systemic levels of the steroid hormone ecdysone, a key coordinator of *Drosophila* developmental progression (Burdette, 1962; Yamanaka et al., 2013). Pulses of ecdysone throughout *Drosophila* lifespan initiate signaling cascades that activate developmental transitions such as larval molts, pupation, and metamorphosis. Therefore, regulating systemic levels of ecdysone is a crucial part of the *Drosophila* regenerative response. Regenerating imaginal discs synthesize and release the relaxin hormone *Drosophila* insulin-like peptide 8 (Dilp8), which signals to the brain and endocrine organs through Lgr3 receptor to limit ecdysone synthesis (Colombani et al., 2012, 2015; Garelli et al., 2012, 2015; Jaszczak et al., 2016; Vallejo et al., 2015). Reduced ecdysone production delays the transition to pupa, providing damaged imaginal discs additional time to regenerate (Halme et al., 2010).

During the L3, one of the earliest targets of the increasing ecdysone signaling is the Broad splice isoform transcription factors (Crossgrove et al., 1996). The spatially and temporally ordered expression of Broad (Br) and the isoform-specific DNA-binding domains allow ecdysone signaling to initiate the tissue-specific cascade of gene

expression and suppression (Crossgrove et al., 1996; D'Avino et al., 1995; Von Kalm et al., 1994). Genetic gain-of-function and loss-of-function experiments in *Drosophila* show that Broad isoforms activate genes specific to the pupal development and suppress larval and adult development programs (Crossgrove et al., 1996; D'Avino et al., 1995; Lynn M. Riddiford et al., 2003; B Zhou & Riddiford, 2001). Broad isoforms have a BTB/POZ domain, allowing for protein-protein interaction to recruit other factors to their target sites. One such interaction is with a subunit of the PcG Repression Complex 1, PRC1 (Lv et al., 2016). This Br-PRC interaction is crucial as loss of regenerative capacity in *Drosophila* is due to the epigenetic-silencing of the "Damage Responsive Maturity-Silenced" enhancers (DRMS or DRE (Harris et al., 2020)). These recently identified DRE are within the regulatory loci of essential regeneration genes such as *wingless* (*wg*), which is necessary for initiating regenerative activity, and MMPs that assist with tissue restructuring during regeneration (Harris et al., 2016, 2020). The DRE loci show H3K27me3 modification by PRC2 and require PRC1 for their silencing at the RRP (Harris et al., 2016). However, it remains unclear how PRC2 is recruited to the suppression sites. Deleting the predicted Pleiohomeotic (Pho) binding site, a presumptive PRC2 targeting site, does not relieve developmental silencing of the *wg*DRE-reporter (Harris et al., 2016). There appears to be a yet unknown factor that recruits the PRC to the DRE locus. I hypothesize that Br recruits PRC to regeneration gene loci, limiting the regenerative capacity of late larval tissues.

Here, I describe how ecdysone signaling cell-autonomously suppresses regenerative activity at the end of larval development. As ecdysone levels increase for pupation, sequential expression of Br splice isoforms is initiated. BrZ1, BrZ2, and BrZ4 coordinate

the suppression of ongoing regenerative activity and the tissue's overall regenerative capacity. They do this by actively suppressing the *wg*DRE. In addition, I show that there is genetic interaction between Broad and PRC.

3.2 Results

3.2.A Regenerative capacity is lost at the end of the larval development

To determine how ecdysone signaling regulates regenerative activity, I assessed how developmental timing and changes in ecdysone titer regulate regenerative outcomes following X-irradiation damage to wing imaginal discs. *Drosophila* larvae exposed to X-irradiation during early third larval instar (L3 - 80hAED @ 25^{o}C) can regenerate their wing tissues almost entirely, with only a few adult wings from irradiated larvae exhibiting minor defects (Fig.3-1A, 2-1A). The regenerated adult wings also match the size of undamaged control wings (Fig.3-1B). In contrast, larvae irradiated later, almost prepupal stage (late L3 – 104hAED @ 25^{o}C) produced adult wings with a greater frequency of malformations in wing veins and margin (Fig.3-1A) and failed to match the undamaged wings size (Fig.3-1B). These differences in regenerative capacity correlate with the ability to activate the regenerative checkpoint. Irradiation at 80hAED produces a robust checkpoint activation and developmental delay, whereas irradiation at 104hAED fails to activate the regeneration checkpoint and delay (Fig.3-1C). These results are consistent with previous observations that identified this regeneration restriction point (RRP), a developmental time point when damage no longer activates the regenerative response (Halme et al., 2010; Smith-Bolton et al., 2009).

This transition through the RRP impacts regenerative signaling in damaged wing discs, as seen in the damage-induced expression of *dilp8*. Dilp8 is a critical regulator of the systemic response to regeneration and a valuable marker for regenerative activity in damaged tissues. Consistent with the reduced regenerative activity, I observe reduced

activation of *dilp8* expression (Dilp8::GFP) in wing discs damaged at progressively later times in larval development (Fig.3-1D).

Figure 3-1: Regenerative capacity is restricted with developmental progression

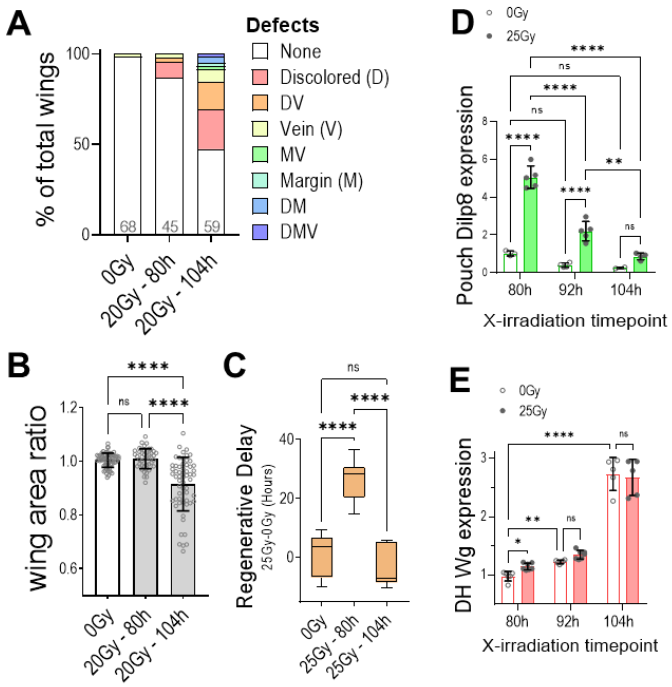

Figure 3-1: Regenerative capacity is restricted with developmental progression - **(A**) Adult wings with single or combination defects increase following late damage. The graph shows the percentage of defective wings undamaged (0Gy), early damaged (25Gy-80h), and late damaged (20Gy-104h) from larvae. Population size is indicated in the graph. **(B)** Quantification of adult wing size following 0Gy, 20Gy-80h, and 20Gy-104h. The wing size (unit area) is normalized to the undamaged wing size of the respective sex. One-way ANOVA with Tukey's test, ****p<0.0001. **(C)** Quantification of regenerative delay in 0Gy, 20Gy-80h, and 20Gy-104h. One-way ANOVA with Tukey's test, ****p<0.0001. **(D)** Quantification of relative Dilp8::GFP expression in wing pouch; normalized to GFP expression in undamaged-92h AED tissues. **p<0.01, ****p<0.0001, two-way ANOVA with Tukey's test. **(E)** Quantification of relative Wg expression in Dorsal Hinge (DH); normalized to DH Wg expression in undamaged-92h AED tissues. *p<0.05, **p<0.01, ****p<0.0001, two-way ANOVA with BK&Y comparisons test.

Genotypes – (A-C, E): w^{1118}, (D) *dilp8*^{MI00727}/+

To further demonstrate changes in regenerative signaling with development in this study, I measured irradiation-induced Wingless (Wg) expression. A critical regenerative morphogen, Wg, is upregulated in the regeneration blastema following targeted damage (Smith-Bolton et al., 2009). In irradiation damaged tissues, Wg expression in the hinge region surrounding the wing pouch defines the radiation-resistant cells contributing to regeneration (Verghese & Su, 2016). Upon quantifying Wg expression in the dorsal hinge of irradiated larvae, I find that irradiation pre-RRP (80hAED) produces a significant increase in Wg expression, which is no longer observed as larvae transit the RRP (Fig.3-1E). These results demonstrate that the loss of regenerative response seen as larvae transit the RRP is accompanied by an inability to activate the expression of Wg and Dilp8, critical mediators of local and systemic regenerative processes.

3.2.B Ecdysone limits regeneration and is necessary for regeneration suppression at the Regeneration Restriction Time-point (RRT)

As larvae approach the L3/pupa transition, circulating levels of ecdysone increase rapidly and promote the exit from the larval period(Lavrynenko et al., 2015; Rewitz et al., 2013). To determine whether increased ecdysone titer is sufficient to limit regenerative activity in wing discs, I ectopically increased ecdysone levels by feeding larvae food containing 20-hydroxyecdysone (20E), an active form of this steroid hormone. Larvae damaged pre-RRP (80hAED) and fed 0.3mg/ml 20E no longer completely regenerate their imaginal discs but instead produce malformed (Fig.3-2A) and smaller (Fig.3-2B) adult wings. Furthermore, feeding low levels of 20E (0.1mg/ml) to larvae irradiated post-RRP (104hAED) produces a synergistic increase in adult wing malformations (Fig.3-2C)

and suppression of regenerative growth (Fig.3-2D). Together these observations support a model that the increasing levels of systemic ecdysone signaling at the end of larval development suppress regenerative signaling and growth in wing imaginal discs.

Figure 3-2: Ecdysone feeding exacerbates damage phenotypes

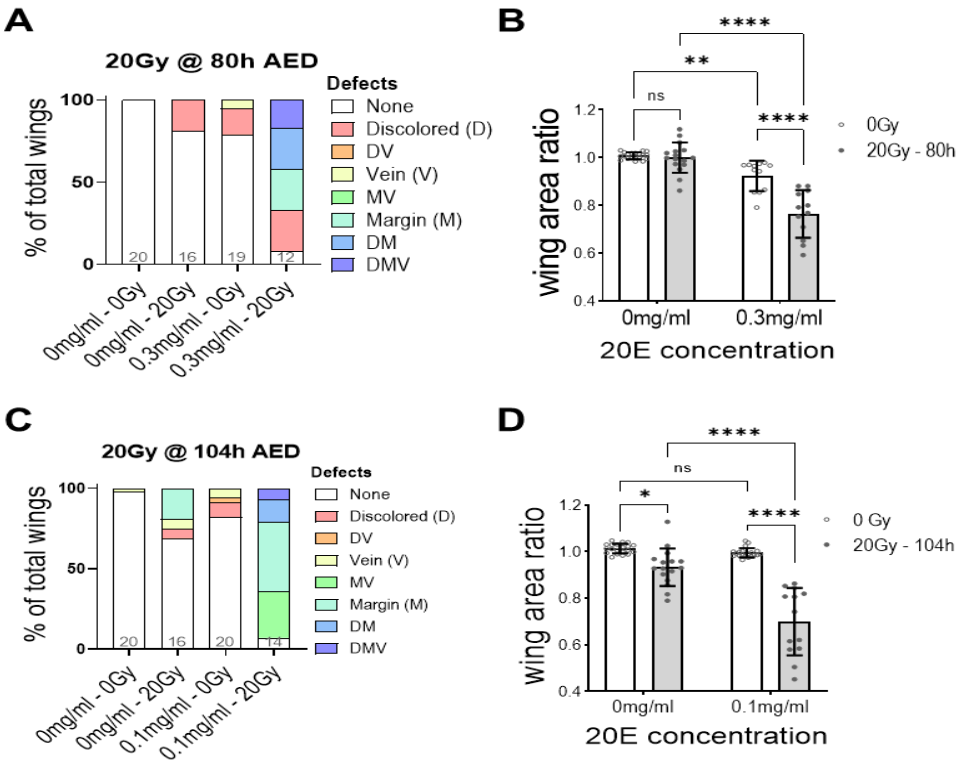

Figure 3-2: Ecdysone feeding exacerbates damage phenotypes - The percentage of adult wings with single or combination defects increases following ecdysone feeding. **(A)** The graph shows the percentage of defective adult wings following no damage (0Gy) and early damage (20Gy-80h) with 0mg/ml or 0.3mg/ml 20E feeding. **(B)** Quantification of adult wing size following 0Gy and 20Gy-80h with 0mg/ml and 0.3mg/ml 20E feeding. Data normalized to the size of the undamaged wing of respective sex. **p<0.01, ****p<0.0001, one-way ANOVA with Tukey's test. **(C)** The graph shows the percentage of defective adult wings following no damage (0Gy) and late damage (20Gy-104h) with 0mg/ml or 0.1mg/ml 20E feeding. **(D)** Quantification of adult wing size following 0Gy and early damage with 0mg/ml or 0.3mg/ml 20E feeding. Data normalized to the size of the undamaged wing of respective sex. *p<0.05, ****p<0.0001, one-way ANOVA with Tukey's test. **Genotypes** – (A-D): w^{1118}

Although the 20E feeding experiments above demonstrate that increasing systemic ecdysone limits the regeneration observed in adult wing tissues, it remained unclear whether ecdysone signaling acts directly on regenerating tissues to suppress regenerative activity or indirectly through other tissues. To test the tissue-autonomous requirement for ecdysone signaling in regenerating wing discs, I expressed a dominant-negative allele of the ecdysone receptor (Cherbas et al., 2003) in the dorsal compartment of the wing pouch using *Beadex*-driven, Gal4-UAS expression (*Bx>EcR.A*^{DN}, Fig.2-2C). After the RRP, regeneration-induced expression of the Dilp8 is limited (Fig.3-1D, 3-3A,C), reflecting the reduced regenerative activity in these tissues. However, I see that targeted inhibition of ecdysone signaling in the dorsal wing pouch significantly increases *dilp8* expression in larvae damaged at 104hAED (Fig.3-3A,C), suggesting that the regenerative Dilp8 expression in these post-RRP tissues is limited by ecdysone signaling.

I also examined the damage-induced expression of Wg at the dorsal hinge region of the wing pouch (Fig.2-2C''). Inhibition of ecdysone signaling in the dorsal pouch leads to an overall decrease in Wg expression at the hinge in undamaged tissues (Fig.3-1E, 3-3A,D), an observation I address more specifically later in Chapter 4. I know that damage in the regeneration incompetent discs post-RRP induces no change in Wg expression at the dorsal hinge (Fig.3-1E, 3-3A,D). However, in contrast to control discs, limiting ecdysone signaling in the wing discs permits a damage-induced increase in dorsal hinge Wg expression in post-RRP wing discs (Fig.3-3B,D). This increase in Wg expression is similar to what I see in regeneration competent discs pre-RRP (Fig.3-1E). These data demonstrate that at the end of larval development, ecdysone signaling acts tissue-

autonomously in wing discs to suppress critical local (hinge-Wg upregulation) and systemic (*dilp8* expression) signaling events associated with regeneration.

Figure 3-3: Ecdysone signaling is necessary for the suppression of regenerative activity at the RRP

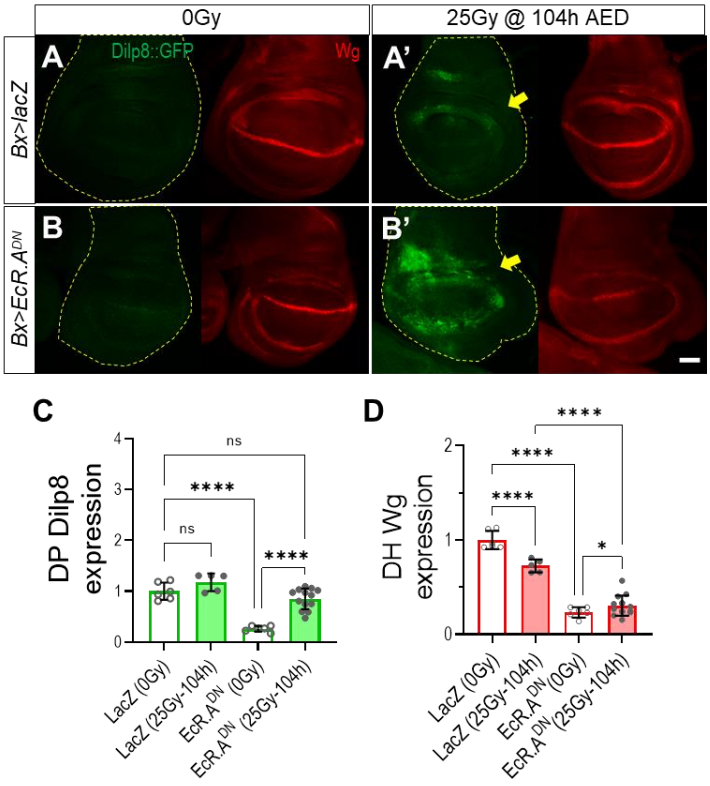

Figure 3-3: Ecdysone signaling is necessary for the suppression of regenerative activity at the RRP - (A-B) Representative images of pouch Dilp8::GFP (green) and dorsal hinge Wg (red) expression in 116hAED wing imaginal discs expressing lacZ (*Bx>lacZ)* as a control (A-A') or EcR.A^{DN} (*Bx>EcR.A^{W650A}*, B-B') in the dorsal wing pouch region, indicated by yellow arrows. Tissues were undamaged (A and B) or damaged late, 25Gy@104hAED (A' and B'), then isolated 12 hours after damage timepoint. Scale bar = 50um. **(C-D)** Quantification of dorsal pouch Dilp8::GFP (C) and DH Wg expression (D) in 116h AED late damaged (25Gy-104h) and undamaged (0Gy), *Bx>lacZ* and *Bx>EcR.A^{DN}* wing discs. Data normalized to *lacZ (0Gy)*, *p<0.05, ****p<0.0001, one-way ANOVA with Tukey's (Dilp8) and DYK (Wg) comparisons tests. **Genotypes** – (A-A'): Bx-Gal4/+; UAS-Dcr2/+; *dilp8*^{MI00727}/UAS-LacZ.NZ, (B-B'): Bx-Gal4/+; UAS-Dcr2/UAS-EcR.A^{W650A}; *dilp8*^{MI00727}/+, (C-D): genotypes in A-B

3.2.C *Expression of broad isoforms coincides with and is necessary for the regeneration restriction point (RRP)*

To determine how ecdysone limits regenerative signaling, I examined the expression of one of the downstream targets of ecdysone signaling, the BTB-POZ family transcription factors, Broad. Broad is one of the earliest targets of the prepupal ecdysone pulse. Splice isoforms of the transcription factor *broad* – (*brZ1, Z2, Z3, and Z4*), named after their respective zinc finger domains ((Bayer et al., 1996; DiBello et al., 1991; Kiss et al., 1988); Fig.3-4B), determine the tissue-specific and temporally-regulated signaling events that are initiated in response to ecdysone (Emery et al., 1994; Von Kalm et al., 1994). In addition, BrZ1 has recently been shown to antagonize Chinmo expression in the wing disc, limiting regenerative activity (Narbonne-Reveau & Maurange, 2019). Using western-blotting of wing disc-derived lysates (Fig.3-4A) and immunofluorescence with Broad-targeting antibodies (data not shown[1]), I specifically characterized the spatial and temporal distribution of Broad expression during wing development. Broad splice isoforms are expressed in all wing disc cells throughout L3 development. Based on their distinct molecular sizes (Emery et al., 1994), I determined that BrZ2 is expressed throughout the L3 stage, but its levels increase as larvae approach pupation. While I could not distinguish BrZ1 and BrZ3 based on size, previous studies have demonstrated a general lack of BrZ3 expression in pre-pupa imaginal discs (Emery et al., 1994). In addition, I could verify the emergence of BrZ1 expression in the tissue using Z1-targeted IF (data not shown). The expression of both BrZ1 and BrZ4 can be detected at 104hAED and dramatically increases as larvae approach pupariation. Following early damage (80hAED), the

[1] Data can be found online: https://www.biorxiv.org/content/10.1101/2021.08.12.456119v1

expression of the Broad isoforms is delayed (Fig.3-4A). Therefore, the expression of Broad isoforms corresponds to the known changes in ecdysone levels during larval development and regeneration, and increases in Broad expression correlate with loss of regenerative capacity.

Figure 3-4: Broad isoforms are expressed at the RRP

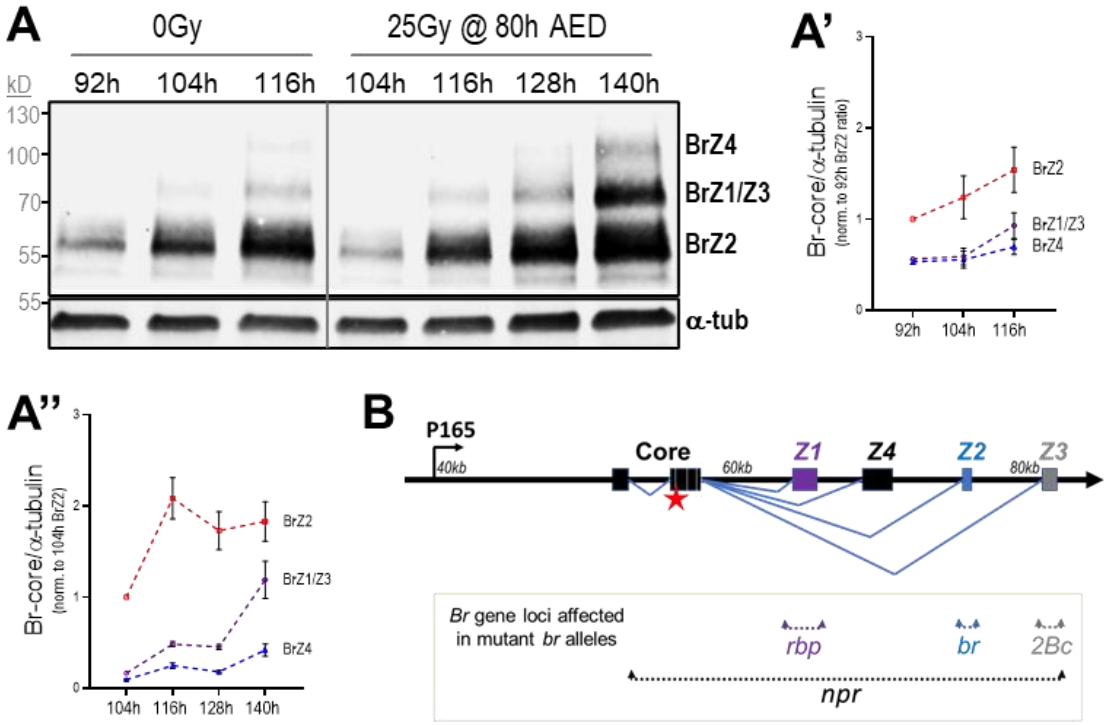

Figure 3-4: Broad isoforms are expressed at the RRP - (A) Western Blot (A) time course of Broad isoform expression in undamaged and early damaged w^{1118} wing imaginal discs. Tissues were isolated in 12-hour intervals. Broad core antibody was used to visualize BrZ4 (~110kD), BrZ1/3 (~90kD), and BrZ2 (~55-65kD), while and a-tub (~50kD) as a loading control. Protein size (kD) ladder - on the left. Quantification of Broad expression in undamaged (A') normalized to 92hAED BrZ2 expression, n=5. Quantification of Broad expression in damaged (A") wing discs normalized to 104hAED BrZ2 expression, n=3. **(B)** Simplified representation of the *br* gene, starting from P120 promoter (0kb - not included in the diagram) to the end of the *br*-encoding locus. The diagram shows alternate splicing targets for br isoforms and the complementation of

their mutant alleles: *npr* (full *br* gene), *rbp (Z1)*, *br (Z2),* and *2Bc (Z3)*. Red star indicates UAS-br^{RNAi} (FBgn0283451) target. **Genotypes** – (A-A"): w^{1118}

To determine whether Broad isoforms participate in the suppression of regenerative capacity at the end of larval development, I examined the effect of isoform-specific or pan-isoform disrupting zygotic *br* mutants (in hemizygous males) (Fig.3-4B, 3-5A-G) or pan-isoform targeting br^{RNAi} expression (data not shown[2]) on regenerative signaling. Loss of all Broad isoforms in npr^{6} (Fig.3-5B',F) or $Bx>br^{RNAi}$ wing discs allows post-RRP discs to express *dilp8*. Using the isoform-specific alleles, I determined that BrZ1 and BrZ2 are necessary for restricting *dilp8* expression at the RRP (Fig.3-5C',D'). Our BrZ3-specific allele $br(2Bc^{2})$ produced little effect on *dilp8* expression at the RRP, consistent with BrZ3 playing a limited role in wing development (Fig.3-5E). I was unable to obtain *BrZ4*-specific mutants to examine BrZ4 loss-of-function phenotypes.

Examining Wg expression in the wing discs of *br* mutants showed additional effects of these mutations on both developmental and regenerative Wg expression. In the npr^{6} mutant, which disrupts all the broad isoforms, I observe a substantial Wg expression reduction in undamaged tissues' hinge region (Fig.3-5B). This reduction is similar to what is seen in the Z2-specific allele br^{28} (Fig.3-5D). In contrast, in rbp^{5} mutant discs where the Z1 isoform is specifically disrupted, the hinge expression of Wg is mainly normal in undamaged discs, but the expression pattern of Wg in the margin is disrupted (Fig.3-5C illustrates a representative example). This phenotype may reflect the role of Broad in regulating Cut expression at the margin (Jia et al., 2016), but I leave the further

[2] Data can be found online: https://www.biorxiv.org/content/10.1101/2021.08.12.456119v1

examination of this phenotype for later studies. When I examine tissues damaged after the RRP, I see that the *rbp*^{5} mutation disabling the Z1 isoform produces a substantial increase in damage-induced Wg expression in the hinge compared to its undamaged controls (Fig.3-5G).

Figure 3-5: Br isoforms are necessary to restrict regenerative activity at the RRP

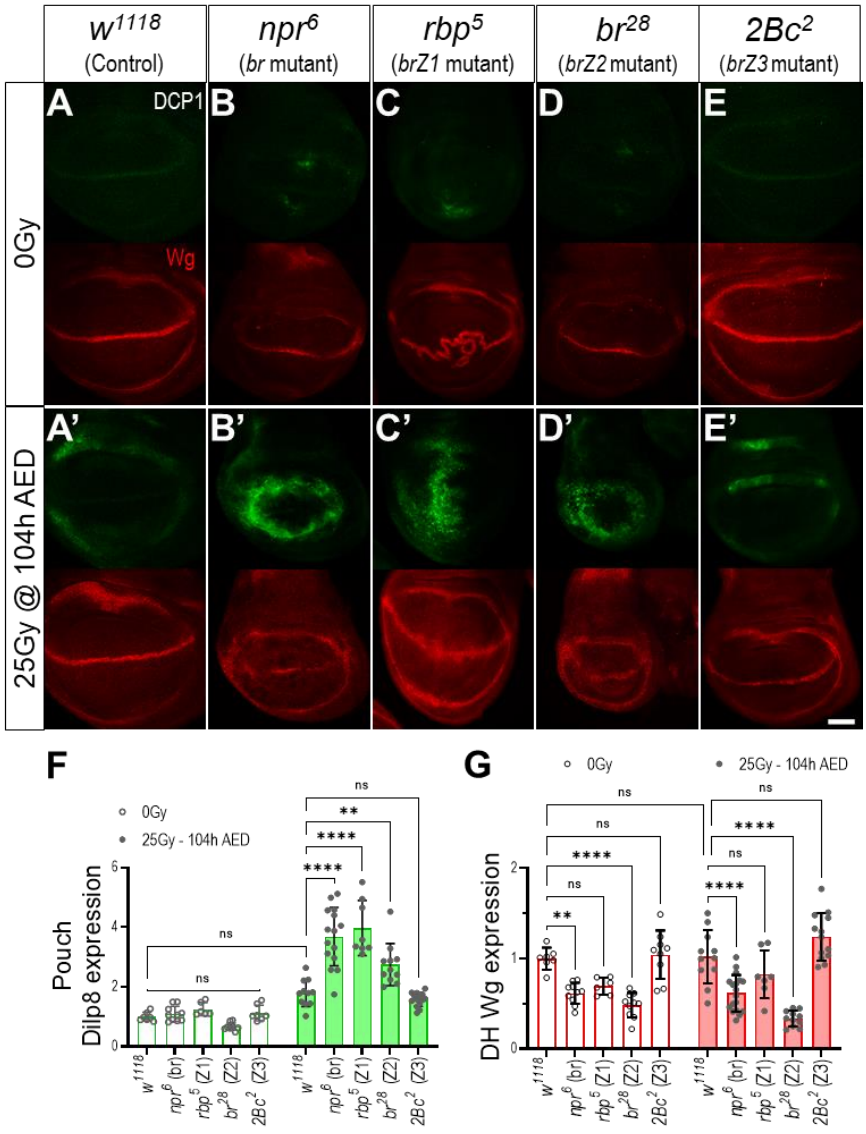

Figure 3-5: Br isoforms are necessary to restrict regenerative activity at the RRP - (A-E). Loss of Broad isoforms allows for activation of regenerative activity post-RRP. Images

show representative examples of pouch Dilp8::GFP (green) and dorsal hinge Wg (red) expression in 116hAED wing discs of undamaged (A-E), and late damage – 25Gy @ 104hAED (A'-E'), control – w^{1118} (A-A'), and *br* mutants: full *br* mutant - npr^{6} (B-B'), *brZ1* mutant - rbp^{5} (C-C'), *brZ2* mutant - br^{28} (D-D'), and *brZ3* mutant - $2Bc^{2}$ (E-E'). **(F-G)** Quantification of regenerative activity in 116h AED tissues using pouch Dilp8::GFP expression in the (F) and dorsal hinge (DH) Wg expression (G) in w^{1118} and *br* mutants wing imaginal discs. All quantifications normalized to undamaged w^{1118}, **p<0.01, ****p<0.0001, two-way ANOVA with Tukey's test.

Genotypes – (A) w^{1118}; +/+; $dilp8^{MI00727}$/+, (B): br^{npr-6}; +/+; $dilp8^{MI00727}$/+, (C): br^{rbp-5}; +/+; $dilp8^{MI00727}$/+, (D): br^{28}; +/+; $dilp8^{MI00727}$/+, (E):br^{2Bc-2}; +/+; $dilp8^{MI00727}$/+, (G-H): genotypes in A-E

In contrast, the Z2-specific mutation br^{28} and the pan-isoform mutation npr^{6} do not produce a significant fold increase in Wg expression following post-RRP damage. I also observe a slight increase in damage-induced Wg expression in Z3-specific mutant $2Bc^{2}$ (Fig.3-5E,G). This increase may reflect a non-autonomous effect of BrZ3 mutation on the regenerating disc. Unfortunately, I could not evaluate whether the increased regenerative signaling activity observed in *br* mutants led to improved tissue repair in adult tissues. All *br* mutants used were either non-pupariating or pupal lethal (D'Avino et al., 1995; Kiss et al., 1988). However, our experiments demonstrate that Broad isoforms mediate the ecdysone-dependent restriction of regenerative signaling in post-RRP L3 wing discs.

3.2.D Broad isoforms restrict the extent and duration of regenerative activity

Since some Broad isoforms are expressed in regenerating tissues (Fig.3-4A), I wanted to determine how the loss of Broad or specific Broad isoforms might affect regenerative activity following early damage (80hAED) of discs. In early damaged discs,

I observe that the pan-isoform mutant *npr*^{6} and the Z2 specific mutant *br*^{28} produce higher *dilp8* expression 12-hours following damage (Fig.3-6A-D,E, 3-7A). In addition to differences in the levels of *dilp8* expression, I also observe substantial differences in the duration of *dilp8* expression between the different mutants, with the pan-isoform mutant *npr*^{6} and the Z1-specific mutant, *rbp*^{5}, producing a more extended *dilp8* expression period compared with control discs, or the Z2-specific mutant *br*^{28} (Fig.3-6A-D, 3-7A). These results suggest that following early damage when the wing disc can initiate a regenerative response, the amount and duration of *dilp8* expression in the disc is regulated by specific Broad isoforms. I confirmed this result by using *br*^{RNAi} to inhibit all the Broad isoforms and demonstrated that *Bx>br*^{RNAi} discs also produce extended *dilp8* expression following damage (Fig.3-7E-G). In contrast to Dilp8 expression, the effects of the Broad isoform mutants on Wg expression during regeneration are less apparent. As described above, the pan-isoform mutant *npr*^{6} and the Z2-specific mutant *br*^{28} produce reduced levels of Wg at the hinge region in undamaged tissues (Fig.3-6A-D,F, 3-7C). However, all the mutants can produce a similar relative increase in Wg expression following damage (Fig.3-7D). Finally, Broad isoforms may also regulate the early events associated with either damage or the initial regenerative response, as I see that the reduction of wing pouch (and overall disc) size following irradiation damage at 80hAED is much greater in all isoform mutants, especially Z1-specific *rbp*^{5} and Z2-specific *br*^{28} mutant discs (Fig.3-6A-D,G, 3-7B).

In summary, our loss-of-function analysis demonstrates that the individual Broad isoforms play distinct roles in regulating the regenerative signaling response of wing discs damaged post-RRP. In addition, the Broad isoforms also regulate the extent and duration

of Dilp8 signaling produced by discs damaged before the regeneration restriction point. However, the Broad isoforms show distinct roles in regulating Wg expression. Based on these observations, I conclude that ecdysone signaling through Broad is necessary to limit both the tissue's competence to produce a regenerative response, as well as the duration of that response.

Figure 3-6: Br isoforms regulate the timing and duration of the regenerative response (Part A)

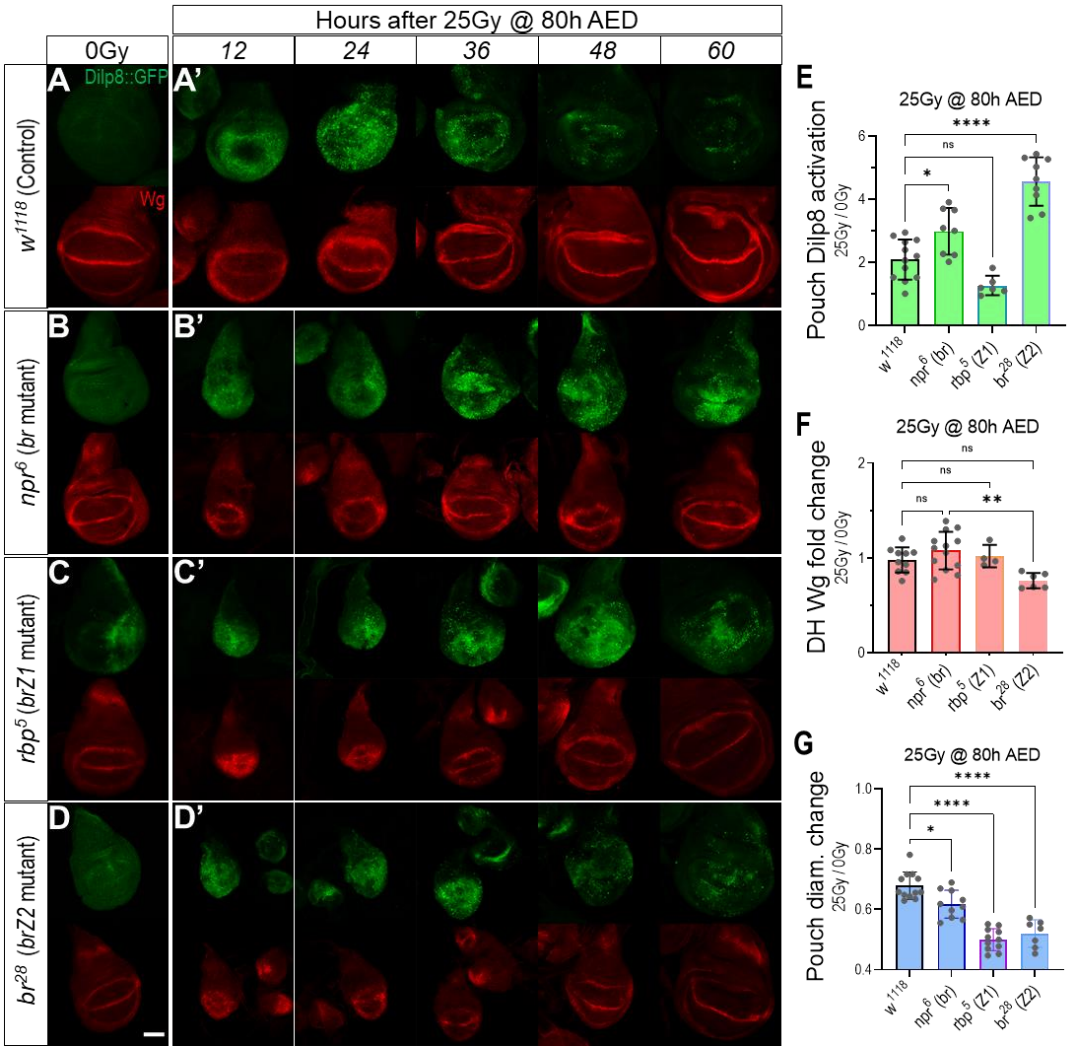

Figure 3-6: Br isoforms regulate the timing and duration of the regenerative response (Part A) - (A-D) Time-course images of Dilp8::GFP (green) and Wg (red) expression

following early damage (25Gy @ 80h AED). Representative undamaged tissues, 12hr after damage timepoint, are shown in A-D. Damaged tissues isolated in 12hr intervals are shown in A'-D'. Tissues used are control – w^{1118} (A-A'), and *br* mutants: total *br* mutant - npr^{6} (B-B'), *brZ1* mutant - rbp^{5} (C-C'), and *brZ2* mutant - br^{28} (D-D'). Scale bar=50um. **(E-G)** Fold change in Dilp8 expression (E), Wg expression (F), and pouch diameter (G) following early damage in w^{1118} controls and *br* mutants: **p<0.01, ****p<0.0001, one-way ANOVA with Tukey's test.

Genotypes – (A) w^{1118}; +/+; $dilp8^{MI00727}$/+, (B): br^{npr-6}; +/+; $dilp8^{MI00727}$/+, (C): br^{rbp-5}; +/+; $dilp8^{MI00727}$/+, (D): br^{28}; +/+; $dilp8^{MI00727}$/+, (E-G): genotypes in A-D

Figure 3-7: Br isoforms regulate the timing and duration of the regenerative response (Part B)

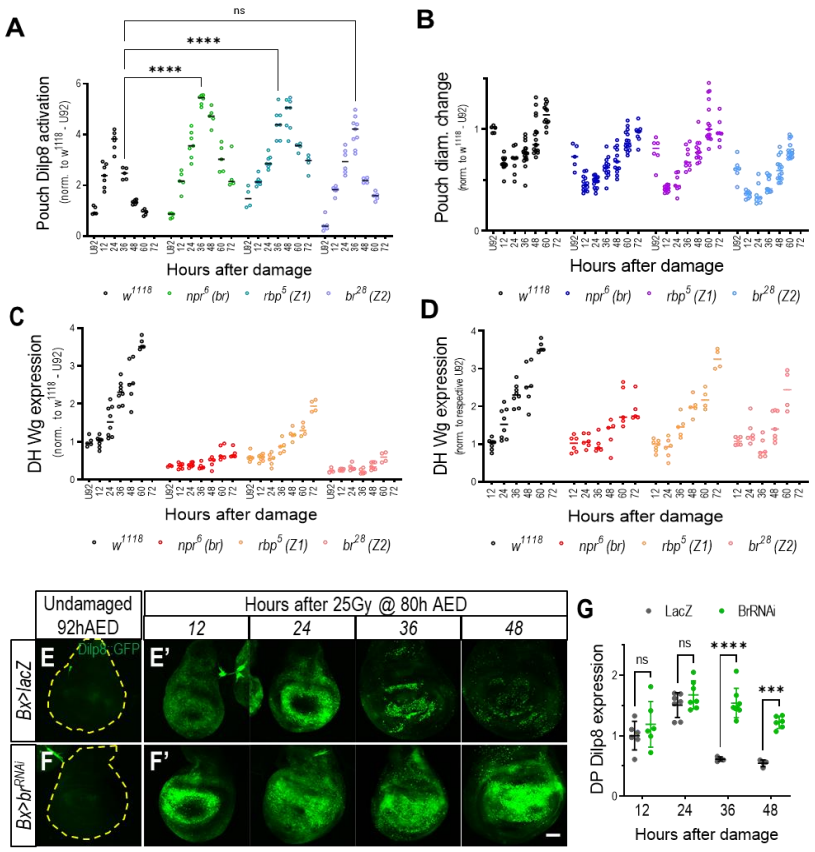

Figure 3-7: Br isoforms regulate the timing and duration of the regenerative response (Part B) - (A-D) Relative expression of Dilp8::GFP (A), pouch diameter (B), and dorsal hinge (DH) Wg expression (C-D). U92's (undamaged 92h AED) are also included in the graph. Data normalized to w^{1118} U92 in (A-C) and respective U92 in (D). 72h npr^{6} and rbp^{5} tissues

are also included in the graph. **(E-F)** Representative images of Dilp8 expression in undamaged (0Gy – E and F) and time-course following early damage (25Gy @ 80h AED – E' and F') in *Bx>lacZ* (E-E') and *Bx>br*^{RNAi} (F-F') tissues. Tissues were isolated in 12-hour intervals following damage. **(G)** Quantification of Dilp8::GFP expression in the dorsal pouch (DP), normalized to expression in *Bx>lacZ* tissues isolated 12 hours after damage. ***p<0.001, ****p<0.0001, two-way ANOVA with Tukey's test.

Genotypes – (A-D): Genotypes in Fig. 3-6, (E): Bx-Gal4/+; UAS-Dcr2/+; *dilp8*^{MI00727}/UAS-LacZ.NZ, (F): Bx-Gal4/+; UAS-Dcr2/UAS-Br^{RNAi}; *dilp8*^{MI00727}/+, (G): genotypes in E-F

3.2.E Broad isoforms are sufficient to suppress regeneration

From our loss-of-function experiments, it appears that expression of Broad isoforms may limit the duration of regenerative response in discs damaged pre-RRP and block the initiation of regenerative activity in discs damaged post-RRP. To examine whether the expression of individual Broad isoforms is sufficient to limit regeneration, I expressed individual Broad isoforms in the wing disc and examined both regenerative signaling and regeneration outcomes in adult wings. When I examine regenerative signaling in 80hAED-damaged discs, I observe that Bx-Gal4-driven expression of BrZ1, BrZ2, and BrZ4 limits both dilp8 and Wg expression in the dorsal compartment of the wing disc, with BrZ1 and BrZ4 producing the most robust inhibition of regenerative Wg expression (Fig.3-8A-F). These distinct effects of Broad isoforms on regenerative Wg and dilp8 expression are also observed in discs with targeted genetic damage via expression of the *Drosophila* TNFα homolog, *eiger* (*Bx>egr*). *Eiger* overexpression in wing discs produces localized damage and elicits strong Wg and Dilp8 expression in the regeneration blastema ((Smith-Bolton et al., 2009), Fig.2-2A'', B''). BrZ1, BrZ2, and BrZ4 all produce a reduction of dilp8 expression in these regenerating tissues. Again, BrZ1 and BrZ4 produce the most robust

inhibition of Wg expression in the eiger damage model (Fig.S-1). Therefore, all three isoforms can limit regenerative signaling in eiger-damage discs. Consistent with this, RNAi-inhibition of all the Broad isoforms produces elevated levels of both Wg and dilp8 in eiger-damaged tissues (Fig.S-1).

Figure 3-8: Br isoform expression is sufficient to suppress regenerative signaling in damaged imaginal discs

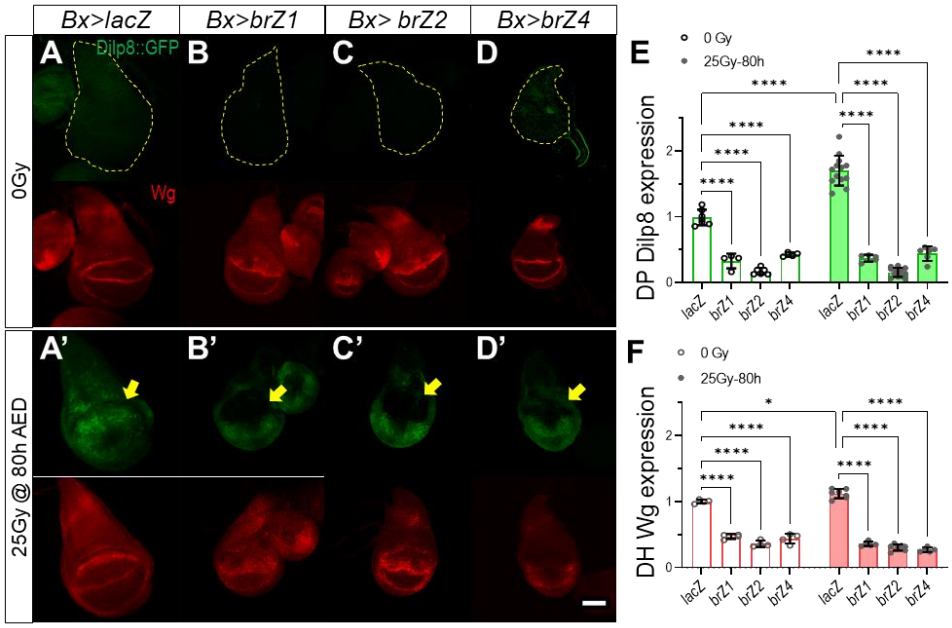

Figure 3-8: Br isoform expression is sufficient to suppress regenerative signaling in damaged imaginal discs - (A-D) Representative images of pouch Dilp8::GFP (green) and Wg (red) expression in 92hAED undamaged (A-E), and early damaged - 25Gy @ 80hAED (A'-E') wing imaginal discs. The yellow dotted line indicates tissue area. Tissues are expressing lacZ (*Bx>lacZ)* as a control (A-A') or br isoforms, *Bx>brZ1* (B-B'), *Bx>brZ2* (C-C'), and *Bx>brZ4* (D-D'). Yellow arrows indicate the primary area of analysis. Scale bar=50um. **(E-F)** Quantification of regenerative activity in 92h AED tissues using dorsal pouch Dilp8::GFP expression in the (E) and dorsal hinge (DH) Wg expression (F) in undamaged and early damaged wing discs and *br* overexpressing wing imaginal discs. All quantifications normalized to undamaged *Bx>lacZ*, ****p<0.0001, *p<0.05, two-way ANOVA with Tukey's test.

Genotypes – (A): Bx-Gal4/+; UAS-Dcr2/+; *dilp8*^{MI00727}/UAS-LacZ.NZ, (B): Bx-Gal4/+; UAS-Dcr2/UAS-BrZ1; *dilp8*^{MI00727}/+, (C): Bx-Gal4/+; UAS-Dcr2/UAS-BrZ2; *dilp8*^{MI00727}/+, (D): Bx-Gal4/+; UAS-Dcr2/UAS-BrZ4; *dilp8*^{MI00727}/+, (E-F): genotypes in A-D.

Constitutive expression of individual Broad isoforms produces substantially deformed adult wings and pupal lethality, making it challenging to assess regenerative outcomes. Therefore, to determine whether expression of the individual Broad isoforms is sufficient to inhibit regeneration, I transiently expressed each of the Broad isoforms in the wing pouch using rn-Gal4 and used tub-Gal80ts expression to limit the timing of expression to a 12-hour window following irradiation. I observe that transient expression of BrZ1, BrZ2, and BrZ4 in undamaged control larvae produces only minor effects on disc patterning and growth (Fig.3-8G,H). However, the transient expression of Broad isoforms following early L3 X-irradiation damage profoundly affects wing regeneration. Expression of BrZ1, BrZ2, and BrZ4 results in a high proportion of incompletely regenerated discs and reduced wing blade size. Of the three splice isoforms, BrZ4 produces the most potent inhibition of regeneration, with all the adult wings mis-patterned and tiny (Fig.3-8G,H).

In summary, our isoform expression experiments demonstrate that the local expression of individual Broad isoforms in damaged tissues is sufficient to block critical local and systemic regeneration signaling events. Even the transient expression of single Br isoforms in regenerating tissues can severely attenuate regeneration in these tissues.

Figure 3-9: Br isoforms limit regeneration

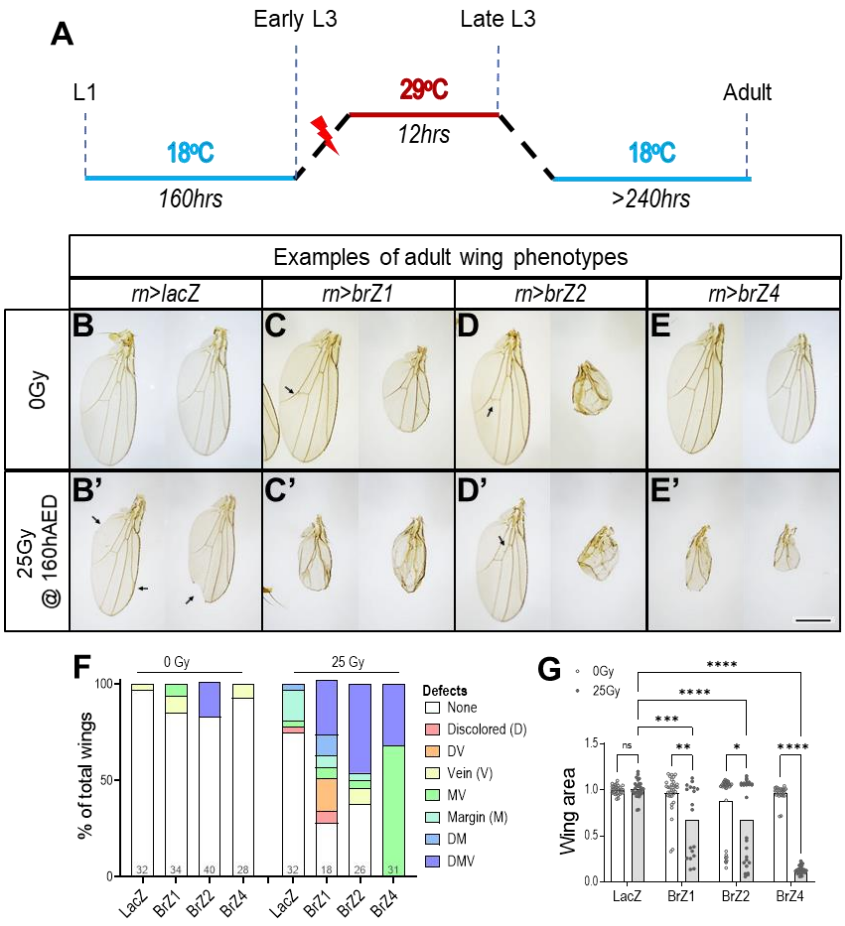

Figure 3-9: Br isoforms limit regeneration - (A) Schematic of transient Br isoform overexpression experiment. rn-gal4 driven expression of UAS-constructs was limited to L3 using temperature-sensitive tubulin-gal80 (tub-gal80^{ts}). Stocks were maintained at 18ºC until early L3, then irradiated (red lightning – 25Gy) or left unirradiated (0Gy). Following a 12hr incubation period at 29ºC, the larvae were returned to 18ºC until the adult stage. **(B-F)** Examples of adult wings arising from 0Gy (B-F) and 25Gy (B'-F') damaged larvae following overexpression of control – LacZ (B-B') and Br isoforms: BrZ1 (C-C'), BrZ2 (D-D'), and BrZ4 (E-E'). Black arrows indicate defects in wings that otherwise appear normal. Examples of normal wings can be found in (B). Scale=500cm **(F)** Adult wings that show individual defects or combinations of defects increase following late damage. The graph shows the percentage of defective adult wings from larvae transiently overexpressing *lacZ*, *brZ1*, *brZ2,* and *brZ4* (*rn-gal4*) with no damage (0Gy) and early damage (25Gy) during wing development. Population size is indicated in the graph. **(G)** Quantification of adult wing size for tissue in (F). Size of wing measured in the unit area and

normalized to undamaged *rn>lacZ* wing size of respective sex. Two-way ANOVA with Tukey's test, ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05.

Genotypes – (B): AP1-GFP/+; rn-Gal4,tub-Gal80^{ts}/UAS-LacZ.NZ, (C): AP1-GFP/UAS-BrZ1; rn-Gal4,tub-Gal80^{ts}/+, AP1-GFP/UAS-BrZ2; (D): rn-Gal4,tub-Gal80^{ts}/+, (E): AP1-GFP/UAS-BrZ4; rn-Gal4,tub-Gal80^{ts}/+, (F-G) genotypes in B-E

3.2.F Broad isoforms regulate wgDRE activation

To better understand how ecdysone produces these distinct effects on regenerative signaling, I started by investigating whether the Br isoforms regulate the JNK-signaling pathway. The JNK-signaling activates both *dilp8* and *wg* expression following damage(Akai et al., 2021; Bergantinos et al., 2010). Using two reporters of the JNK signaling pathway in *Drosophila*, I found that the BrZ1 has no significant effect on JNK signaling (data not shown). Due to experimental complications, such as poor disc growth following BrZ2 overexpression, we did not continue the JNK signaling assessments. Instead, I examined how ecdysone regulates the enhancer region located ~8 kb downstream of the *wg* coding region (Fig.3-11A), which was previously named BRV118 (Schubiger et al., 2010) but has more recently been described as *wg* Damage Responsive Enhancer (*wg*DRE, (Harris et al., 2020)). The *wg*DRE (Fig3-11A) is activated by JNK signaling and is critical for the regenerative activation of *wg* expression following damage (Harris et al., 2016; Smith-Bolton et al., 2009). Epigenetic changes at the *wg*DRE towards the end of larval development lead to the loss of regenerative capacity following the RRP (Harris et al., 2016). However, it is unclear how these epigenetic changes are facilitated. Using a transgenic reporter of *wg*DRE activity (*wg*DRE-GFP, (Harris et al., 2016)), I observe the attenuation of damage-induced *wg*DRE activity as larvae develop across the

RRP, similar to our observation in Dilp8 expression. Figure 3-10A&E show examples of the activation of the DRE (GFP expression) following late damage and early damage, respectively.

Figure 3-10: Br isoforms limit wgDRE activation

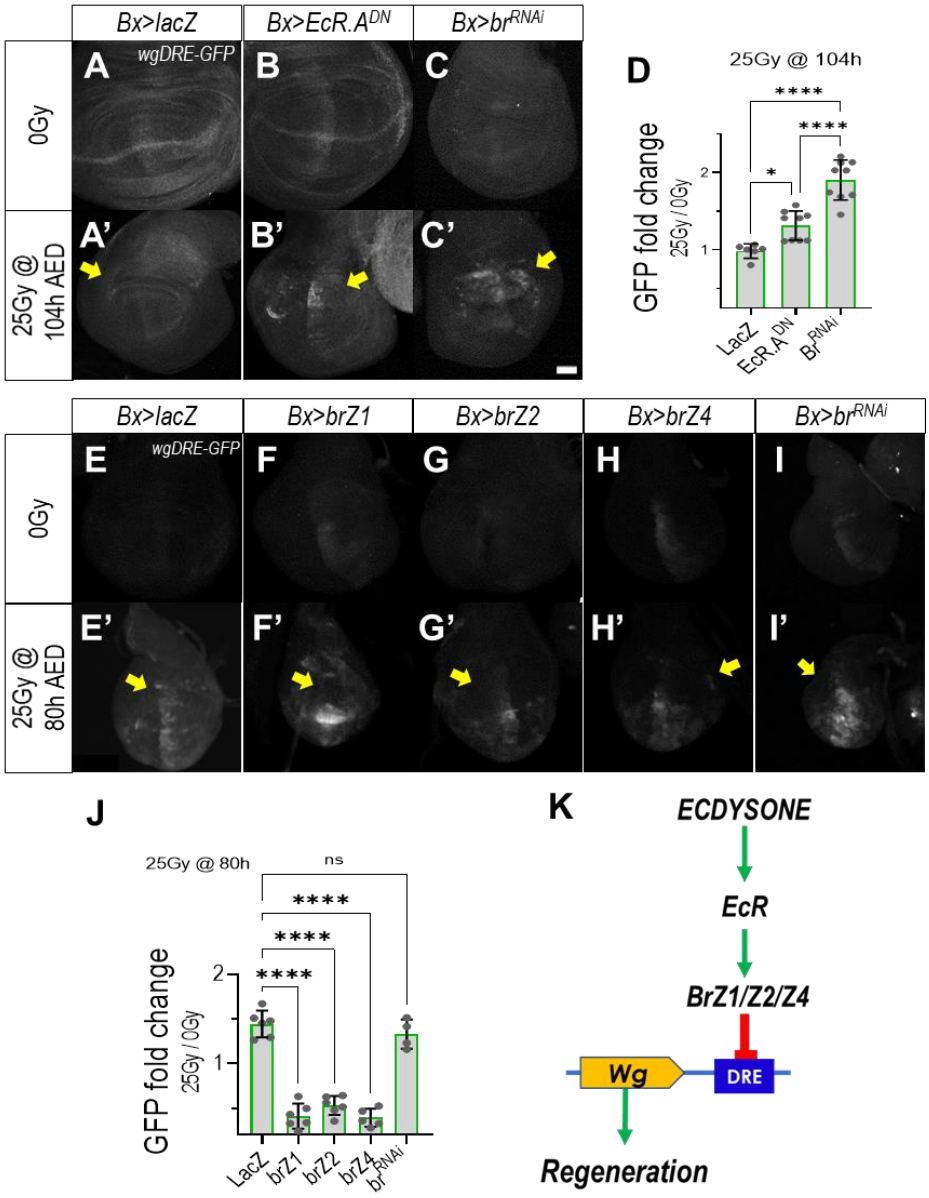

Figure 3-10: Br isoforms limit *wg*DRE activation - (A-C) Representative images of *wg*DRE-GFP (grey) expression in 116hAED undamaged (A-C) and late damaged - 25Gy @ 104hAED (A'-C') wing imaginal discs. Yellow arrows indicate the primary area of Gal4-UAS

expression. Tissues are expressing lacZ (*Bx>lacZ)* as a control (A-A), *Bx>EcR.A*^{DN} (B-B), and *Bx>br*^{RNAi} (C-C). Scale bar=50um. **(D)** Quantification of dorsal pouch *wg*DRE-GFP expression fold change following late damage (25Gy-104h), control - *Bx>lacZ, Bx>EcR.A*^{DN} and *Bx>br*^{RNAi} overexpressing wing imaginal discs. Quantification normalized to respective undamaged tissues. ****p<0.0001, *p<0.05, One-way ANOVA with Tukey's test. **(E-I)** Representative images of *wg*DRE-GFP (grey) expression in 92hAED undamaged (E-I) and early damaged - 25Gy @ 80hAED (E'-I') wing imaginal discs. Tissues are expressing lacZ (*Bx>lacZ)* as a control (F-F) or br isoforms, *Bx>brZ1* (G-G), *Bx>brZ2* (H-H), *Bx>brZ4* (I-I), *Bx>br*^{RNAi} (J-J), and *bx> EcR.A*^{DN} (K-K). Yellow arrows indicate the primary area of expression—scale bar=50um. **(J)** Quantification of dorsal pouch *wg*DRE-GFP expression fold change following early damaged, control - *bx>lacZ*, *br* isoform overexpressing, *br* knockdown, and *EcR*^{DN} expressing wing imaginal discs. Data normalized to respective undamaged tissues, ****p<0.0001, **p<0.01, One-way ANOVA with Tukey's test. **(K)** Summary of how ecdysone signaling suppresses regenerative activity via Br isoforms.

Genotypes – (A,D,E): Bx-Gal4/+; BRV118-GFP/+; UAS-Dcr2/UAS-LacZ.NZ, (B,D): Bx-Gal4/+; BRV118-GFP/UAS-EcR.A^{W650A}; UAS-Dcr2/+, (C,D,I,J): Bx-Gal4/+; BRV118-GFP/UAS-Br^{RNAi}; UAS-Dcr2/+, (F): Bx-Gal4/+; BRV118-GFP/UAS-BrZ1; UAS-Dcr2/+, (G): Bx-Gal4/+; BRV118-GFP/UAS-BrZ2; UAS-Dcr2/+, (H): Bx-Gal4/+; BRV118-GFP/ UAS-BrZ4; UAS-Dcr2/+

To determine whether the limitation of regenerative activity by ecdysone at the RRP is mediated through the *wg*DRE, I measured reporter expression in *Bx>EcR.A*^{DN} discs. I see that blocking ecdysone signaling increases the damage-induced *wg*DRE reporter activity in the dorsal pouch of discs damaged post-RRP (Fig.3-10A,B,D). Therefore, the inhibition of the *wg*DRE in late-damaged tissues is dependent on ecdysone signaling in regenerating disc tissues. Consistent with our earlier observations, the inhibition of the *wg*DRE post-RRP is also dependent on Broad, as late damage can also activate the *wg*DRE in *Bx>br*^{RNAi} expressing discs (Fig.3-10A,C,D). To determine which Broad isoforms are sufficient to suppress *wg*DRE activity, I examined whether expression

of Broad isoforms can suppress the pre-RRP damage-induced activation of *wg*DRE. I see that expression of BrZ1, BrZ2, and BrZ4 can suppress *wg*DRE activation following early damage (Fig.3-10E-J). Based on these results, I conclude ecdysone, via Broad isoform expression, can limit regenerative activity by suppressing the damage-induced activity of the *wg*DRE. Finally, to assess whether Br isoforms suppress the *wg*DRE during regeneration, I knocked down Br (*Bx>br*^{RNAi}) and looked for any increases in *wg*DRE activation. Loss of Br isoforms does not affect *wg*DRE activation following early damage (Fig. 3-10I,J). From our findings, I can conclude that ecdysone signaling suppresses regenerative activity by silencing the *wg*DRE (Fig.3-10K)

3.2.G Broad isoforms have genetic interaction with PRC

For tissues to lose regenerative capacity, the *wg*DRE is epigenetically silenced at the RRP by the Polycomb Repressive Complexes (PRC1 and PRC2, (Harris et al., 2016)). One of the PRC recruitment methods is through cis-regulatory elements called PRC response elements (PREs), enabling them to bind to chromatin and maintain the state of transcriptional silencing over many cell divisions (Franke et al., 1992; Van Kruijsbergen et al., 2015). There is a putative PRE site on the *wg*DRE, but it is not necessary for silencing the locus at the end of larval development (Fig3-11A (Harris et al., 2016)). It remains unclear how PRC is recruited to the *wg*DRE.

There is evidence that Broad and Pc (essential subunit of PRC1 complex) physically interact during the wing disc development (Lv et al., 2016). I hypothesized that Br isoforms recruit PRC to the *wg*DRE. Using publicly available Br isoform-specific binding motif data, I identified several putative Br binding sites in the DRE section necessary for its

suppression (Fig3-11A). I confirmed using Pc heterozygous amorphic allele of Pc, *Pc*$^{15-/+}$ (J. Simon et al., 1992), and three Pc-targeting RNAi's (*Bx>Pc*^{RNAi}) that PRC1 is also necessary to suppress *dilp8* expression at the RRP. After the loss of Pc, which is responsible for the stable gene repression (H2AUb1 modification (Aranda et al., 2015)), tissues damaged post-RRP can express Dilp8 (Fig.3-11B-D).

Figure 3-11: Br isoforms regulate polycomb repressive activity

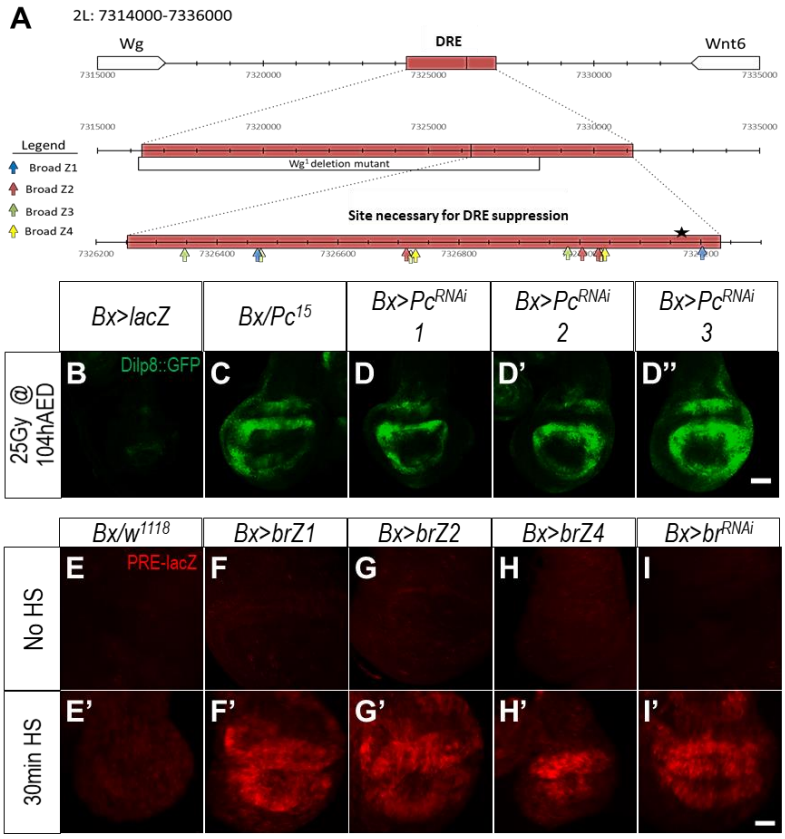

Figure 3-11: Br isoforms regulate polycomb repressive activity - (A) Simplified representation of the *wg*-DRE-*wnt6* locus. The diagram shows the deletion site of the *wg*^{1} mutation (used in Appendix 1) and the site of the H3K27me3 modification (suppression) on the DRE. The putative PRE site is marked by a black star, while Br isoform binding sites on the DRE are indicated using color-coded arrows. **(B-D)** Representative images of pouch Dilp8::GFP (green) expression in 116hAED following late damage - 25Gy @ 104hAED (A-C'') wing imaginal

discs. Tissues are expressing lacZ (*Bx>lacZ)* as a control (A), $pc^{15-/+}$ polycomb mutant background, or expressing three different Pc^{RNAi} lines ((C-C''), (Perkins et al., 2015)). Scale bar=50um. **(D-H)** Representative images of polycomb reporter enhancer activation through heat shock (PRE-lacZ expression) following Br isoform overexpression. The images show the tissues that have not undergone heat shock (No HS: D-H) and those that have undergone 30minutes of 37ºC heat shock (D'-H'). The tissues are expressing lacZ (*Bx>lacZ)* as a control (D-D') or br isoforms, *Bx>brZ1* (E-E'), *Bx>brZ2* (F-F'), *Bx>brZ4* (G-G'), and *Bx>brZ4* (H-H'). Scale bar=50um.

From our study and the findings of Harris et al., 2016, I know that both PRC and Br are essential for suppressing regeneration genes. Lv et al. 2016 did not provide any information about which Br isoform interacts with Pc. To identify which Br isoform interacts with PRC complexes, I investigated whether overexpression of the Broad isoforms regulates PRC suppression activity. The PRC binding reporter uses a Polycomb Response Element (PRE; gene sequence recognized PRC-recruiting protein, bithoraxoid, identified initially through their role in silencing bithorax-complex genes), to repress the expression of a heat-shock promoter-driven LacZ (*PRE-hs-LacZ*, (Dellino et al., 2004; Franke et al., 1992)). When the PRC is active, it binds to the PRE and suppresses the Hsp locus, thus inhibiting lacZ expression upon heat shock (Fig.3-11E). I find that BrZ1, BrZ2, and BrZ4 expression allows hs activation and lacZ expression (Fig.3-11F-H). I also expressed BrZ3, and it had no effect on the suppression of the PRE site (data not shown). Surprisingly, knocking down Br elicits a similar derepression of the PRE site (Fig.3-11I). I hypothesize that overexpressed Br isoforms sequester PRC subunits to other sites, causing the PRE reporter's derepression. However, the derepression in $Bx>br^{RNAi}$ tissues suggests a more complex interaction between Br and PRC. Although I provide evidence of a robust genetic interaction between Br and PRC, further

investigation is needed to identify which PRC subunit interacts with the Br isoforms and understand how this interaction regulates *wg*DRE activity at the RRP.

3.3 Discussion

3.3.A *Summary of findings*

The role of hormones in the suppression of regeneration has been hypothesized and demonstrated in many animals (Bubenik et al., 1987; Halme et al., 2010; Hirose et al., 2019; Marshall et al., 2019). However, the mechanism through which hormone signaling suppresses regeneration remained unknown. I used *Drosophila* to demonstrate a signaling mechanism for the epigenetic silencing of regeneration genes. Consistent with previous findings(Halme et al., 2010), Elevated ecdysone levels at the end of larval development suppress the expression of the patterning gene Wg by silencing the wgDRE locus. The wgDRE is silenced by downstream targets of 20E signaling, Broad splice isoforms BrZ1, BrZ2, and BrZ4.

3.3.B *The roles of the Broad isoforms in regeneration*

Broad isoforms are previously known collectively as the Broad Complex due to the complementation groups that identified them, *broad (br)*, *reduced bristles on palpus (rbp)*, *l(1)2Bc* and *l(1)2Bd*), and non-pupariating (*npr1*) alleles ((Kiss et al., 1988), Fig.3-4B). They were later identified as isoforms from the same gene locus(DiBello et al., 1991). The Br isoforms are essential for pupation and imaginal disc development. The *npr* mutants fail to pupate, and their imaginal discs begin to form swollen vesicles, which seems to be a failure to initiate metamorphosis (Kiss et al., 1988; Baohua Zhou et al., 2009). We still do not know the role of Br in most tissues, but we know that all tissues in *Drosophila* express Br. Our study demonstrates that Br isoforms BrZ1, BrZ2, and BrZ4

are sequentially expressed in the wing imaginal disc as development progresses (Fig.3-4A) and seem to have varying effects on their suppression of regenerative activity.

a) Broad-Z1

The role of BrZ1 in the development of the wing disc is induction of cell cycle exit (Guo et al., 2016) and differentiation of cells at the margin (Jia et al., 2016; C. K. Mirth et al., 2009). This role is reflected in how margin Wg expression at 116hAED is distorted in rbp5 mutants (Fig.3-5C). The Maurange lab demonstrated that BrZ1 acts as an antagonist to Chinmo and argues that the differentiation induced by BrZ1 shuts down the “stemness” induced by Chinmo (Narbonne-Reveau & Maurange, 2019). Our findings support the Maurange lab findings. I demonstrate that BrZ1 signaling silences *wg*DRE, causing a loss in regenerative capacity. BrZ1 expression also ends any ongoing regenerative activity (Fig.3-6, 3-7). However, it is unclear whether BrZ1 binds the *wg*DRE directly. Unlike the findings of the Maurange lab, I found that other Br isoforms are expressed in the wing disc and contribute to the suppression of regenerative activity.

b) Broad-Z2

In imaginal discs cultured in 20E, BrZ2 is the first isoform to respond to increased ecdysone levels (Emery et al., 1994). Our findings are consistent with this observation as BrZ2 is expressed first in L3 wing discs. Similar to BrZ1, BrZ2 significantly suppresses regenerative activity. However, in addition to increased Wg and Dilp8 expression, br^{28} mutant tissues show increased apoptosis and a more significant decrease in tissue size after early damage (Fig.3-6, 3-7, A3-2). BrZ2’s suppression of regenerative activity also includes complete loss of apoptotic cells in the area of expression (Fig.A3-3). However, Brz2 overexpression seems to have little to no effect in the eiger damage model (Fig.A2-

1). This discrepancy may be due to the differences in damage models (*see Appendix A2*). However, BrZ2 may be cell-autonomously limiting Jnk signaling to limit cell death or protect the tissues from irradiation damage. JAK/STAT signaling has been shown to define irradiation-resistant cells, which replace the damaged tissue when Wg is expressed (Verghese & Su, 2016). BrZ2 may be promoting JAK/STAT activity, but there is no regeneration since Wg expression is suppressed. If the tissue does not “register” the damage caused, there would be no initiation of a regenerative response. In the absence of BrZ2, the tissue has an overactive response to irradiation damage.

c) Broad-Z3

BrZ3 is necessary for head and thorax development. BrZ3 plays a critical role in CNS metamorphosis(Baohua Zhou et al., 2009) and *2Bc* (Z3 specific mutation) mutants showing early pupa developmental arrest due to incomplete of the head and thorax epidermis fusion (Kiss et al., 1988). Although BrZ3 is not expressed in the wing disc, I found that overexpression of BrZ3 strongly affects Wg expression and apoptosis (Appendix: A3). The over-activation of Wg may be the cause of increased cell death in the tissue. Overexpression of Wg induces cell competition and apoptosis due to steep signaling differences(Vincent et al., 2011) or through Myc activation (De La Cova et al., 2014; Moreno & Basler, 2004).

d) Broad-Z4

The last Br isoforms to be discovered was BrZ4 (Bayer et al., 1996). Very little is known of its function. Due to sequence similarities to BrZ1, it is presumed to function similarly or bind to similar DNA sites. From our study, BrZ4 is the last isoform to be expressed and has the most potent effect on regeneration (Fig.3-8, 3-9). In the CNS, BrZ4 acts as a

marker for cell-fate commitment (Baohua Zhou et al., 2009), which may be the role BrZ4 plays in the wing discs. BrZ4 may be a more potent driver of differentiation and metamorphosis than BrZ1. Without a BrZ4-specific loss of function assays, it is difficult to accurately determine the importance and role of BrZ4 at the end of larval development.

3.3.C Broad isoforms and regulation of epigenetic landscape

Ecdysone pulses initiate developmental transitions (Yamanaka et al., 2013). Developmental transitions involve a change in the epigenetic landscape that allows activation of some genes while silencing others. I have yet to identify how ecdysone signaling regulates the epigenetic landscape. Broad isoforms are the first effectors of ecdysone signaling in L3. However, I still know very little of the regulation of Br isoform expression and the genes or loci that Br regulates. The primary role of Br isoforms appears to be the restriction of expression of downstream genes to discrete developmental periods (Bayer et al., 1996). However, there are cases of Br isoforms acting on the same locus with opposing functions (activation and suppression). For example, in Dopa decarboxylase expression, Z2 transgene activates transcription, while Z1 or Z4 represses this gene (Andres et al., 1993; Bayer et al., 1996). From our binding site predictions, all Broad isoforms are predicted to bind the *wg*DRE (Fig.3-11A). The overlap seen in the binding sites suggests that multiple isoforms may act on the same site. The temporal expression and their cofactors would instead determine which isoform binds at which time. If isoforms share similar expression profiles, cofactors, and binding sites, then I would take into account the binding affinity of each isoform. Although BrZ1, BrZ2, and BrZ4 all regulate PRC activity (Fig.3-11), it is unclear from Lv et al.'s findings

which Br isoform binds to PRC proteins (Lv et al., 2016). The suppression mechanism of PRC is a three-step process: recruitment, histone methylation by Pc2, and subsequent histone ubiquitination by Pc1(Franke et al., 1992; J. A. Simon & Kingston, 2009; Van Kruijsbergen et al., 2015). Each of these steps requires targeted recruitment. Therefore, Br isoforms may regulate PRC activity at any of these steps.

Chapter 3 Appendices

A1: *Broad Z3 data*

In addition to BrZ1, BrZ2, and BrZ4, I also did BrZ3 overexpression experiments. BrZ3 is not expressed in the wing disc (*See Section 3.3*). Therefore, the data I obtained may give insight into signaling pathways that BrZ3 regulates, but the findings are a neomorphic phenotype for the wing disc. The data below (Fig.A1-1) shows how BrZ3 affects the activation of Wg, Dilp8, and *wg*DRE following early damage. BrZ3 causes increased Wg and Dilp8 expression as well as apoptosis (Fig.A3-3H). However, this increase does not lead to increased activation following damage.

Figure A1-1: BrZ3 overexpression phenotypes

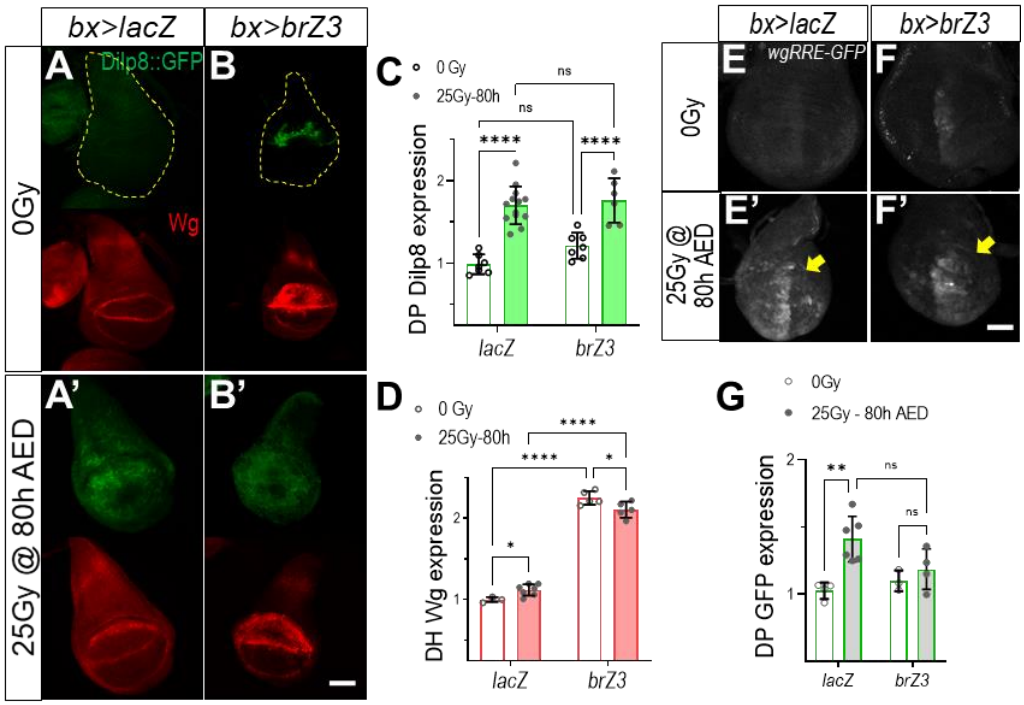

Figure A1-1: BrZ3 overexpression phenotypes - (A-B) Representative images of pouch Dilp8::GFP (green) and dorsal hinge Wg (red) expression in 92h AED undamaged (A-B), and early damaged - 25Gy-80h AED (A'-B') wing imaginal discs. The tissues are expressing lacZ

(*bx>lacZ)* as a control (A-A') and *bx>brZ3* (B-B'). Scale bar=50um. **(C-D)** Quantification of regenerative activity in 92h AED tissues using dorsal pouch Dilp8::GFP expression in the **(C)** and dorsal hinge (DH) Wg expression (D) in undamaged and early damaged wing discs. All quantifications normalized to undamaged lacZ ****p<0.0001, *p<0.05, two-way ANOVA with Tukey's test (Dilp8) and BK&Y test (Wg). **(E-F)** Representative images of *wg*DRE-GFP (grey) expression in 92h AED undamaged (E-F) and early damaged - 25Gy-80h AED (E'-F') wing discs. Tissues are expressing lacZ (*Bx>lacZ)* as a control (E-E') and *Bx>brZ3* (F-F'). Primary area of expression indicated by yellow arrows. Scale bar=50um **(G)** Quantification of dorsal pouch *wg*DRE-GFP expression following early damaged, control - *lacZ* and *brZ3* overexpressing wing discs. Data normalized to undamaged control tissues, **p<0.01, One-way ANOVA with Tukey's multiple comparisons tests.

Genotypes – (A-A'): Bx-Gal4/+; UAS-Dcr2/+; *dilp8*^{MI00727}/UAS-LacZ.NZ, (B-B'): Bx-Gal4/+; UAS-Dcr2/UAS-BrZ3; *dilp8*^{MI00727}/+, (C-D): genotypes in A-B, (E-E'): Bx-Gal4/+; BRV118-GFP/+; UAS-Dcr2/UAS-LacZ.NZ, (F-F'): Bx-Gal4/+; BRV118-GFP/UAS-BrZ3; UAS-Dcr2/+, (G): genotypes in E-F.

A2: *Genetic damage data for chapter 3*

In addition to causing damage using irradiation, I validated our findings by causing genetic damage in the tissue. This section shows the levels of Wg and Dilp8 I observed following eiger overexpression in the tissue (Fig.A1-1).

Figure A2-1:Br isoforms suppress Wg expression in the blastema

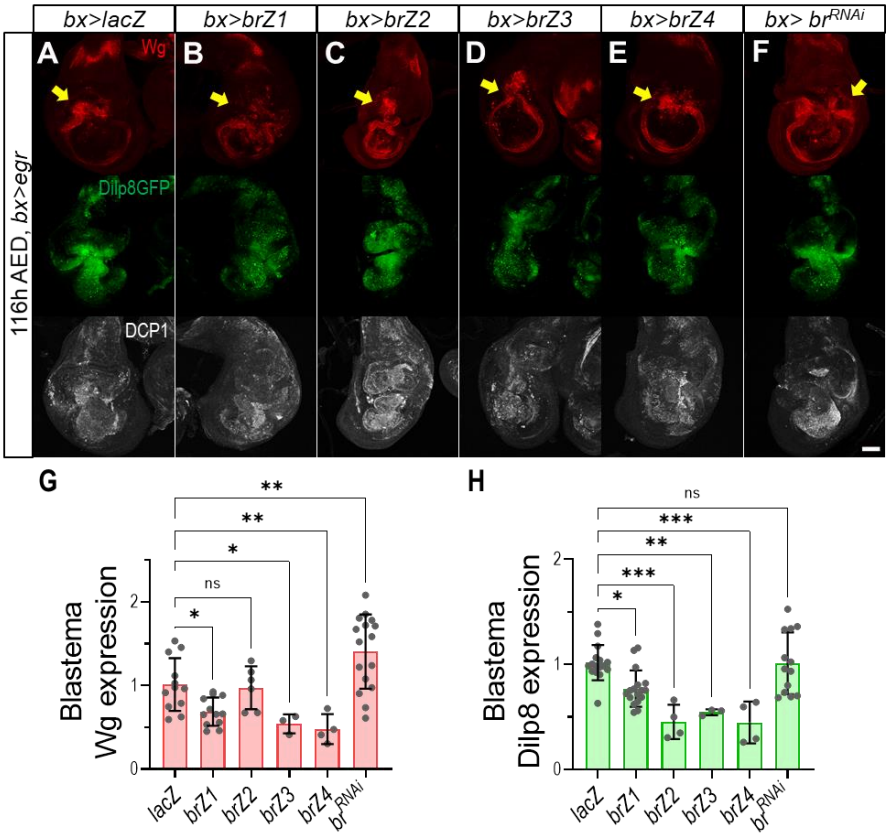

Figure A2-1: Br isoforms suppress Wg expression in the blastema - (A-F) Representative images of Wg (red), and Dilp8 (green), and DCP1 (grey) expression in eiger damaged tissues *(Bx>eiger*). The eiger damaged tissues co-expressed *lacZ* (A), *brZ1* (B), *brZ2* (C), *brZ3* (D), *brZ4* (E), and *br*^{RNAi} (F). **(G-H)** Wg and Dilp8 expression in the blastema. Broad knockdown leads to increased Wg expression in the blastema. Yellow arrows indicate the area of *eiger* expression (the regeneration blastema). Wg (G) and Dilp8 (H) expression in the blastema is quantified and normalized to *lacZ* controls. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, one-way ANOVA with BK&Y (Wg) and Tukey's test (Dilp8).

Genotypes – (A): Bx-Gal4/+;UAS-Eiger/+;*dilp8*^{MI00727}/UAS-LacZ.NZ, (B): Bx-Gal4/+;UAS-Eiger/UAS-BrZ1;*dilp8*^{MI00727}/+, (C): Bx-Gal4/+;UAS-Eiger/UAS-BrZ2; *dilp8*^{MI00727}/+, (D): Bx-Gal4/+;UAS-Eiger/UAS-BrZ3; *dilp8*^{MI00727}/+, (E): Bx-Gal4/+;UAS-Eiger/UAS-BrZ4; *dilp8*^{MI00727}/+, (F): Bx-Gal4/+; UAS-Eiger/UAS-Br^{RNAi}; *dilp8*^{MI00727}/+, (G-H): genotypes in A-F

BrZ3 expressing tissues seem to have diminished regenerative activity following eiger damage, while irradiation damage had no effect (Appendix A1-1). I noticed a discrepancy in our Dilp8 expression findings following irradiation damage vs. genetic damage, and Br isoform-induced suppression of Dilp8 following eiger damage is not as significant as that seen with irradiation damage. This discrepancy may be due to the persistence of the eiger damage causing increased activation of Jnk and Jak-Stat signaling. In addition, eiger is secreted and may be inducing cell death outside the area of Br expression.

A3: *Apoptosis data for chapter 3*

I included DCP1 staining in our assessments. Although cell death is a complex topic to broach during regeneration (*see Appendix A7*), I still wanted to see the changes in apoptosis in the tissue. Fig.A3-1 and A3-2 show apoptosis in Br mutant lines. The data includes *2Bc²* mutant (BrZ3 specific) tissues. Fig.A3-3 shows apoptosis in EcR.A^{DN} and Br isoform overexpression experiments

Figure A3-1: Apoptosis in late-damaged *br* mutant wing discs

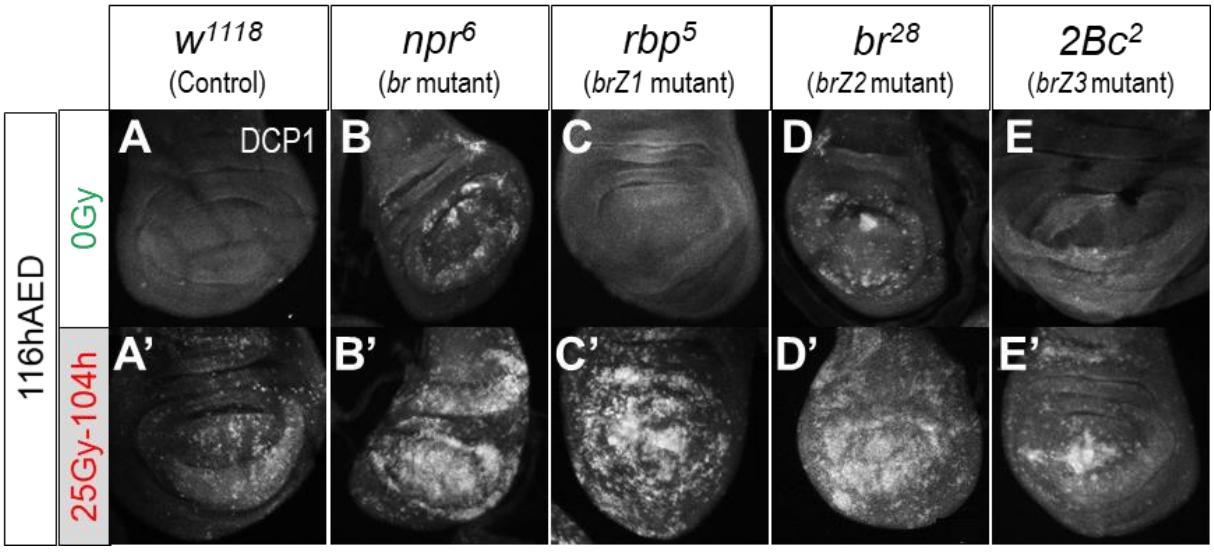

Figure A3-1: Apoptosis in late-damaged *br* mutant wing discs - The figure shows representative images of DCP1 staining in control (w1118, A) and br mutant tissue (B-E & G-J). **(A-E)** shows late L3 (116hAED) tissues that are undamaged (A-E) or damaged post-RRP (A'-E'). **Genotypes** – (A) w^{1118};+/+;*dilp8*^{MI00727}/+, (B): br^{npr-6};+/+;*dilp8*^{MI00727}/+, (C): br^{rbp-5};+/+;*dilp8*^{MI00727}/+, (D): br^{28}; +/+; *dilp8*^{MI00727}/+, (E):br^{2Bc-2}; +/+; *dilp8*^{MI00727}/+,

Figure A3-2: Apoptosis in early-damaged br mutant wing discs

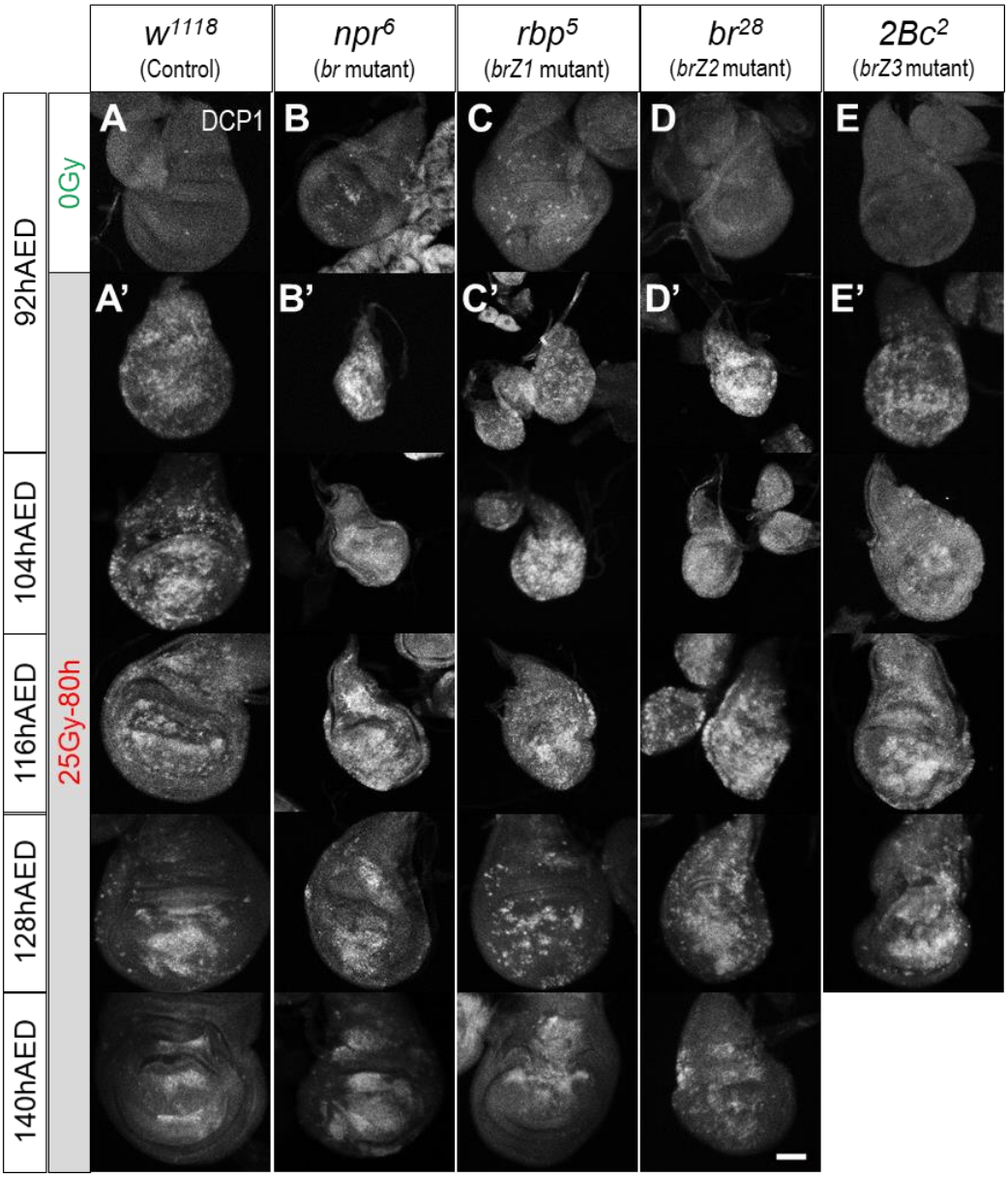

Figure A3-2: Apoptosis in early-damaged *br* mutant wing discs - The figure shows representative images of DCP1 staining in control (w1118, A) and br mutant tissue (B-E). **(A-E)** shows undamaged tissues (A-E) and a time-course, collecting tissues every 12 hours, following pre-RRP damage (A'-E'). No $2Bc^2$ tissues were obtained at 140hAED due to lethality /premature pupation.

Genotypes – (A) w^{1118}; +/+; *dilp8*^{MI00727}/+, (B): br^{npr-6}; +/+; *dilp8*^{MI00727}/+, (C): br^{rbp-5}; +/+; *dilp8*^{MI00727}/+, (D): br^{28}; +/+; *dilp8*^{MI00727}/+, (E):br^{2Bc-2}; +/+; *dilp8*^{MI00727}/+,

Figure A3-3: Cell death in EcR^{DN} and Br overexpression experiments

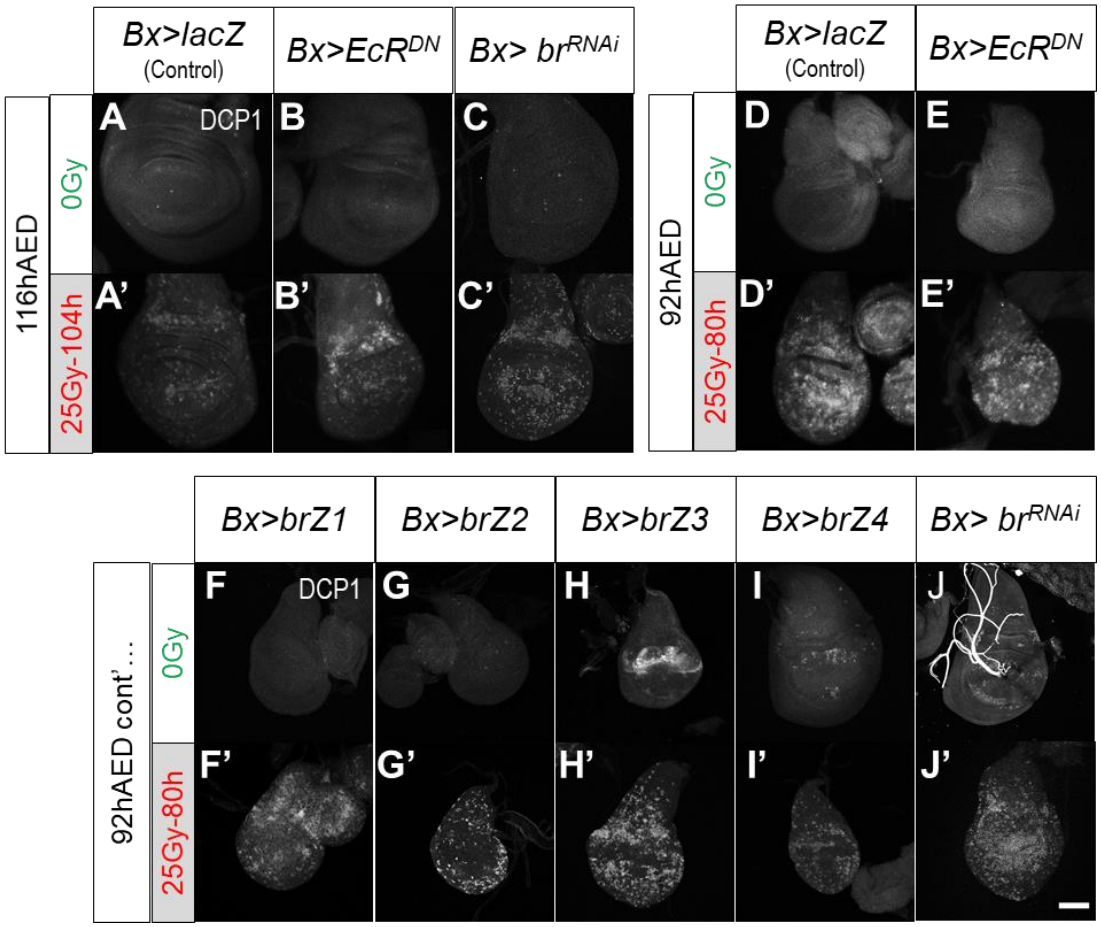

Figure A3-3: Cell death in EcR^{DN} and Br overexpression experiments- Representative images of apoptosis (DCP1 staining) levels seen in undamaged (**A-J**) and irradiation damaged tissues (**A'-J'**) of control (*Bx>lacZ*, **A**, **D**), EcR^{DN} (*Bx>EcR.A^{DN}*, **B**, **E**), Br^{RNAi} (*Bx>Br^{RNAi}*, **C**, **J**), BrZ1 (*Bx>brZ1*, **A**), BrZ2 (*Bx>brZ2*, **G**), BrZ3 (*Bx>brZ3*, **H**), and BrZ4 (*Bx>brZ4*, **I**) overexpressing tissues. The damaged tissues were irradiated post-RRP (25Gy@104hAED, A'-C') and pre-RRP (25Gy@80hAED, D'-J').

Genotypes – (A, D): Bx-Gal4/+; BRV118-GFP/+; UAS-Dcr2/UAS-LacZ.NZ, (B, E): Bx-Gal4/+; BRV118-GFP/UAS-EcR.A^{W650A}; UAS-Dcr2/+, (C, J): Bx-Gal4/+; BRV118-GFP/UAS-Br^{RNAi}; UAS Dcr2/+, (G): Bx-Gal4/+; BRV118-GFP/UAS-BrZ1; UAS-Dcr2/+, (H): Bx-Gal4/+; BRV118-GFP/UAS-BrZ2; UAS-Dcr2/+, (I): Bx-Gal4/+; BRV118-GFP/UAS- BrZ4; UAS-Dcr2/+

CHAPTER 4: Hormone signaling activates and promotes regenerative activity

Abstract

In regenerating tissues, the overarching goal is to restore the tissue with normal development seen in the undamaged tissues. In this chapter, I demonstrate that this restoration of developmental progressions is coordinated by hormone signaling. I identified that *dilp8*$^{-}$ mutants, in which ecdysone levels are not limited during regeneration, show accelerated tissue growth and successful complete regeneration without the checkpoint. I identified that hormone signaling not only suppresses regenerative capacity (*see chapter 3*), it is also necessary to activate regenerative activity. The regenerative activity has a positive response to increases in systemic levels of ecdysone. Ecdysone signaling regulated *wg* expression independent of the *wg*DRE locus. By accelerating the regenerative process, hormone signaling ensures the damaged tissues are fully regenerated by pupation. However, regeneration comes at the cost of viability.

4.1 Introduction

From the findings in Chapter 3, I have evidence that hormone signaling can regulate multiple aspects of the regenerative activity. Ecdysone signaling suppresses regenerative capacity, as well as the duration and extent of regenerative activity. Thyroid hormone in vertebrates and ecdysone in *Drosophila* have been associated with loss of regenerative capacity. However, both hormones are present at lower levels during the regeneration-competent periods of development (Hirose et al., 2019; Hodgetts et al., 1977; Lavrynenko et al., 2015; Marshall et al., 2019). There is a possibility that these hormones play some role in regenerating tissues. In moths (Madhavan & Schneiderman, 1969), *Sarcophaga* (Kunieda et al., 1997), fiddler crabs (S. Das & Durica, 2013; Hopkins, 1989), and newts (Goss, 1969c), loss of ecdysteroids leads to incapability to regenerate. *In-vitro* experiments of *Sarcophaga* leg discs demonstrate that the absence of ecdysone in culture limits Wingless (Wg) expression at the wound site (Kunieda et al., 1997).

In addition, other models such as deer antler regeneration also show the complexity of hormone signaling during regeneration. In antler regeneration in *Cervidae* family male deer, elevated testosterone levels determine the end of the regenerative growth cycle (Bartos et al., 2012). Testosterone suppresses the activity of thyroid hormone and growth hormone (GH/IGF), which promote regenerative growth (Price & Allen, 2004; Shi & Barrell, 1994). Loss of testosterone production (as seen in castrated “peruke” male deer) during the regenerative growth phase results in the persistent dysmorphic overgrowth of the antlers (Goss, 1969a; Kierdorf et al., 2004). However, male deer cannot initiate antler growth if castrated during the resting phase between regenerative cycles (Goss, 1969a). These observations suggest that individual hormones

or combinations of hormones can regulate multiple aspects of the regenerative response, such as the activation, patterning, and regenerative growth duration.

Therefore, in this chapter, I examine what the role of hormones is in regeneration-competent *Drosophila* tissues. To understand how hormones regulate the ongoing regenerative activity, I examined how manipulating ecdysone signaling affects both systemic and local regenerative pathways in the *Drosophila* wing imaginal disc. Here I demonstrate that although ecdysone signaling is limited during regeneration, it remains necessary for the regenerative response. This dual role for ecdysone in promoting and limiting regeneration helps explain a phenotype I had observed in *dilp8*$^{-}$ mutants. Despite the lack of the regenerative checkpoint delay following damage, *dilp8*$^{-}$ mutants can still regenerate their wing discs during the shorter regenerative period. I also establish that ecdysone signaling is cell-autonomously essential for regenerative activity and Wg expression in the disc. Regenerating discs exhibit a positive signaling response to increasing ecdysone levels promoting Wg expression through a Br-independent and *wg*DRE-independent pathway. Ecdysone's biphasic regulation of regenerative activity gives *Drosophila* larvae the ability to coordinate regeneration completion with the end of the larval period. At the cost of pupa viability, ecdysone signaling ensures that the regeneration target is met before the larva-pupa developmental transition.

4.2 Results

4.2.A *dilp8$^{-/-}$ larvae can regenerate their tissues*

Drosophila insulin-like Protein 8 (Dilp8) expression is activated by JNK signaling in regenerating tissues. It acts remotely by binding to Lgr3 receptors in the brain and PG to inhibit ecdysone production (Fig.4-1, (Colombani et al., 2015; Garelli et al., 2015; Vallejo et al., 2015)). By limiting systemic ecdysone levels, Dilp8 delays the late third instar (pupariation) ecdysone peak, extending the larval developmental period and providing regenerating tissues ample time to repair damage and meet target size(Colombani et al., 2012; Garelli et al., 2012, 2015). Our lab and others concluded that Dilp8-Lgr3 signaling is necessary for the proportional growth and the maintenance of body symmetry after damage as it ensures that the growth of damaged and undamaged tissues is coordinated (Andersen et al., 2013; Boone et al., 2016; Jaszczak et al., 2015, 2016).

Figure 4-1: Dilp8 signaling following tissue damage

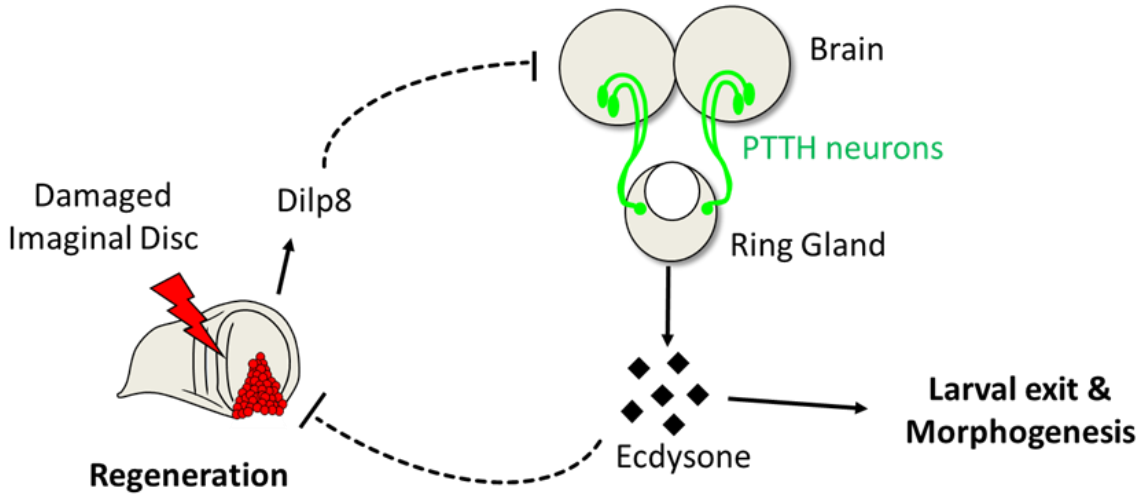

Figure 4-1: Dilp8 signaling following tissue damage – The tissue produce and secrete Dilp8 within 2-4 hours of the damage. Dilp8 signaling and ecdysone signaling have feedback

communication; Dilp8 suppresses ecdysone production, while ecdysone suppresses Dilp8 expression (*see Chapter 3*).

Unexpectedly, adult flies arising from X-irradiated dilp8- larvae could regenerate their wing discs as successfully as control *dilp8*$^{+}$ (w1118) adults. The lack of regeneration checkpoint delay (Fig.4-2A) produces no significant impact on tissue repatterning (Fig.4-1B). I see that the *dilp8*$^{-}$ larvae have smaller wings in general (Fig.4-2C). However, *dilp8*$^{-}$ larvae produce regenerated adult wings closer to their undamaged target size than *dilp8*$^{+}$ larvae in which the checkpoint is intact (Fig.4-2C).

Figure 4-2: *dilp8*$^{-/-}$ mutants can regenerate their tissues following damage

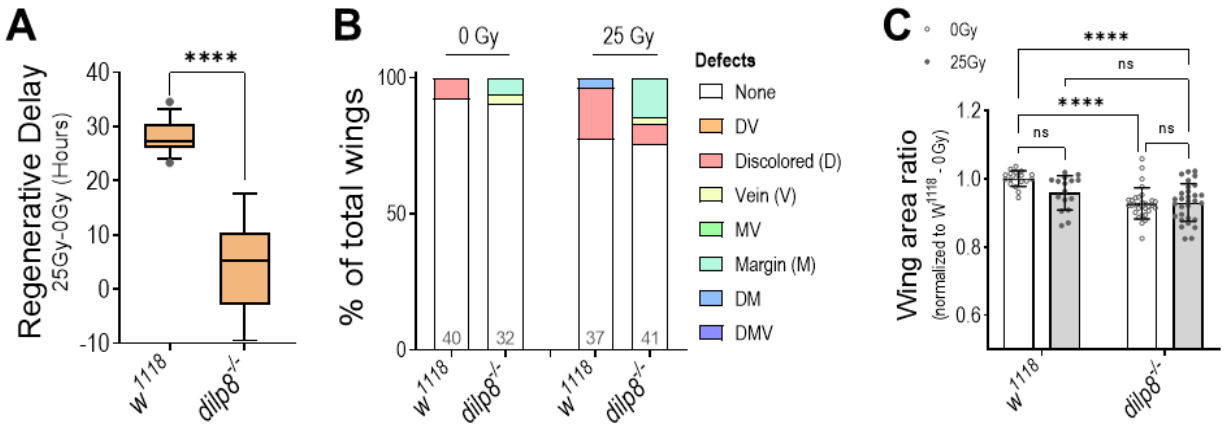

Figure 4-2: *dilp8*$^{-/-}$ mutants can regenerate their tissues following damage - (A) Quantification of regenerative delay following early damage (20Gy-80h) in *w*^{1118} (*dilp8*$^{+}$) and *dilp8*$^{-/-}$ larvae. *dilp8*$^{-/-}$ show no little or no regenerative delay following early damage. Unpaired t-test, ****p<0.0001. **(B-C)** *dilp8*$^{-/-}$ adult wings show no increase in defects (B) or size mismatch (C) following early damage. (B) shows the percentage of defective adult wings following no damage (0Gy) and early damage (25Gy-80h) in *w*^{1118} and *dilp8*$^{-/-}$ adults. Population size is indicated in the graph. (C) Quantification of adult wing size for tissue in adult wings following no damage (0Gy) and early damage (25Gy-80h) in *w*^{1118} and *dilp8*$^{-/-}$ adults. Size of wing measured in the unit area

and normalized to undamaged w^{1118} wing size of respective genotype and sex. 2-way ANOVA with Tukey's test, ****p<0.0001.

Genotypes – (A-C): w^{1118} and *dilp8*^{MI00727} / *dilp8*^{MI00727}

To better understand how *dilp8*$^{-}$ larvae can regenerate damaged wing discs despite the attenuated regenerative period, I measured disc size in undamaged and regenerating discs through L3 in *dilp8*$^{+}$ and *dilp8*$^{-}$ larvae. In control larvae, damage produces a delay in disc growth, with regenerating imaginal discs being measurably smaller than undamaged controls between 92 and 116hAED, just before unirradiated larvae typically end their larval period. However, during the extended larval period produced by activation of the regenerative checkpoint, the growth of the regenerating imaginal discs rapidly reaches the target size (final size of undamaged discs) by 128hAED. It remains at that size until the end of the larval period (Fig.4-3A). In *dilp8*$^{-}$ larvae, I still observe a growth lag in damaged and regenerating tissues, with regenerating tissues being significantly smaller between 92 and 104hAED. However, unlike *dilp8*$^{+}$ larvae, the regenerating imaginal discs in dilp8- larvae rapidly grow after 104hAED, reaching target size by 116hAED, just before both control and dilp8- larvae pupate (Fig.4-3B). When I directly compare the growth of control and *dilp8*$^{-}$ imaginal discs, I see that undamaged discs grow at approximately the same rate (Fig.4-3C), whereas regenerating *dilp8*$^{-}$ imaginal discs grow much faster than control discs (Fig.4-3D).

In summary, I observe that in the absence of the Dilp8 regeneration checkpoint, ecdysone synthesis is no longer limited, and the regenerative growth of imaginal discs is accelerated such that target disc size is still reached by the end of the shortened larval

period. The role of Dilp8 in regeneration is to decrease the levels of the steroid hormone ecdysone in the hemolymph, which delays pupation. This finding suggests that in the absence of Dilp8-Lgr3 signaling, the higher titer of ecdysone leads to accelerated tissue regeneration.

Figure 4-3: *dilp8*$^{-/-}$ mutants show accelerated tissue growth

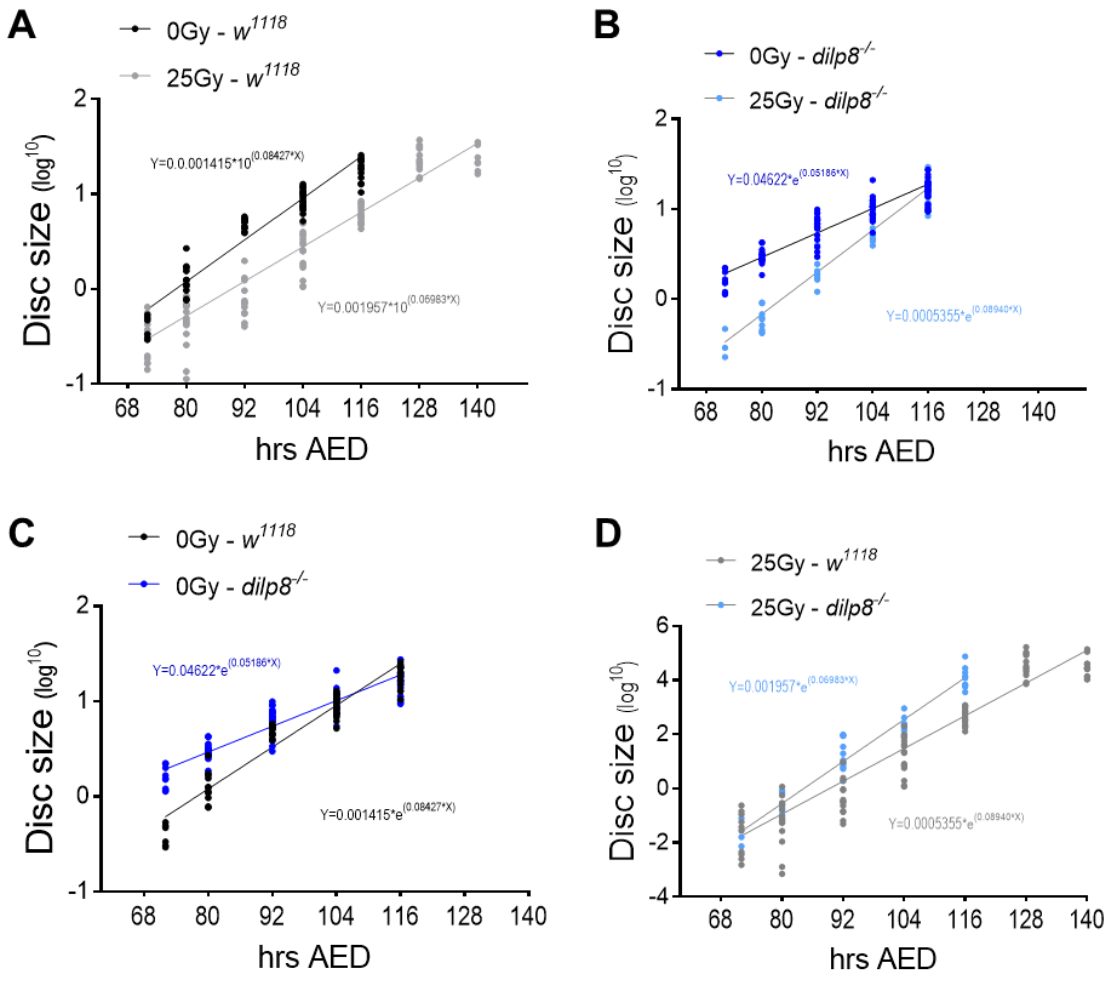

Figure 4-3: *dilp8*$^{-/-}$ mutants show accelerated tissue growth - **(A-B)** Exponential growth analysis of wing discs following no damaged tissues (0Gy) and damage (25Gy) at 48hAED in w^{1118} (A) and *dilp8*$^{-/-}$ (B) larvae. Data sets normalized to 72h undamaged w^{1118} tissues. **(C-D)** Comparison of wing disc growth between w^{1118} and *dilp8*$^{-/-}$ tissues following no damage - 0Gy (C), and damage - 25Gy-48h AED (D). Exponential growth: w^{1118} R^2= 0.8726 (0Gy) and 0.8537

(25Gy), *dilp8*$^{-/-}$ R^2= 0.9533 (0Gy) and 0.9784 (25Gy). Data sets normalized to 72h undamaged *w*^{1118} tissues. **Genotypes** – (A, C, D): w^{1118} and (B,C,D): *dilp8*^{MI00727} / *dilp8*^{MI00727}

4.2.B Regenerating tissues have a biphasic response to systemic levels of ecdysone

The findings in *dilp8*$^{-/-}$ larvae suggest that ecdysone has a role in promoting regenerative growth. Ecdysone signaling is known to be necessary for tissue growth and developmental transitions during normal tissue development. Given our findings in Chapter 3, I hypothesized that ecdysone signaling plays a dual role (promotion and suppression) in regulating regenerative activity. During the final larval instar, pulses of ecdysone synthesis increase the systemic levels of circulating ecdysteroids in the larvae before a final surge of ecdysone synthesis at the end of larval development activates pupariation pathways and initiate metamorphosis(Lavrynenko et al., 2015). Based on this, I hypothesized that the dual activities of ecdysone signaling could depend on the differences in ecdysone levels during L3 development. To test this hypothesis, I manipulated circulating 20E levels in larvae by supplementing their food with increasing doses of 20E following X-irradiation damage at 80hAED. I then measured the regenerative activation of Wg and Dilp8 expression in wing discs 12 hours after X-irradiation (Fig.4-4A).

Consistent with our hypothesis, I find that feeding larvae ecdysone generally promotes regenerative activity following X-irradiation damage. At all concentrations of 20E feeding, I observe an increase in Dilp8 and Wg expression in damaged wing discs

compared with control larvae with no 20E supplement in their food (Fig.4-4B-F,G,H). Following damage, apoptotic cells are extruded from the primary epithelium as the tissue repairs(Bergantinos et al., 2010). I see a similar progression in the 20HE fed larvae, where tissues seem to undergo accelerated repair with increasing 20HE concentration (Appendix, Fig A4-1A-E).

Figure 4-4: Ecdysone regulates regenerative signaling in a biphasic, concentration-dependent manner

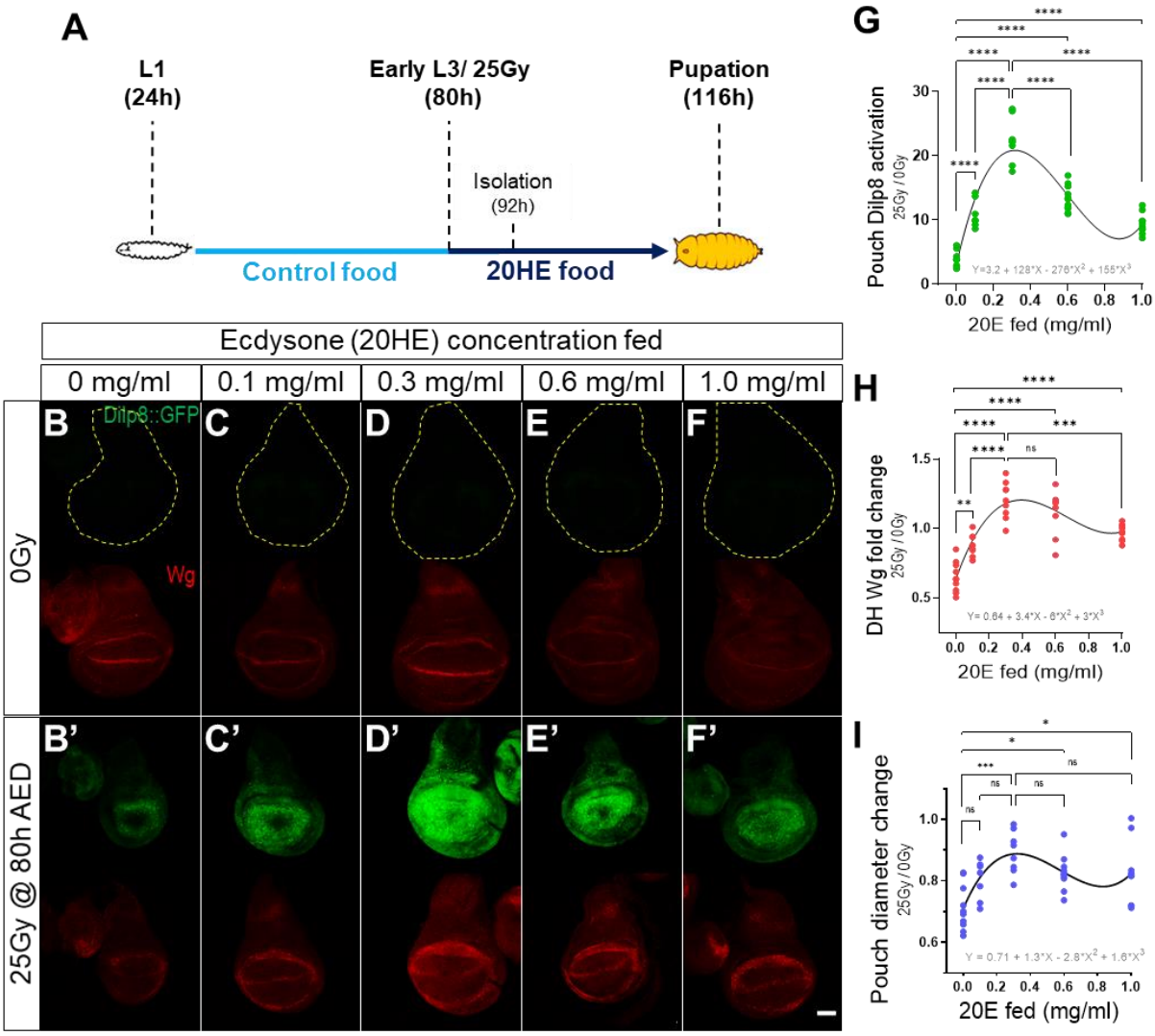

Figure 4-4: Ecdysone regulates regenerative signaling in a biphasic, concentration-dependent manner - (A) Schematic of ecdysone (20E in ethanol) feeding experiment. w^{1118} larvae were fed various 20E concentrations in early L3 (80hAED) immediately after irradiation damage (25Gy @ 80hAED) or no damage (0Gy). Tissues were isolated 12 hours after damage and feeding (92hAED). **(B-F)** Representative images of pouch Dilp8::GFP (green) and dorsal hinge Wg (red) expression in 92hAED undamaged (B-F) and early damaged – 25Gy @ 80hAED (B'-F') wing imaginal discs. The yellow dotted line indicates tissue area. w^{1118} larvae were fed 20E: 0mg/ml (B-B'), 0.1mg/ml (C-C'), 0.3mg/ml (D-D'), 0.6mg/ml (E-E') and 1.0mg/ml (F-F'). Scale bar = 50um. **(G-I)** Quantification of relative regenerative activity using fold change of Dilp8::GFP expression in the wing pouch (G), dorsal hinge (DH) Wg expression (H), and pouch diameter (I) in 20E fed and early damaged w^{1118} wing imaginal discs. Fold change determined by normalizing to respective undamaged tissues, Polynomial regression: Dilp8 $R^2 = 0.86$, Wg $R^2 = 0.76$, and Diam. $R^2 = 0.64$. Statistical analysis: one-way ANOVA with Tukey's test, ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05. **Genotype** – w^{1118}

Similar to the observations in $dilp8^{-/-}$ larvae, I observe that 20E feeding increased the size of the regenerating tissue (Fig.4-4I). However, the effect of ecdysone feeding on regenerative signaling was maximized at 0.3mg/ml 20E concentration. Higher concentrations of ecdysone (0.6 or 1.0mg/ml) produce a substantial reduction in Dilp8 and Wg expression (Fig.4-4B-F,G,H) and produce no additional increase in growth (Fig.4-4I). Therefore, 20E feeding produces a biphasic regenerative signaling response in irradiated tissues.

These findings suggest that the biphasic effect of ecdysone signaling on disc regeneration can coordinate disc regeneration with the duration of the larval period. However, the biphasic effect of increasing 20E concentrations through feeding is not seen in *eiger*-induced Dilp8 and Wg expression. Only a modest and not statistically significant increase is seen for both Wg and Dilp8 expression with increasing ecdysone levels

(Appendix, Fig.A5-1). The differences in ecdysone sensitivity from X-irradiated tissues may reflect the persistence and the intensity of the damage produced in the *Bx>eiger* tissues, which likely maximizes regenerative signaling.

4.2.C Ecdysone signaling is necessary for regeneration activation

Since circulating ecdysone promotes regeneration, I wanted to determine whether ecdysone is necessary for regeneration signaling in wing discs damaged pre-RRP (80hAED). To assess this, I X-irradiated control and *Bx>EcR.A^{DN}* larvae when regenerative activity is high (80hAED, Fig.4-5A,A',C). I observe that ecdysone signaling is necessary to activate regenerative signaling pathways following early damage. There is a clear inhibition of *dilp8* expression in the regenerating dorsal wing of *Bx>EcR.A^{DN}* larvae compared to controls (Fig.4-5B,B',C). Ecdysone signaling is also necessary for the increased expression of Wg in the dorsal hinge following damage as I observed reduced expression of Wg at the dorsal hinge of tissues expressing *Bx>EcR.A^{DN}* compared to controls (Fig.4-5A,B,D).

This requirement of ecdysone signaling in the activation of regenerative activity is also seen in targeted genetic damage via expression of the *Drosophila* TNFα homolog, *eiger* (*Bx>egr*). *Eiger* overexpression in wing discs produces localized damage and elicits strong Wg and Dilp8 expression in the regeneration blastema ((Smith-Bolton et al., 2009), Appendix Fig.A5-1). I see that expression of *EcR.A^{DN}* significantly decreases *eiger*-induced Wg and Dilp8 expression in the damage blastema formed in the dorsal compartment of the wing pouch (Appendix Fig.A5-1A). In some tissues

Figure 4-5: Ecdysone signaling is necessary for activation of regenerative signaling

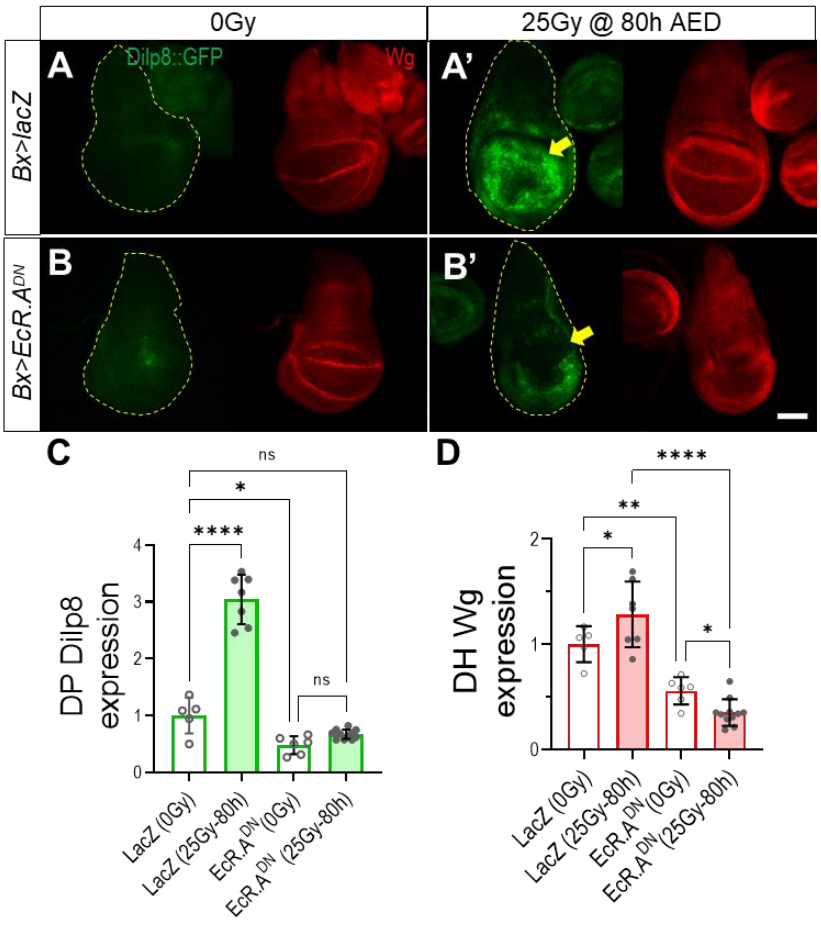

Figure 4-5: Ecdysone signaling is necessary for activation of regenerative signaling - (A-B) Representative images of pouch Dilp8::GFP (green) and DH Wg (red) expression in 92hAED wing imaginal discs. The yellow dotted line indicates tissue area. Tissues are expressing lacZ (A-A') or *EcR.A^{DN}* (B-B') in the dorsal wing pouch region, indicated by yellow arrows. Tissues were either left undamaged (A and B) or damaged early, 25Gy@80hAED (A' and B'), then isolated 12 hours after damage timepoint. Scale bar = 50um. **(C-D)** Quantification of Dilp8::GFP expression in the dorsal wing pouch (C) and dorsal hinge Wg expression (D) in 92h AED early damaged (25Gy-80h) and undamaged (0Gy), *Bx>lacZ* and *Bx>EcR.A^{DN}* wing imaginal discs. Quantification normalized to *lacZ (0Gy)*, *p<0.05, **p<0.01, ****p<0.0001, one-way ANOVA with Tukey's test (Dilp8) and DYK (Wg) tests.

Genotypes – (A-A'): Bx-Gal4/+; UAS-Dcr2/+; *dilp8*^{MI00727}/UAS-LacZ.NZ, (B-B'): Bx-Gal4/+; UAS-Dcr2/UAS-EcR.A^{W650A}; *dilp8*^{MI00727}/+, (C-D) genotypes in A-B

Our findings support the *in-vitro* observations in *Sarcophaga* leg discs demonstrating that ecdysone's absence in culture limits Wingless (Wg) expression at the wound site (Kunieda et al., 1997). Together, these findings suggest a dual (activation and suppression) role for ecdysone in regulating regenerative activity. During regenerative competence, ecdysone signaling in damaged discs is required to activate Wg and Dilp8 expression, critical signaling events that coordinate the local and systemic regenerative responses, respectively. Following development past the RRP, when imaginal disc regenerative capacity is lost, ecdysone signaling in the disc is required to suppress the activation of these regenerative pathways.

4.2.D Ecdysone regulates Wg expression independent of the wgDRE

To assess how lower levels of ecdysone function to promote regenerative Wg expression, I first examined how the loss of ecdysone signaling affects Wg expression in undamaged wing discs. I observed in Fig.3-3 and Fig.4-5, *Bx>EcR.A^{DN}* expression appears to suppress hinge Wg expression in undamaged tissues. To examine this more carefully, I used MARCM to generate GFP-labeled clones that expressed EcR.A^{DN}. I observed that EcR.A^{DN} expression produced clones that cell-autonomously inhibited Wg expression at the hinge regions of the developing wing disc but not at the margin (Fig.4-6A,B). this hinge-specific suppression is unique to EcR.A^{DN}, EcR.B1^{DN} suppresses both the hinge and margin *wg* while EcR.B2^{DN} does not affect either (Appendix A6). This inhibition appears to be a transcriptional regulation of *wg* expression as I see a similar effect of EcR.A^{DN} expression on a *wg* transcriptional reporter line (*wg*-lacZ, Fig.4-6C). Ecdysone may exert both its inhibitory and activating effects on Wg expression through the *wg*DRE.

However, when I inhibit ecdysone signaling (*Bx>EcR.A*^{DN}, Fig4-6D-F) or Broad isoform expression (*Bx>br*^{RNAi}, Fig.3-10J) in early-damaged discs, I see that neither of these manipulations limit *wg*DRE activation. Therefore, ecdysone signaling is required for regenerative Wg expression but regulates *wg* transcription independently of Broad and through a regulatory region that is not part of the *wg*DRE.

Figure 4-6: Ecdysone signaling regulates Wg expression

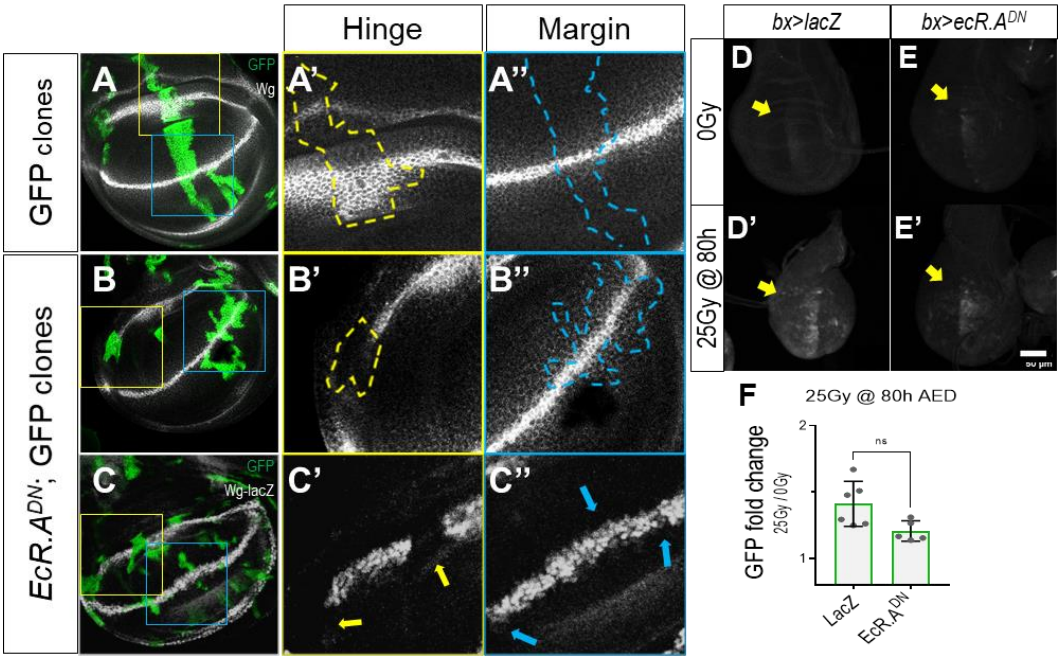

Figure 4-6: Ecdysone signaling regulates Wg expression - **(A-B)** Representative images of Wg (grey) expression in control, *UAS-GFP* alone (A), and *UAS-EcR.A*^{DN}*;UAS-GFP* (B), MARCM clones. Larvae were heat-shocked at 60hAED, and tissues were isolated at 104hAED. Zoom-in images of clones at the hinge (A'-A'') and margin (B'-B'') are shown on the right. Arrows indicate the region where clones cross the Wg expression at the hinge (yellow) and margin (blue). **(C)** Wg locus transcriptional activity - *UAS-EcR.A*^{DN}*;UAS-GFP* clones in *wg*-lacZ background. C' and C'' show clones at hinge and margin, respectively. **(D)** Ecdysone signaling cascade for the regulation of Wg expression.

Genotype – (A): UAS-mCD8-GFP,hs-Flp; tub-Gal4/+; FRT82B,tub-Gal80/FRT82B, (B): UAS-mCD8-GFP,hs-Flp; tub-Gal4/UAS-EcR.A^{W650A}; FRT82B,tub-Gal80/FRT82B, (C): UAS-mCD8-

GFP,hs-Flp;tub-Gal4/*wg*$^{en-11}$,UAS-EcR.A^{W650A};FRT82B, tub-Gal80/FRT82B, (D,F): Bx-Gal4/+; BRV118-GFP/+; UAS-Dcr2/UAS-LacZ.NZ, (E,F): Bx-Gal4/+; BRV118-GFP/UAS-EcR.A^{W650A}; UAS-Dcr2/+

As a transcription factor, EcR may directly regulate *wg* transcription. Previous work in our lab (Rajan Bhandari) demonstrates that several *wg* regulatory loci respond to systemic ecdysone levels. Figure 4-7 shows a summary of the findings of the study. We used lacZ-reporters of these loci to identify which loci are transcriptionally active in the wing disc and respond to increases (20E feeding) or decreases (erg6$^{-/-}$ feeding) in systemic ecdysone levels (β-gal staining images not shown). Of the 23 enhancer regions tested, three showed no transcriptional activity in the wing disc. We identified four (three, if I consider the overlapping loci as one) ecdysone-responsive loci. The *wg*DRE also responds to systemic ecdysone levels The overlapping enhancer loci (25C6 and 25B9) upstream of the *wg*DRE is the site of EcR enrichment (Gauhar et al., 2009) during L3 wing disc development. These findings support the hypothesis that EcR may act directly on a *wg* regulatory locus.

Figure 4-7: 20E-responsive and EcR binding sites on the wg regulatory locus

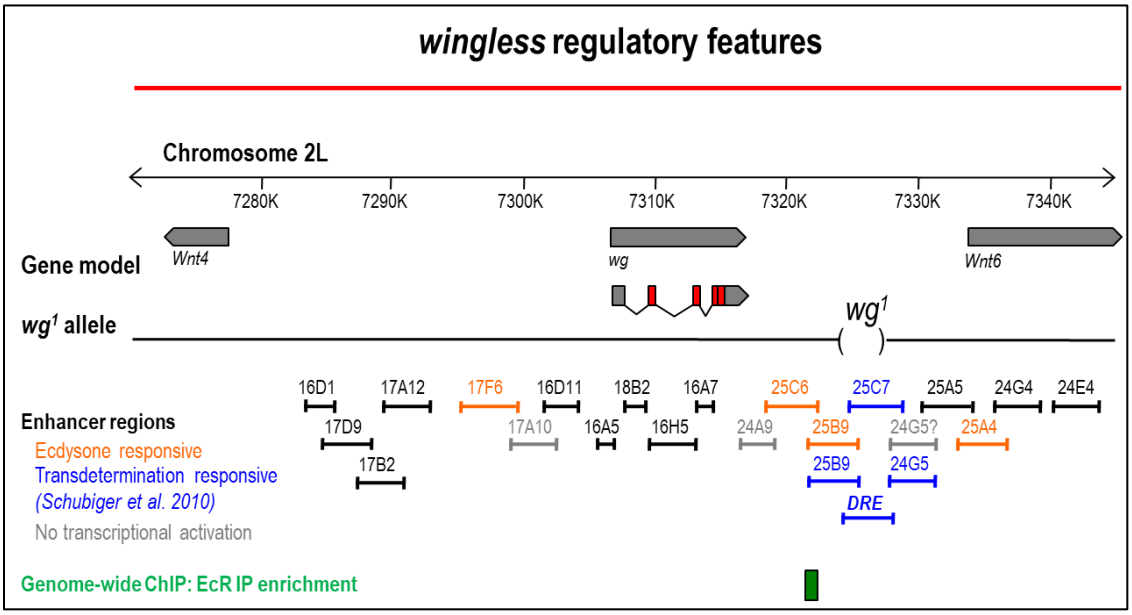

Figure 4-7: 20E responsive and EcR binding sites on the *wg* regulatory locus – The diagram represents the *wnt4-wg-wnt6* gene locus on the left arm of the second chromosome (2L). The diagram shows the location of the wg1 allele and the enhancer-transcriptional reporter. The enhancers associated with specific roles are color-coded; orange enhancers respond to systemic ecdysone levels, blue enhancers are activated during transdetermination events, and grey shows no transcriptional activity in the wing disc. A known EcR binding site is indicated by the green bar.

4.2.E Dilp8 promotes larvae viability, not tissue regeneration

I identified that ecdysone signaling both promotes and suppresses regenerative activity coordinating regeneration with the timing of developmental progression. However, it remained unclear what role Dilp8 played in regeneration. I identified that while Dilp8 and checkpoint activation is not necessary for providing additional time to accommodate regenerative growth, checkpoint activity is essential for maintaining the viability of pupae following regeneration.

Figure 4-8: *dilp8*$^{-/-}$ mutants show decreased viability

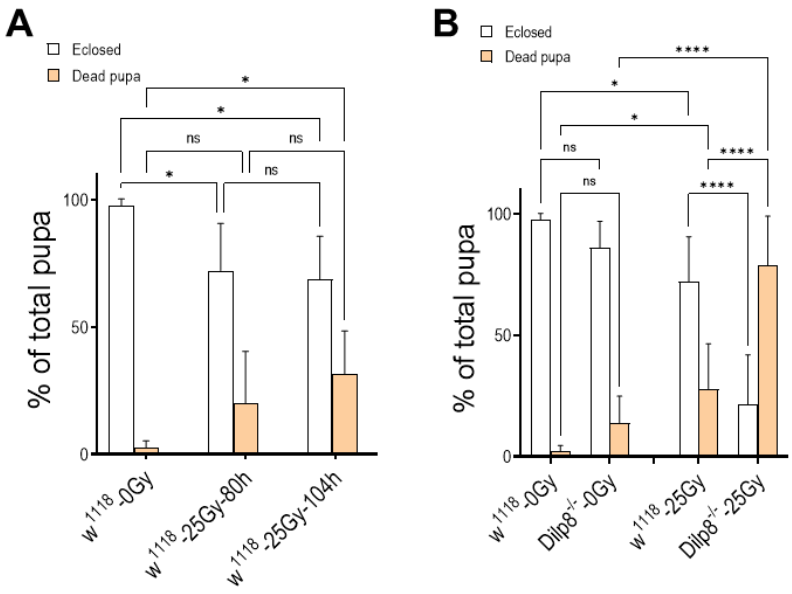

Figure 4-8: *dilp8*$^{-/-}$ mutants show decreased viability - **(A)** Quantification of population viability in *w*^{1118} following no damage -0Gy, early damage 25Gy-80h, and late damage 25Gy-104h. Irradiation damage decreases viability following both early and late damage *p<0.05, two-way ANOVA with Tukey's test. **(B)** *dilp8*$^{-}$ mutants showed decreased viability following early damage. The graph compares population viability following early damage (20Gy-80h) in *w*^{1118} and *dilp8*$^{-/-}$ larvae. ****p<0.0001, *p<0.05, 2-way ANOVA with Tukey's test.
Genotypes – (A): w^{1118} and (B): w^{1118} and *dilp8*^{MI00727} / *dilp8*^{MI00727}

The frequency of pupal lethality (pupal cases where the adults fail to eclose) in control larvae is relatively low, typically ~25% for larvae irradiated at 25Gy (Fig.4-8A). Irradiation damage has a similar level of lethality following early or late damage. However, pupal lethality of irradiated *dilp8*$^{-}$ larvae is much higher, ~78% (Fig.4-8B). I conclude that the increase in pupal lethality is a consequence of regenerative activity in *dilp8*$^{-}$ larvae. This conclusion is drawn from the late-irradiated (pos-RRP) control larvae, which fail to initiate a regenerative response but still produce a relatively low rate of pupal lethality

(~30%, Fig.4-8A). Therefore, Dilp8 checkpoint activation appears to play an essential role in preserving the future pupal viability of animals undergoing disc regeneration.

4.3 Discussion

4.3.A *Summary of Findings*

Regenerating imaginal discs release Dilp8, which circulates in the hemolymph and signals through Lgr3 in the brain and PG to limit ecdysone production (Colombani et al., 2012, 2015; Garelli et al., 2012; Jaszczak et al., 2016; Vallejo et al., 2015). By delaying the ecdysone increase that signals the end of larval development, Dilp8 extends the larval development period for regeneration (Colombani et al., 2012; Garelli et al., 2012). However, I demonstrate here that even in the absence of Dilp8 signaling, damaged wing imaginal discs are capable of the repatterning and regrowth required to reach their regeneration target in an attenuated regenerative period (Fig.4-2). However, I see that the accelerated regeneration, seen in *dilp8*$^{-}$ larvae, is also accompanied by a substantial increase in pupal lethality (Fig.4-7B). This pupal lethality does not appear to result from unrepaired damage, as larvae irradiated post-RRP when a regenerative response cannot be initiated do not show increased pupal lethality (Fig.4-7A). Therefore, the role of Dilp8 and regenerative checkpoint may be primarily to preserve viability in the presence of regenerating tissues instead of providing adequate regeneration time. In this study, I did not examine how regeneration checkpoint activation preserves pupal viability. One possibility is that an extended larval feeding period allows larvae with regenerating tissues the ability to store up sufficient energy reserves for both regeneration and completion of metamorphosis. Further study is necessary to determine how regeneration impacts pupal viability.

4.3.B EcR regulation of Wg expression

Ecdysone signaling activates gene expression for developmental transitions (Beckstead et al., 2005; Bender et al., 1997; McBrayer et al., 2007). In this chapter, I demonstrate that ecdysone signaling promotes Wg transcription through binding outside the *wg*DRE (Figure 4-6D-F). However, EcR may be regulating the *wg*DRE indirectly. Our reporter only tells us if EcR regulates the *wg*DRE locus directly as the transgenic construct only includes the DRE sequence (Harris et al., 2016). Given that an EcR binding site is adjacent to the *wg*DRE (Fig.4-8), it is possible that EcR may regulate *wg*DRE accessibility following damage.

Our study used EcR^{DN} mutant, which tells us how the cells respond to changing ecdysone levels. We used EcR^{DN} while bearing in mind that EcR can have gene-regulating activity without binding its ligand, 20E (Mansilla et al., 2016; Schubiger et al., 2003). The EcR-USP complex may bind to a locus at one developmental stage and release the DNA binding when EcR binds 20E. This explains the phenotypic discrepancies in studies that opt for EcR^{DN} vs. EcR^{RNAi}. For example, expression of EcR^{DN} limits Br expression, while expression of EcR^{RNAi} promotes Br expression (Uyehara & McKay, 2019), suggesting that EcR suppresses Br expression until 20E is bound. EcR alone acts as a temporal gate to block precocious gene expression and entry to the next developmental. 20E acts as the temporal trigger to promote developmental progression programs (Uyehara & McKay, 2019). Whether EcR-USP is actively suppressing *wg* transcription (during early L3) before ecdysone levels increase is unclear. Further investigation is needed to verify the timing of EcR binding.

In *Drosophila,* the ecdysone receptor has three isoforms, EcR.A, EcR.B1, and EcR.B2. EcR.A and B1 are both expressed in the wing disc(Cherbas et al., 2003; Jindra et al., 1996). The temporal and spatial dynamics of EcR isoforms during L3 wing disc development are not yet known. I primarily studied the effects of EcR.A on regenerative activity due to its spatially regulated control over *wg* expression. Unlike EcR.B1, which regulates overall Wg expression (hinge and margin), EcR.A only regulates Wg expression at the hinge (*see Appendix A6*). It is unclear if EcR.B1 regulation of Wg is via similar or unique mechanisms. A caveat of using $EcR.A^{DN}$ is that overexpression of one dominant-negative (DN) may be masking the EcR isoform-specific roles. The overexpressed DN isoform may be binding to DNA loci that would normally preferentially bind other isoforms. Therefore, further studies are needed to delineate EcR.A and EcR.B1 specific roles.

4.3.C Mechanisms of promoting regenerative growth

Ecdysone signaling promotes regenerative growth (Fig.4-4I), but it is unclear with the growth promotion is through Wg signaling. Recently published experiments have observed that damage of the *Drosophila* hindgut during L2 or early L3 produces Dilp8, delaying the onset of pupariation. In contrast, damage to the hindgut of wandering L3 larvae no longer produces Dilp8 or developmental delay. However, regeneration is still completed in this attenuated period through accelerated mitotic cycling, which allows the tissue to meet the regeneration target within the mitotic regeneration window (Cohen et al., 2021). In this example, while ecdysone signaling regulates the end of mitotic regeneration, the mechanisms of producing accelerated mitoses remain unknown.

The biphasic, concentration-dependent regulation of regenerative activity allows ecdysone to coordinate regeneration with the duration of larval development. This coordinating role may be similar to how ecdysone synchronizes imaginal disc patterning with the larval development period (M. M. Oliveira et al., 2014). The mechanism that ensures this coordination is still unknown but maybe through the promotion of Wg expression. However, I see only a moderate increase in Wg expression in undamaged tissues following 20E feeding (Fig.4-4H). This suggests that ecdysone may be activating additional Wg expression following damage or that regenerating tissues are more sensitive to ecdysone signaling.

Although my findings show that ecdysone signaling influences Wg expression, it is possible that the increased growth rate during regeneration is not through the activation of Wg but an alternative pathway. The most likely target of EcR signaling would be the Hippo signaling pathway. Hippo signaling is the primary regulator of tissue growth and final tissue size (Boone et al., 2016) and is also regulated by JNK signaling during regeneration (Sun & Irvine, 2011). There is evidence that EcR coactivator Taiman interacts with the Hippo transcriptional coactivator Yorkie (Yki) and promotes the expression of canonical Yki-responsive genes that promote growth in somatic tissue (C. Zhang et al., 2015). Taiman requires EcR to support Yki-driven tissue growth, making it a plausible target for ecdysone-induced tissue growth during regeneration.

Chapter 4 Appendices

A primary concern with the increased activation of Dilp8 and Wg in 20HE feeding experiments (Fig.4-4) is that the increased levels of ecdysone sensitized the tissue to irradiation damage. To address this, I assessed the level of apoptosis in the ecdysone-fed tissues (Appendix A4) and validated the findings using an alternative (genetic) damage model (Appendix A5).

A4: *Apoptosis data for chapter 4*

When ecdysone levels are high, tissues show higher numbers of apoptotic cells. Undamaged 20HE fed tissues show no increases in apoptosis (Fig.A4-1A-E). However, damaged tissues in 20HE fed larvae show increased cell death (Fig.A4-1A'-E'). This 20HE induced increase in cell death is also seen in *dilp8*^{KO} and *lgr3*^{KO} knockout lines. Although *dilp8*^{KO} and *lgr3*^{KO} mutants have more apoptotic cells, the dead cells are already extruded from the primary epithelium (Fig.A4-1F'', I'', J''). The increased apoptosis may be a part of the regenerative response. With increasing 20HE concentration, the tissue seems to be further along in the repair process, showing an increase in the number of cells extruded from the primary epithelium. The cells are extruded at the margin as seen in Fig.A4-1D' and E.'

Figure A4-1: Tissues show increased cell death during regeneration when ecdysone levels are high

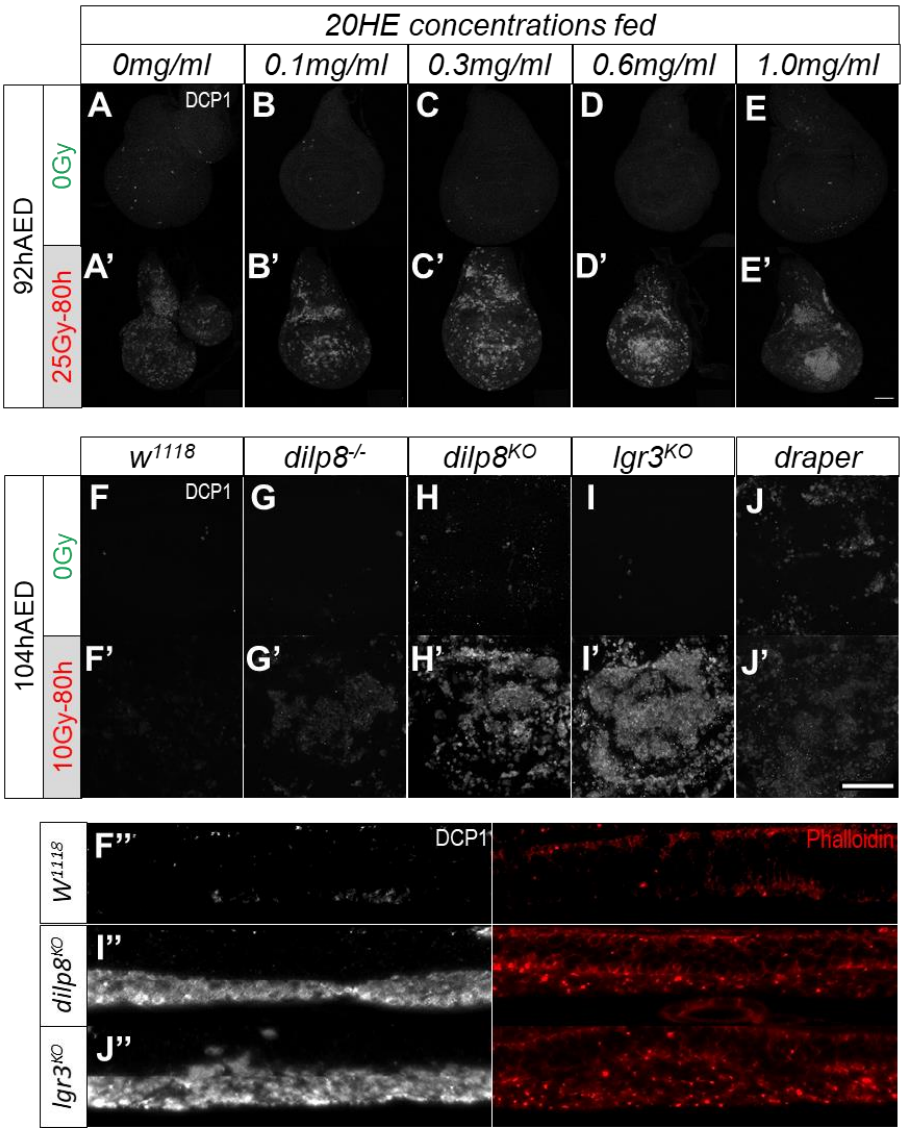

Figure A4-1: Tissues show increased cell death during regeneration when ecdysone levels are high – (A-E) shows the DCP1 staining in 92hAED w^{1118} tissues following 20HE feeding. w^{1118} larvae were fed 20E: 0mg/ml (A-A'), 0.1mg/ml (B-B'), 0.3mg/ml (C-C'), 0.6mg/ml (D-D') and 1.0mg/ml (E-E'). **(F-J)** shows apoptotic cells in control (F), *dilp8* knockout lines (G-H), *lgr3* knockout line (I), and *draper* mutant line (J). Scale bar = 50um. F'', I'' and J'' show cross-sections through the wing pouch. The epithelium can be seen in phalloidin (red) stained tissues

Genotypes – (A-F): w^{1118}, (G): $dilp8^{MI00727}$/ $dilp8^{MI00727}$, (H):$dilp8^{KO-/-}$ (I): $lgr3^{KO-/-}$ (J):$draper^{D5-/-}$

A5: *Genetic damage (eiger) data for chapter 4*

Figure A5-1: Ecdysone regulates activation of regenerative signaling in the eiger damage model.

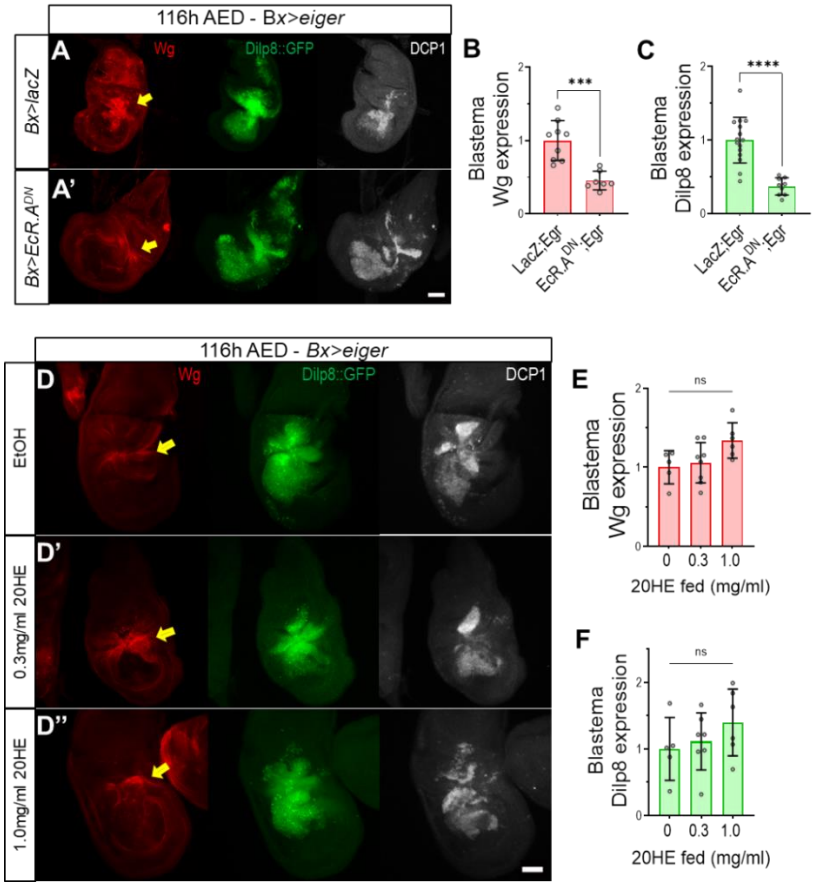

Figure A5-1: Ecdysone regulates activation of regenerative signaling in the eiger damage model - (A-C) Representative images of Wg, Dilp8 (Dilp8::GFP), and DCP1 (marker for apoptotic cells) expression in eiger damaged tissues *(Bx>eiger*). The eiger damaged tissues co-express *lacZ* (A) and *EcR.A*^{DN} (A'). Yellow arrows indicate the area of eiger expression (the regeneration blastema). Blastema Wg and Dilp8 were quantified in (B) and (C), respectively. Loss of ecdysone signaling leads to decreased Wg and Dilp8 in the blastema. ***p<0.001, ****p<0.0001, Mann Whitney t-test. **(D-F)** Representative images of Wg, Dilp8, and DCP1 expression in *Bx>eiger* tissues of larvae fed EtOH-0mg/ml (D), 0.3mg/ml (D'), and 1.0mg/ml (D'') of 20E. Yellow arrows indicate the regeneration blastema. Wg and Dilp8 were quantified in (E) and (F), respectively. Data normalized to 0mg/ml tissues, One-way ANOVA with Tukey's test. **Genotypes** – (A-F): Bx-Gal4/+; UAS-Eiger/+; Dilp8^{MI00727}/UAS-LacZ.NZ

A6: *Regulation of wg expression by other EcR^{DN}*

I wanted to investigate whether the regulation of *wg* expression was unique to the EcR.A isoform. I found that the regulation is spatially unique. EcR.A regulated Wg expression at the hinge and not the margin. EcR.B1 regulated Wg expression in both the hinge and the margin. EcR.B2, which is not expressed in the wing disc, did not affect Wg expression

Figure A6-1: EcR regulation of Wg expression (hinge vs. margin)

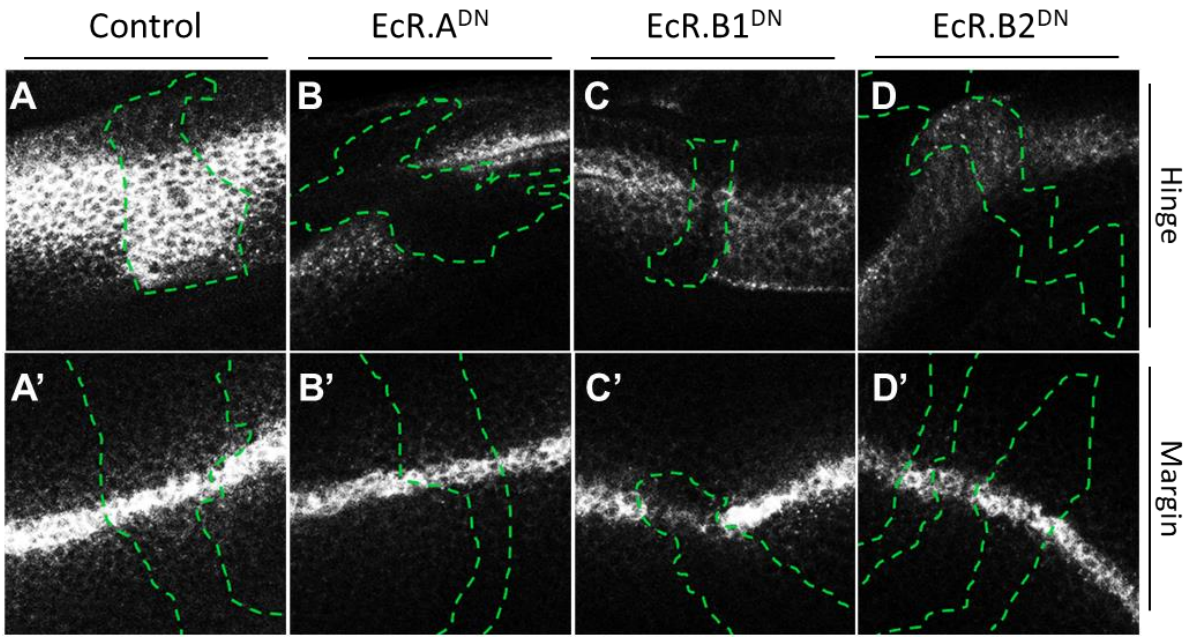

Figure A6-1: EcR regulation of Wg expression (hinge vs. margin) – The figure shows zoom-in images of Wg expression in control (**A** - GFP alone) and EcR^{DN} isoform (**B** – EcR.A^{DN}, **C** – EcR.B1^{DN}, and **D** – EcR.B2^{DN}) mutants. The isoforms have similar mutations in the ecdysone binding domain. (A-D) shows Wg expression at the hinge, while (A'-D') shows Wg expression at the margin. The green dotted lines outline the area of transgene expression.

Genotype – (A): UAS-mCD8-GFP,hs-Flp; tub-Gal4/+; FRT82B,tub-Gal80/FRT82B, (B): UAS-mCD8-GFP,hs-Flp; tub-Gal4/UAS-EcR.A^{W650A}; FRT82B,tub-Gal80/FRT82B, (C): UAS-mCD8-GFP,hs-Flp; tub-Gal4/UAS-EcR.B1^{W650A}; FRT82B,tub-Gal80/FRT82B, (D): UAS-mCD8-GFP,hs-Flp; tub-Gal4/UAS-EcR.B2^{W650A}; FRT82B,tub-Gal80/FRT82B,

CHAPTER 5: Discussion and Future Directions

5.1 Discussion

5.1.A Summary of combined findings

For most organisms, regenerative capacity varies at different stages of development. Previous studies have demonstrated that changes in hormone signaling contribute or coincide with changes in regenerative capacity. The findings seemed contradictory, with some animals requiring hormone signaling for regeneration, whereas others require hormone signals to suppress regeneration. Here I show that steroid hormone signaling is necessary to activate and suppress regenerative activity in *Drosophila* imaginal discs (Fig.5-1A).

The goal of regeneration is to restore the tissue pattern and function while catching up with the development of undamaged tissues. My work shows that hormone signaling is necessary for restoring tissue patterns and helps restore inter-organ growth coordination. Regenerating tissues have a biphasic response to hormone signaling that is dependent on hormone concentration and developmental stage. Lower concentrations such as those found in early L3 development promote regenerative activity. As 20E concentrations increase, the regenerative activity also increases. During this stage, ecdysone signaling ensures imaginal discs are regenerated and catch up with the development of undamaged tissues whether the larval developmental period is short (*dilp8*$^{-}$) or long (*dilp8*$^{+}$). However, once ecdysone levels reach a certain threshold, any ongoing regeneration is brought to a close, and cells lose regenerative capacity. In the next section, I will discuss the sequence of hormone signaling-induced events that facilitate ecdysone's dual role in regulating regeneration.

Figure 5-1: Model of biphasic coordination of regenerative capacity and activity

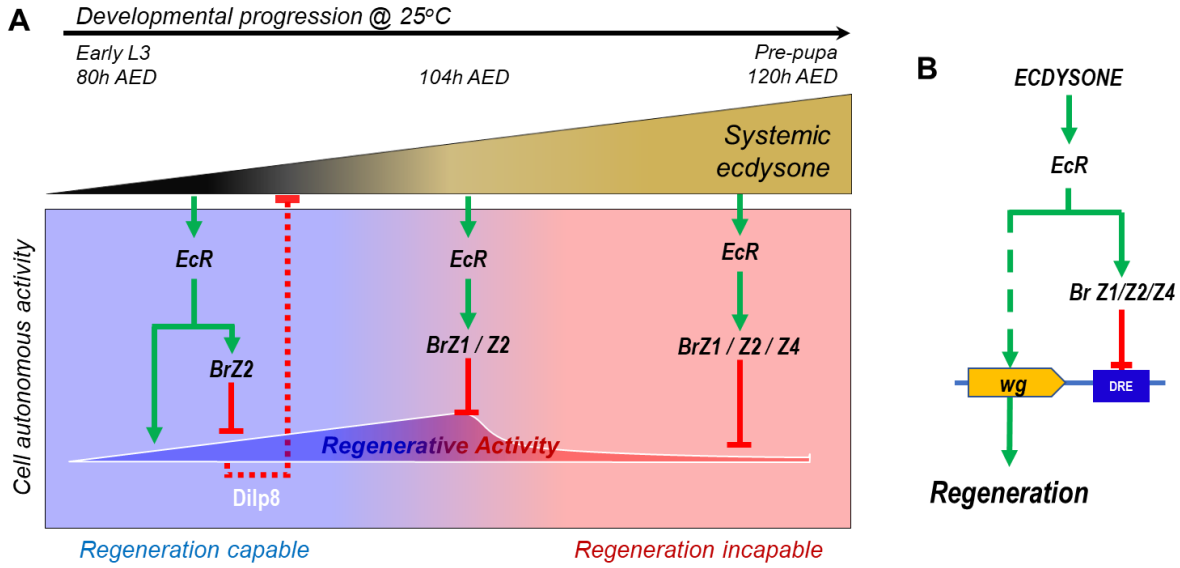

Figure 5-1: Model of biphasic coordination of regenerative capacity and activity – (A) - During early L3, lower ecdysone concentrations promote activation of regenerative signaling following damage. However, as ecdysone levels increase with the progression of development, the expression of Br splice isoforms leads to suppression of regenerative signaling. Eventually, the capacity to activate a regenerative response following damage is lost. **(B)** - Schematic of how ecdysone signaling achieves the dual regulation of *wg* expression

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5.1.B Breaking down hormone signaling during regeneration

Similar to the observations in deer antler regeneration, *Drosophila* imaginal disc regeneration seems to have a complex response to changing systemic hormone levels. Here I discuss how ecdysone regulates the regenerative activity and the possible interaction of signaling from ecdysone and other hormones present during regeneration.

In *Drosophila,* the Prothoracic glands (PG) act as the signaling center for coordinating developmental progression in response to environmental cues (McBrayer et al., 2007;

Shimell et al., 2018). The PG produces pulses of ecdysone that coordinate developmental progression and the rate of tissue growth. Apart from the pulses that initiate larval molts, pupation, and metamorphosis, other ecdysone pulses are produced in larvae (Fig.1-2). During L3, *Drosophila* larvae produce three small pulses of ecdysone before the final large pulse that initiates pupation (M. M. Oliveira et al., 2014). Most imaginal discs' growth and development occur during this L3 phase, through the progressive increase of systemic 20E from these four pulses (Lavrynenko et al., 2015; Warren et al., 2006). From my findings, I presume that regenerative activity is also limited to the period between three if not all four of the L3 ecdysone pulses. In our experiments, wing discs show gain and loss of regenerative capacity between the estimated critical weight and wandering periods (*discussed below*). Therefore, I will discuss the role of hormone signaling in regeneration according to the timing of these pulses and what they might mean for regenerative activity and coordination of developmental progression.

a) <u>Early-L3 pulse – Critical Weight pulse</u>

The first L3 ecdysone pulse, called the critical weight (CW) pulse, is controlled by nutrient storage in the fat body (Davidowitz et al., 2003; Warren et al., 2006). In our experiment set-up, the timing of the critical weight pulse is approximately 72hAED. Once the critical weight is reached, starvation will no longer delay the onset of pupation (Warren et al., 2006). CW pulse changes the body metabolism promoting storage over the consumption of different metabolites to ensure the animal has sufficient nutrients to undergo metamorphosis (Yamada et al., 2020). I hypothesize that this CW pulse is the signaling that allows activation of regenerative signaling. Lack of ecdysone signaling in

early-L3 limits the activation of local (Wg) and systemic (Dilp8) regenerative signaling (*see Chapter 4*).

At the same time, ecdysone is promoting the expression of BrZ2, which actively suppresses regenerative activity. At the CW, Juvenile hormone (JH) production stops, and systemic levels would taper out (C. Mirth et al., 2005; Lynn M. Riddiford et al., 2010). In the general epidermis of *Manduca*, pupal commitment and Br expression are induced by 20E acting in the absence of JH. JH is known to antagonize ecdysone activity, maintaining the imaginal discs in a developmental arrest during the intermolt periods (Lynn M. Riddiford et al., 2010). Very little is known of JH's mechanism of action; however, JH regulates the expression of some 20E regulated genes *in-vitro* (Beckstead et al., 2007; Baohua Zhou et al., 1998). JH's regulation of ecdysone signaling may be transcriptional (EcR coactivators) or by regulating the conversion of ecdysone to its active form (20E) by the P450 enzyme, *shade*. Either way, the drop in JH levels may allow ecdysone signaling to both promote regenerative activity and express BrZ2, which limits regeneration.

At CW, expression of the BTB transcription factor Chinmo is suppressed in the wing disc (Narbonne-Reveau & Maurange, 2019). Chinmo is associated with the regenerative potential of cells. Narbonne-Reveau & Maurange argue that ecdysone signaling suppresses Chinmo expression through BrZ1 expression and that the transition from self-renewing tissues to differentiated tissues begins at CW. Taking their findings together with mine, it is unclear how Chinmo interacts with EcR to maintain regenerative capacity, especially when Chinmo also promotes EcR expression in other tissues (Marchetti & Tavosanis, 2017). However, Chinmo is associated with preventing

alternative splicing (Grmai et al., 2018) and may regulate the alternative splicing of BrZ2 to BrZ1. This could explain why BrZ1 increases as Chinmo decreases.

b) Mid-L3 pulse – Glue secretion pulse

As the larvae approach pupation, they begin to prepare for the developmental transition. This preparation includes producing the glue used to adhere to a surface during pupation (Kaieda et al., 2017). An ecdysone pulse initiates the glue secretion (GS) process in mid-L3 (Warren et al., 2006). For imaginal discs, this means an increase in ecdysone signaling and no JH signaling. During this time, the communication between the larvae and the imaginal discs is essential for growth coordination. As ecdysone levels increase, the regenerating tissues accelerate their growth. This response to systemic ecdysone levels seems more pronounced in damaged tissues than undamaged tissues (Fig.4-4), suggesting that regenerating tissues may be more sensitive to ecdysone signaling. In both damaged and undamaged tissues, ecdysone signaling plays the role of a wind-up clock that signifies the approaching end of the larval development period. In turn, the regenerating tissues are secreting the peptide hormone Dilp8 according to the extent of damage in the tissue. Dilp8 signaling is part of a feedback loop that coordinates the regenerating tissue's development.

c) Late-L3 pulse – Wandering pulse

Another major step in the preparation of pupation is finding a dry surface to perch for the process. The larvae exit the food and begin wandering to find a suitable spot for pupation. A pulse of ecdysone precedes this period of wandering (Warren et al., 2006). In our experiment set-up, wandering starts around 110hAED in undamaged discs, and I see a loss of regenerative capacity at 104hAED. The wandering pulse is most likely the

pulse that pushes systemic levels of ecdysone over the ‘threshold.’ At this threshold, ecdysone signaling switches from regeneration-capable to regeneration-incapable. BrZ1 expression is more pronounced at this stage (Fig.3-4A). BrZ1 and BrZ2 have a shared promoter, so, understandably, both isoforms would increase with increasing ecdysone levels. However, the drastic increase in BrZ1 levels suggests changes in *br* mRNA splicing. During this wandering ecdysone pulse, BrZ1 signaling begins the process of cell differentiation and epigenetic silencing of Damage Responsive Enhancers (DREs). This pulse limits any ongoing regenerative activity in regenerating tissues, explaining why the loss of Br isoforms leads to extended regenerative periods (Fig.3-6, 3-7).

d) Pre-pupa ecdysone pulse

Once larvae have found a suitable pupation spot, the cuticle begins hardening to form the pupal casing. Proliferation and morphogenesis of larval tissues continue until pre-pupa at approximately 120hAED. The larva-to-prepupa transition is initiated by an ecdysone pulse greater than the previous L3 pulses (Warren et al., 2006). In addition to ecdysone signaling, holometabolous insects show a reemergence of JH signaling (Hiruma, 2003; Lynn M. Riddiford & Ashburner, 1991). JH signaling prevents premature adult differentiation in imaginal structures (L. M. Riddiford, 1972). Similar to early L3, this combined JH and ecdysone signaling may affect the loss of regenerative capacity and development transition. I see the expression of BrZ4 at 116hAED, suggesting that the high hormone levels promote BrZ4 expression. BrZ4 shares its promoter with BrZ1, so this is likely a splicing regulation for BrZ4 expression. BrZ4 may be the main effector of the larva-pupa transition in the wing disc as it had the most robust suppression of regenerative activity.

5.1.C The role of Dilp8 in regeneration

For survival and sufficient growth, each developmental stage's duration and tissue growth rate must be precisely regulated in response to environmental conditions. *Drosophila* Insulin-like proteins (Dilps) produced by peripheral tissues in response to nutrient availability influence the timing of ecdysone pulses from the prothoracic gland (PG) (Koyama et al., 2014; C. K. Mirth et al., 2009; Nässel et al., 2015). Recent findings have demonstrated that the Dilps communicate different messages to the PG depending on developmental timing (Kannangara et al., 2021). Prior to the CW pulse, Dilp-signaling promotes ecdysone production (Colombani et al., 2005; C. Mirth et al., 2005; Shingleton et al., 2005); however, after CW, Dilp-signaling limits ecdysone production, extending the larval development period (Shingleton et al., 2005; Stieper et al., 2008). Correspondingly, the ecdysone biosynthesis pathway genes produce different transcriptional and translational responses pre- and post-CW, respectively (Gibbens et al., 2011). Our findings suggest that Dilp8's role is similar to Dilps as a regulator of post-CW energy homeostasis.

So far, Dilp8 has been set aside from other Dilps due to its close resemblance to mammalian relaxins, its signaling through the relaxin receptor Lgr3, and its stage (L3) specific expression following damage or tumor formation (Colombani et al., 2012; Garelli et al., 2012). From our findings, overall regenerative activity and Dilp8 expression appear to be limited to the period between the CW and wandering ecdysone pulses. The CW-to-wandering period is when larvae have stored sufficient resources to fuel metamorphosis but can still consume more food if any energy-consuming activities (e.g., tissue repair)

arise. If food is unavailable, the decreased Dilp signaling enhances ecdysone production, and the larvae pupate prematurely. During this stage, Dilp8 plays a similar role to the Dilps in slowing down ecdysone production and lengthening the CW-to-wandering development period. Dilp8 is produced following perturbations in the development of imaginal discs, extending the larval period. It was previously presumed that the purpose of this extension was only to provide additional time for disc repair and growth. However, I determined that regeneration is completed even without the Dilp8 induced developmental delay (Fig4-1). Dilp8 is instead necessary for survival through metamorphosis to adult. Therefore, the extension of the CW-to-wandering developmental period is likely to be necessary for additional nutrient consumption. This way, Dilp8 signaling acts as an indicator of an energy-consuming event, extending the developmental period to accommodate additional nutrient consumption by the larva.

Although I identified a primary role of Dilp8 in nutrient homeostasis during regeneration, Dilp8-Lgr3 signaling also regulates developmental symmetry. Previous studies have shown that *dilp8*$^{-/-}$ mutants have asymmetric wings (Colombani et al., 2012). In our study, the damaged wings match undamaged wing size, but I have not determined whether the left and right wings match in size. In addition, to symmetry coordination, *Dilp8* transcript levels increase following development-transition ecdysone pulses (J. B. Brown et al., 2014). However, it is unclear if these transcripts are translated into a functional Dilp8 protein. Therefore, the function of Dilp8 at these developmental stages remains unclear. Unlike the hypomorphic *dilp8* allele we use in our experiments (Dilp8::GFP), we find that larvae homozygous for a null allele (*dilp8*^{KO}) die during pupation, even in the absence of tissue damage. This suggests that dilp8 is likely to have required functions

during metamorphosis. Further studies are necessary to understand the role of Dilp8 throughout *Drosophila* development.

5.1.D The cost of regeneration

In most animals, regenerative capacity is lost with developmental progression, and it is unclear why, evolutionarily, developmental progression restricts regeneration. In arthropods, regenerative capacity is tied to the molting process, suggesting that the exoskeleton may limit regenerative potential (Gontijo & Garelli, 2018). The most recent theory is that the suppression of regenerative capacity by hormones may be a trade-off for acquiring other mechanisms vital to survival. Our study speculates that imaginal disc regeneration is an energy-intensive process that takes more energy than larvae have to spare. Completion of regeneration within the standard developmental period would cost the larvae their lives. Regenerative capacity is lost when pupation is near (following the wandering ecdysone pulse) as the larvae opt to save the accumulated energy resources for the energy-intensive metamorphosis.

In mammals, viability is also the cost of regeneration. In mammals, where regenerative capacity is lost soon after birth, the timing coincides with increases in systemic levels of thyroid hormone (Hirose et al., 2019; Marshall et al., 2019). The thyroid hormone regulates metabolism and allows for endothermy (warm-bloodedness) (Hirose et al., 2019). These dual roles for thyroid hormone have led to the hypothesis that mammals trade regenerative capacity for endothermy. Rapid growth occurs soon after birth; therefore, mammals reserve their acquired energy stores for maturation and the

generation of body heat. A similar argument has been made for the loss of regenerative capacity in the Xenopus heart (Liao et al., 2018).

5.2 Future Directions

Although I delineated the significant events of hormone signaling during the wing disc's development and regeneration, I am still far from understanding the cellular dynamics of hormone signaling. Here, I discuss the major questions that should be addressed in the future.

5.2.A How does ecdysone signaling activate regeneration?

My thesis research demonstrates that ecdysone signaling activates and promotes regenerative signaling. However, the mechanism by which ecdysone signaling does this remains unclear. Although we know that ecdysone signaling regulates Wg expression, this does not necessarily mean that the regulation is direct. To better understand how ecdysone regulates regenerative capacity, we need to assess whether the regulation is direct or indirect.

From our identification of ecdysone responsive enhancers and the EcR ChIP data, we have probable cause to believe that EcR regulates Wg expression directly at the *wg* locus. In addition, previous work demonstrates that trx, a subunit of gene activator Trithorax Group (TrxG), is necessary for the developmental checkpoint that allows regeneration to occur, and reducing trx copies impairs regeneration (Skinner et al., 2015). To determine how EcR regulates the *wg* regulatory locus, we need to do a detailed

assessment of EcR enrichment and TrxG-induced chromatin modifications in the wing discs throughout the L3 development period. After identifying the binding sites, targeted deletions would determine whether they are necessary for regeneration. There is a possibility that there would be no changes in EcR enrichment. If this is the case, we would need to assess how other factors may contribute to the changes in EcR signaling. We would start with assessing hormone signaling-induced events that are known to change at this time, and downstream targets EcR and JH signaling would be a good starting point.

EcR.A may be regulating Wg expression at the hinge through the regulation of other genes. The regulation of *wg* expression at the hinge is activated and maintained by the cooperative signaling of Nubbin (*nub*), Vestigial (*vg*), and Rotund (*rn*) during development (Rodríguez et al., 2002). Loss of expression in any of these genes leads to complete loss of Wg at the hinge (Rodríguez et al., 2002). I hypothesize that EcR.A may be regulating *wg* expression at the hinge through the regulation of one of these genes. Therefore, I would encourage the investigation of whether EcR.A influences the expression of the genes *nub, vg,* or *rn.*

Ecdysone may also be activating the expression of Dilp8 independent of Wg. Our experiment with wg^{1} mutant wings was inconclusive (Appendix 7) and needed to be redone using MARCM clones of wg^{1}. However, studies show that a pulse of ecdysone precedes increases in *dilp8* mRNA throughout *Drosophila* development. If Dilp8 expression is activated independent of Wg, a similar assessment of EcR binding sites and possible *dilp8* transcription regulators should be done.

5.2.B How do Br isoforms regulate gene expression?

Three important questions should be addressed when studying the Br isoforms. The first is how the expression of the Broad isoforms is temporally regulated. The broad isoforms share common promoter sites; therefore, isoform regulation is likely at the RNA splicing level. However, we have no information on which genes regulate *broad* splicing. Given the expression profile of Chinmo and its known role in regulating differential splicing, I hypothesize that it might regulate the splicing between BrZ2 and BrZ1. However, a more extensive search for splicing-regulation candidates is needed.

The second question we should address is how the Broad isoforms suppress the wgDRE and DRE's of other regeneration genes. Although we found possible *broad* binding sites on the wgDRE, we still need to verify if Br isoforms bind to the site during wing development. If the isoforms do not bind to the wgDRE, further investigation into the genes that mediate Br induced wgDRE suppression is needed.

The last question to address is how Broad regulates PcG activity. In addition to regeneration genes, Br induces other gene expression changes involving trxG and PcG regulation. However, very little is known on how Br isoforms mediate these changes. Our findings indicate that Br regulates PcG activity and that this interaction may be direct. However, it is unclear which isoforms interact with and regulate the PcG proteins during wing disc development

5.2.C *How does Dilp8 regulate Drosophila viability?*

Our lab and others previously assumed we understood the role of Dilp8 in regeneration; however, my findings shed new light on its role. In future studies, we would focus on identifying if Dilp8 regulates energy homeostasis during regeneration. We would assess this by manipulating aspects of the energy homeostasis during regeneration. For example, we would assess whether limiting feeding in regenerating wild-type (W^{1118}) larvae have the same effect on pupa viability as we see in *dilp8*$^{-}$ mutants. Alternatively, we could assess the inverse by feeding a high caloric diet to dilp8- mutants and assessing pupa viability. Since L3 is a growing phase for larvae, manipulating metabolic pathways may affect growth and survival, independent of regeneration. Aside from energy homeostasis, it is possible that Dilp8 signaling is necessary for other pupa-specific mechanisms. Therefore, if the energy homeostasis hypothesis is rejected, we would screen for genes that alleviate pupa lethality in regenerating *dilp8*$^{-}$ mutants.

5.3 Conclusions

The goal of regenerative medicine is to use the host's body systems, sometimes with the help of clinically introduced cells, scaffolds, or signaling molecules, to rebuild tissues and organs (Griffith, 2002; Lutolf & Hubbell, 2005). Although understanding molecular mechanisms involved in the regeneration process is essential, it does not explain the varying degrees of regeneration quality, timing, or differences in regenerative ability depending on extrinsic or intrinsic factors. In this study, I demonstrate that hormone

signaling from the damaged tissues (Dilp8) and the whole organism (ecdysone) play a crucial role in determining the regeneration outcomes of the tissue. As systemic levels of ecdysone increase, the rate of regenerating tissue growth increases. However, past a certain threshold, ecdysone initiates signaling that restricts regeneration. We are still a long way from fully understanding how hormone signaling regulates regenerative activity. Much more research is needed to investigate the role of hormones that vary with age, sex, and states of stress and disease, as the efficacy of any treatment to promote regeneration in human patients could vary with each of these factors.

Additional Appendices

A7: *Dilp8 activation requires Wg expression*

The *wg*^{1} mutants cannot regenerate their tissues; however, it is unclear whether systemic signaling for regeneration depends on *wg*DRE activation. Using *wg*^{1} mutants with a Dilp8::GFP background, I assessed whether Dilp8 expression is dependent on Wg (*wg*DRE). *Wg*^{1} mutants show no Wg expression in the pouch-like region (Fig.A6-1B). Following damage, I see little to no activation of Dilp8 in the *wg*1 tissues (Fig.A6-1B'). Although the findings are promising and support the hypothesis that Dilp8 expression is dependent on Wg, there are still points of contention. It is unclear whether the small area of Dilp8 expression (Fig.A6-1B') is a miniaturized pouch due to *wg* misexpression.

Figure A7-1: Dilp8 expression is dependent on Wg expression

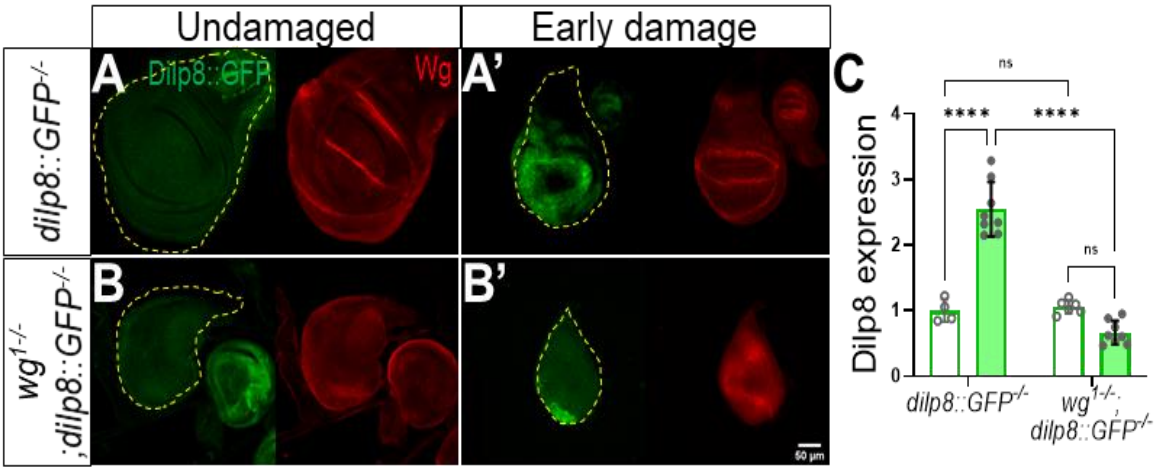

Figure A7-1: Dilp8 expression is dependent on Wg expression - (A-B) WgDRE is necessary for Dilp8 activation following irradiation damage. Representative images of pouch Dilp8::GFP (green) and dorsal hinge Wg (red) expression in 104h AED wing imaginal discs in undamaged (A-B) and early damage – 2.5kR @ 92h AED (A'-B'). Loss of *wg*DRE (*wg*$^{1-/-}$*;dilp8::gfp*$^{-/-}$) (B-B') leads to decreased Dilp8::GFP expression compared to controls - *dilp8::gfp*$^{-/-}$ (A-A'). **(C)** Quantification of Dilp8::GFP expression following early damage in *dilp8::gfp*$^{-/-}$ and *wg*$^{1-/-}$*;dilp8::gfp*$^{-/-}$. ****p<0.0001, Mann Whitney t-test.

A8: *Apoptosis is an essential part of the regenerative response*

As part of collaborative work in the lab (Cristina DÁncona), we investigated the effects of knocking down cell death genes. We had presumed that loss of apoptosis genes would lead to loss of regenerative activity; however, the opposite is true. Loss of apoptotic activity in the tissue leads to neoplastic tissue overgrowth and over-activation of regeneration pathways. We experimented using both eiger damage and irradiation. We found that tumor formation occurs closer to the end of the larval developmental period in both cases. The timing of tumor formation suggests an association with ecdysone signaling at the time.

Figure A8-1: Inhibiting apoptosis during regeneration induces neoplasia

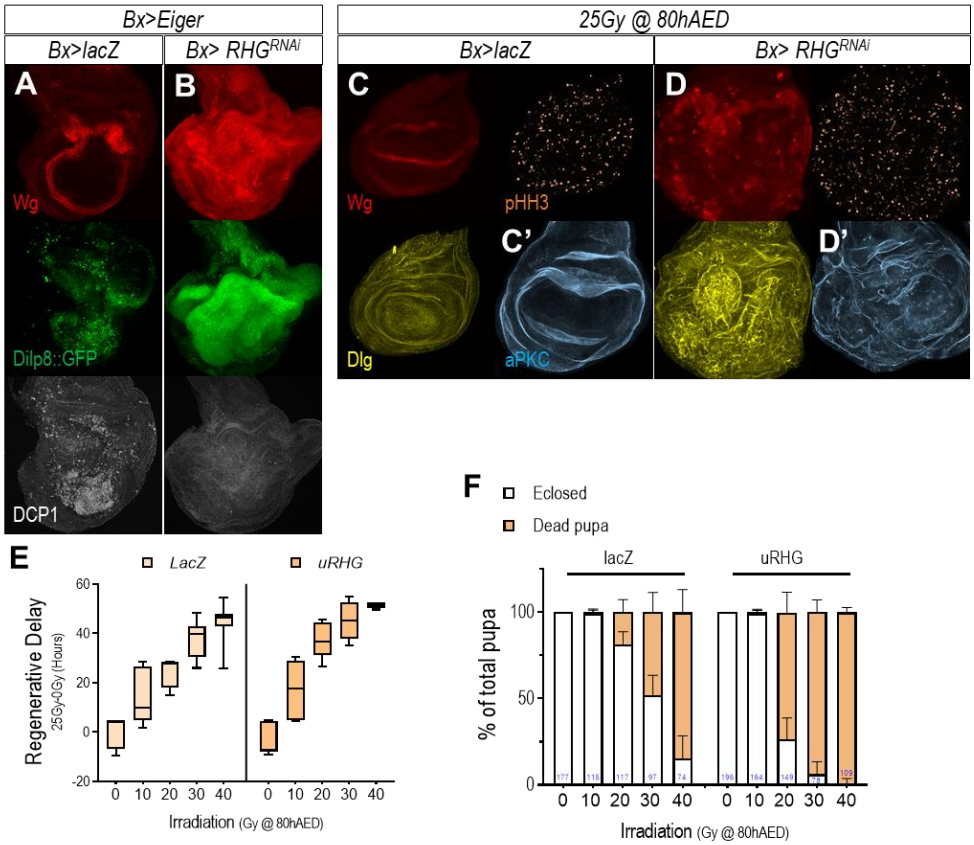

Figure A8-1: Inhibiting apoptosis during regeneration induces neoplasia - (A-B) Representative images of 140hAED wing discs of Wg (red), Dilp8 (green), and DCP1 (grey) expression in eiger damaged tissues *(Bx>eiger)* expressing *lacZ* (A) as control or *uRHG* (B). Loss of apoptosis genes *rpr, hid,* and *grim* cause neoplastic tumor formation **(C-D)** Representative images of 140hAED wing discs with Wg (red), and Dlg (yellow), pHH3 (pastel), and aPKC (blue) staining following irradiation damage in *lacZ* (C) and *uRHG* (D) expressing tissues. Similar to eiger damage, irradiation damaged tissues form neoplastic tumors. (**E-F**) Quantification of regenerative delay (E) and pupa viability (F) following increasing irradiation damage in *lacZ* and *uRHG* expressing tissues. Loss of apoptosis induced increased regenerative delay and a rapid decrease in pupa viability with increasing irradiation damage.

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