THE RIGHT SIZE: THE HORMONAL AND TRANSCRIPTIONAL REGULATION OF GROWTH IN *DROSOPHILA MELANOGASTER*

By

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ABSTRACT

THE RIGHT SIZE: THE HORMONAL AND TRANSCRIPTIONAL REGULATION OF GROWTH IN *DROSOPHILA MELANOGASTER*

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Regulation of final organ size is a complex developmental process involving the integration of systemic, organ-specific and environmentally regulated processes. Together these processes enable co-ordination of organ growth with body growth and the achievement of correct organ size. Dysregulation in these processes causes over- or under growth of organs resulting in compromised organ function. Understanding the mechanisms contributing to regulation of organ size is therefore key to understanding organ function.

My research has focused on understanding two distinct aspects of developmental growth control. Firstly, what role does systemic hormonal signaling play in regulating final organ size? Second, what is the role of transcriptional regulation of the Insulin Receptor (InR) gene in regulating final organ size? I have addressed these questions through a variety of genetic and biochemical tools in the model system of the common fruit fly *Drosophila melanogaster*.

In order to understand the role of hormonal signaling, I used the model of the wing imaginal disc to show that growth retardation in one part of the imaginal disc results in coordinated reduction in growth rate in the unperturbed part. Further, I show that this is mediated through systemic signaling by the insect hormone 20-hydroxyecdysone. Lastly, I demonstrate that systemic ecdysone signaling interacts with organ-autonomous insulin signaling to mediate growth coordination across the imaginal disc.

To understand the role of transcriptional regulation of *InR*, I generated transgenic flies with increased dosage of *InR*. Using this transgenic fly line, I identified the minimal *cis*-

regulatory region of *InR* and the effects of increased dosage of *InR* on aspects of fly physiology. I further show that the *InR cis*-regulatory region consists of multiple enhancers, which are capable of driving tissue-specific reporter gene expression. Lastly, I outline a strategy to test the role of modified transcriptional feedback to *InR* by the transcription factor dFoxo.

Together, my results would be of broad interest to developmental biologists and contribute to our understanding of the mechanisms organ and body size regulation.

This thesis is dedicated to

My parents, Hemant Moreshwar Gokhale and Sarita Hemant Gokhale
My brother, Nachiket Hemant Gokhale,
Without whose support and encouragement I would never have begun,

And

My husband, Jayesh Hariprasad Kakade,
Without whom I would never have finished.

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I would also like to acknowledge the members of my thesis committee: Dr. Lee Kroos, Dr. Christina Chan, Dr. Kyle Miller and Dr. Jeff Mackeigan for their valuable input throughout my PhD. Many thanks to the former members of the Shingleton lab here at MSU for providing an excellent environment for the first half of my PhD, as well as the current and former members of the Arnosti lab for many interesting conversations over the years. In particular, I would like to thank my partners-in-crime Yiliang Wei and Anne Sonnenschein—all three of us joined the Arnosti lab at the same time and it was very helpful having someone else in the same boat as me throughout this time.

I was fortunate to be surrounded by some wonderful friends here at MSU, many of whom were in the middle of finishing their graduate school degrees as well. Shared conversations over coffee, long rants about failed experiments over a drink and shared celebrations of Indian festivals together provided the much needed social fuel to finish this thesis. I am glad to say that that the friendships formed here would last me lifelong.

I would also like to take this opportunity to thank my extended family here in the US: Dr. Mrunalini Dhamdhere and Dr. Jaydeep Kulkarni, and Dr. Ramesh and Seema Bhave for being a home away from home. Lastly, none of this would have been complete without the support of my amazing family—my husband Jayesh, who more often than not was my punching bag at the end of a long day, my parents for their unflinching love and support, my brother for many pearls of wisdom gained during his own doctoral studies, and my in-laws for their love and blessings. I will always be grateful to them.

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KEY TO ABBREVIATIONS

20E	20 hydroxyecdysone
cDNA	complementary deoxyribonucleic acid
ci	cubitus interruptus
DNA	deoxyribonucleic acis
FLP	flippase
FOXO	Forkhead box, sub-group O
FRT	flippase recongnition target
IIS	Insulin/Insulin-like growth factor (IGF) Signaling
JNK	c-Jun N-terminal kinase
MAPK	Mitogen Activated Protein Kinase
RNA	ribonucleic acid
TOR	Target of Rapamycin
WT	Wild Type

CHAPTER I: INTRODUCTION

[1]Part of the work described in this chapter is used in the following manuscript: Gokhale, R. H., & Shingleton, A. W. (2015). Size control: the developmental physiology of body and organ size regulation. *Wiley Interdisciplinary Reviews: Developmental Biology*, *4*(4), 335-356

Introduction

Body size is perhaps the most fundamental of phenotypic traits. Body size impacts multiple aspects of an animal's biology, from its anatomy and physiology, to its behavior and ecology. The control of body and organ size is therefore a key developmental process that ensures an animal grows to a body size that is characteristic of its species and where the size of individual organs matches the size of the body as a whole . Research over the last two decades has revealed a multitude of signaling pathways, genes and hormones that regulate body and organ size in response to environmental and developmental perturbations. Misregulation in these pathways results in multiple pathologies, from dwarfism and gigantism, to hypo-and hyperplasia of individual organs, to cancer. Understanding the mechanisms contributing to regulation of size is therefore, key to understanding function.

Much of what we know about size regulation has been elucidated in non-mammalian model organisms, in particular the fruit fly *Drosophila melanogaster*. These studies have revealed the crucial role of developmental nutrition and hormone signaling in regulating organ growth. However, we still lack a clear understanding of the organ specific mechanisms that contribute to growth regulation in response to these systemic inputs. The goal of my thesis research is to bridge this gap and understand these key aspects of organ-specific growth control in *D. melanogaster*.

Before I describe my efforts to address these questions, I provide in the following chapter an in-depth review of the current state of knowledge of size regulation. I first outline the signaling pathways involved in size control and then detail how these pathways regulate five aspects of size control (i) growth rate, (ii) growth duration, (iii) target size, (iv) apoptosis, and (v) systemic growth coordination. Finally, I describe how these aspects relate to natural variation in body size

observed between and within species. I would like to point out, however, that while I discuss each of these size control aspects individually, they do not function completely independent of each other. For example, growth duration may be regulated in part by growth rate. Size control therefore involves integration and crosstalk at every level of organization in a growing animal. Moreover, while most of the research described below was conducted in *D. melanogaster,* size regulatory mechanisms are highly conserved among animals, and therefore, the concepts and mechanisms the research reveals apply broadly to animals in general, including humans. For simplicity and where possible I use the mammalian gene nomenclature.

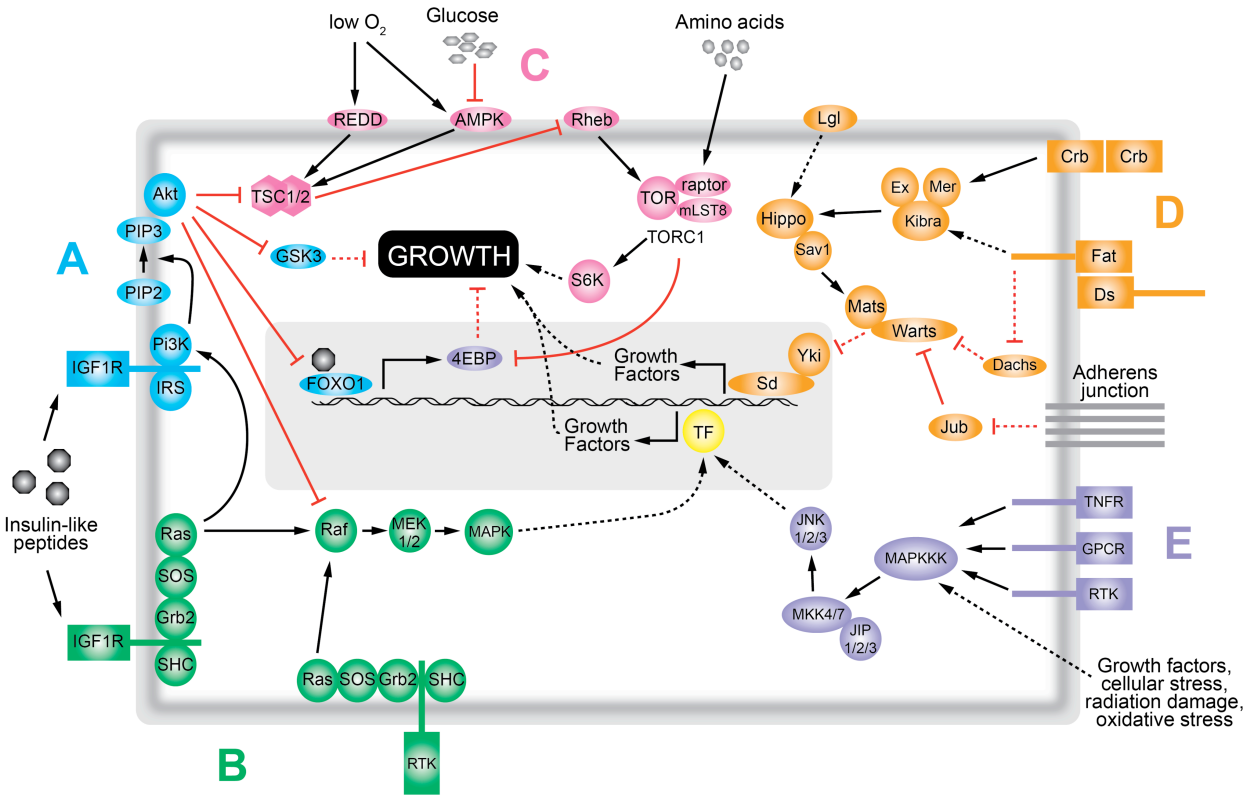
Signaling Pathways that Control Size

A number of signaling pathways have been shown to regulate growth rate, growth duration and final body and organ size. Figure 1-1 illustrates the key components of these pathways and how they are integrated, but is by no means comprehensive. These pathways have been well described elsewhere, however, and I will point the interested reader to relevant reviews. Nevertheless, a brief introduction of the major components of each pathway is necessary to fully understand how they interact to control growth regulatory processes.

Insulin/IGF1-Signaling

The Insulin/Insulin-like growth factor (IGF) Signaling (IIS) pathway regulates growth in response to insulin-like growth factors that are, in part, released in a nutrition-dependent manner (Figure 1-1A). Insulin-like Growth Factors bind to the IGF1 receptor (IGF1R) of dividing cells, which initiates the phosphorylation of the Insulin Receptor Substrate (IRS). IRS phosphorylation recruits the PhosphoInositide 3-Kinase (PI3K) to the membrane and the resulting complex phosphorylates membrane-bound $PI(4,5)P_2$ to $PI(3,4,5)P_3$. This then binds Phosphoinositide-Dependent Kinase (PDK) and Akt to the membrane and leads to the

Figure 1-1 Size regulatory signaling pathways



(A) The insulin/IGF1-signaling (IIS) pathway. (B) The RAS/RAF/MAPK-signaling pathway. (C) The TOR-signaling pathway. (D) The Hippo-signaling pathway. (E) The JNK-signaling pathway. Transcribed growth factors targeted by MAPK, Hippo and JNK-signaling pathways include secreted factors, protein synthesis and cell-cycle regulators and anti-apoptotic factors. Note that any individual growth factor may be regulated by only one pathway. IGF1R: Insulin-Growth Factor 1 Receptor; Pi3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase; IRS: IGF1R Substrate; PIP2/3: Phosphatidylinositol bi/triphosphate; GSK3: Glycogen Synthase Kinase 3; FOXO1: Forkhead transcription factor 1; SHC: Src Homology 2 domain Containing protein; Grb2: Growth factor receptor-bound protein 2; SOS: Son-Of-Sevenless; MEK: Mitogen/Extracellular signal-regulated Kinase; MAPK: Mitogen-Activated Protein Kinase; TSC1/2: Tuberous Sclerosis proteins 1/2; Rheb: Ras homolog enriched in brain; AMPK: 5' Adenosine Monophosphate-activated Protein Kinase; TOR: Target of Rapamycin; mLST8:

mammalian Lethal with SEC13 protein 8; S6K: Ribosomal protein S6 kinase beta-1; Crb: Crumb; Mer: Merlin; Ex: Expanded; Sav1: Salvador; Mats: Mob as tumor suppressor; Yki: Yorkie; Sd: Scalloped; TNFR: Tumor Necrosis Factor Receptor; GPCR: G Protein-Coupled Receptors; RTK: Receptor Tyrosine Kinase; MAPKKK: MAPK Kinase Kinase; MKK4/7: Dual specificity Mitogen-activated protein Kinase Kinase 4/7; JIP: JNK-Interacting Protein; TF: Transcription Factor.

phosphorylation of Akt [1-5].PI3K/Akt can also be activated by other receptors, including the Fibroblast Growth Factor Receptor (FGFR), Epidermal Growth Factor Receptor (EGFR) family receptors, and interleukin receptors [6-8]. Akt is a phosphokinase and has a number of downstream targets, FOXO1 and TSC2 being amongst the more important ones. Activated Akt is transported to the nucleus where it phosphorylates and deactivates the forkhead transcription factor FOXO1[9-11], a negative regulator of growth which targets the expression of myriad growth regulators [12]. Akt also regulates the TOR signaling pathway by phosphorylation of TSC2, thereby inactivating the TSC1/2 complex (see below) [13-17]. For a more detailed review of the IIS-pathway see [18, 19].

Ras/Raf/MAPK-Signaling

Binding of insulin-like growth factor to IGF1R also initiates the phosphorylation of a second substrate, Shc [20, 21] (Figure 1-1B). Upon phosphorylation, Shc binds Grb2, which is complexed with SOS. This brings SOS to the membrane, where it activates Ras through binding of GTP. Ras activation in turn activates Raf kinase, which phosphorylates and activates MEK1/2, which phosphorylates and activates MAPK [20, 21]. Activated MAPK then either regulates targets in the cytosol or is translocated to the nucleus where it phosphorylates a number of transcription factors, both activating and suppressing their activity [22]. A number of other receptor tyrosine kinases can also activate Ras, including epidermal-, fibroblast-, and vascular endothelial-growth factor receptors [23]. The role of Ras/Raf/MAPK signaling on size regulation is complex and context dependent. For example, while many of the nuclear and cytosolic targets of Ras/Raf/MAPK are regulators of cell-cycle progression, over-expression of activated Raf proteins can lead to such diverse responses as cell growth, cell cycle arrest or even apoptosis, as well as the expression of autocrine and paracrine growth factors [23]. This complexity is compounded by the fact that there is considerable crosstalk between Ras/Raf/MAPK and other

growth regulatory pathways. For example, Ras can also activate Pi3K [24], while Akt can inhibit the activity of Raf [23]. For more details about the MAPK signaling pathway see [25].

TOR-Signaling

Like the Insulin/IGF1-signaling pathway, the TOR-signaling pathway also regulates growth with respect to nutrition, cellular energy and oxygen (Figure 1-1C). At the centre of the pathway is the Target of Rapamycin (TOR), so called because mutations of *TOR* confer resistance to the growth inhibitory effects of rapamycin. TOR forms two complexes, TORC1 and TORC2, both of which have kinase activity. Signaling through TORC1 is better understood than signaling through TORC2.

TORC1 has two key targets that regulate growth: 4EBP and the p70 ribosomal protein S6 Kinase (S6K). 4EBP suppresses growth by inhibiting eIF4E, a translational initiation factor. Deactivation of 4EBP by TORC1 therefore promotes growth by de-repressing protein synthesis. At the same time, TORC1 promotes translation and growth more directly by phosphorylating and activating S6K, a regulator of ribosomal protein production. In addition to its effects on 4EBP and S6K, TORC1 indirectly promotes the translation of additional growth regulators, including cyclin D1[26], HIF-1α (see below) and c-Myc[27]. For more details of TORC1 signaling see [28].

TORC2 signaling is less well elucidated. The best-characterized function of TORC2 is the phosphorylation and activation of Akt [29, 30]. In contrast to TORC1, TORC2 is positively regulated by TSC1/2 (itself negatively regulated by Akt, Figure 1-1A), which binds directly to the TORC2 complex and is required for its activation [31]. In yeast and mammals, TORC2 is involved in actin cytoskeleton organization of the cell and so may be involved in regulating spatial aspects of cell growth [32]. For more details of TORC2 signaling see [33].

Hippo-Signaling

Hippo-signaling is the most recent addition to the family of signaling pathways that are involved in size control (Figure 1-1D). At the core of the pathway is Hippo (Mst1/2 in mammals), Salvador (Sav1 in mammals), Warts (Lats1/2 in mammals), and Mats (Mob1A/B in mammals). When active, MST1/2 binds Sav1 and together they phosphorylate Lats1/2 and its co-factor Mob1A/B, stimulating Lats1/2 kinase activity [34-36]. Active Lats/Mob then phosphorylate the downstream effectors YAP and TAZ – which have a single homolog Yorkie (Yki) in *Drosophila* – and inhibits their activity as transcriptional coactivators [37-41]. In mammals, the major binding partners of YAP/TAZ are four TEA-domain-containing proteins (TEAD1-4), while in *Drosophila* the major binding partner of Yki is Scalloped (Sd), the single homolog of TEAD1-4 [42-46]. Yap/Taz/TEAD1-4 (or Yki/Sd in *Drosophila*) promotes transcription of a number of growth-regulating genes, including *cyclin E*, *Myc* and the microRNAi *bantam* in *Drosophila.* [47-49]. Thus Hippo-signaling regulates growth by inhibiting the activity of growth-promoting YAP/TAZ and Yki. Consequently, loss of Mst1/2 or Hippo or over-expression of YAP or Yki in individual organs leads to overgrowth [37, 50, 51], although this is highly tissue-specific[52].

While the core of the Hippo-signaling pathway and many of the genes that it regulates have been relatively well elucidated, its upstream regulation is less clear. What is known has been largely elucidated in *Drosophila* and suggest that Hippo-signaling appears to regulate growth in response to a variety of different cell-cell interactions[53]. For example, the interaction of Fat and Dachsous at the surface of neighbouring cells appears to negatively regulate growth by activating Warts via Dachs and the Expanded (Exp)/Merlin (Mer)/Kibra complex[54-57]. The transmembrane protein Crumbs (Crb) also regulates Warts, as do a number of other membrane associated proteins including Lethal giant large (Lgl), which organizes apical-basal cell polarity [58], and

Ajuba (Jub), a protein that localizes to adherens junctions upon cell-cell contact [59, 60] . For a more details of the Hippo signaling pathway see [61].

JNK-Signaling

The JNK-signaling pathway functions mainly in response to cellular stress [62] (Figure 1-1E)**.** The JNK pathway is a mitogen activated protein kinase pathway (MAPK), with the c-Jun Kinase as its terminal activator [63]. The JNK pathway is conserved from flies to mammals, and is crucial in regulating cell death, tissue regeneration, wound healing and many other morphogenetic processes [64]. In mammals, there are three JNK proteins encoded by the genes *jnk1*, *jnk2*, and *jnk3*[62, 63]. JNK signaling is perhaps the most complex MAPK signaling pathway, owing mainly due to its number of inputs. These include, but are not limited to, Tumor Necrosis Factor-signaling (TNF/TNFR)[65, 66], Platelet Derived Growth Factor-Signaling (PDGF/PDGFR)[67] and Wnt-signaling[68, 69]**.** Signaling through the JNK pathway is initiated via small GTPases of the Rho family (Rac, Rho, cdc42)[70, 71]. This leads to the phosphorylation and activation of MAPKKKs such as MEKK1-4, Apoptosis Induced Kinases (ASKs)) and Mixed Lineage Kinases (MLKs)[72, 73]**.** These activate the MAPKKs MEK4 and MEK7, which phosphorylate and activate JNK1/2/3([72]). Activation of JNKs also depends on the scaffold proteins JIP1-3[74-76]. Phosphorylation of JNK1/2/3 causes their activation and translocation to the nucleus where they phosphorylate and activate transcription factors such as c-Jun [63, 77], Fos[78], and p53[79, 80], among others. An important class of proteins that are phosphorylated by JNKs are anti-apoptotic proteins such Bcl2 [73, 81] and heat shock proteins such as Hsf1 [62]. However, JNK also plays an important role in regulating growth in response to stress, in particular as a result of tissue damage and localized growth perturbation [64], as discussed below. For a more detailed review of the JNK pathway see [72].

Signaling pathways that control patterning

Another group of pathways that contribute to the final size of an individual are those involved in tissue patterning. Patterning pathways regulate overall organ shape and the number and organization of each individual cell type within a tissue. Deregulation of these pathways result in changes in the overall shape and patterning of organs, with corresponding changes in cell growth and proliferation. Patterning genes relay positional information to cells in a tissue by either forming a diffusible protein gradient (as in the case of Hh, Wnt and TGF-beta signaling in imaginal discs in *Drosophila*) or via cell-cell interactions (as in the case of Notch-signaling and Fat-Dachsous signaling). These pathways then regulate growth by interacting with canonical growth-regulatory pathways, although the details of these interactions are poorly understood. Nevertheless, because of their central role in development, the major components of patterning pathways and how they function have been very well elucidated. For more details see [82-84]

Aspects of Size Control

While the pathways described above all regulate cell growth and proliferation, this is not sufficient to explain how they control size. Understanding body size regulation requires an appreciation of the physiological and developmental context within which cells grow and proliferate. At a very basic level body size is the product of growth rate and growth duration. Growth rate is regulated by signaling pathways that control the rate of cell growth and proliferation. Growth duration is regulated by systemic hormonal signals that coordinate the cessation of growth across the whole body, as well as by organ-autonomous processes that ensure organs stop growing when they achieve their final size. Body size is not only regulated by positive growth, however, but also by negative growth, and there is increasing evidence that apoptosis plays a major role in size control. Finally, all these processes must be coordinated

across the body to ensure that each organ achieves its correct final size by the end of development. The remainder of this chapter will deal with each of these different aspects of size control and how they are regulated by the pathways detailed above.

Growth Rate

Organismal growth rate is regulated by the processes that control the rate of cell growth and cell division. Over a given time period, an increase in the rate of cell growth and division will result in an increase in final cell size and number, leading to an increase in final body and organ size. Nevertheless, the relationship between the rate of cell growth and division and final cell size and number will depend on how the rates of cell growth and division are coordinated [85]. Changes in the rate of cell growth but not division will increase final cell size but not cell number. In contrast, changes in both the rate of cell growth and cell division, in parallel, changes cell number but not cell size. Historically, there has been a tendency to confound 'cell growth' with 'cell division'. However, the processes that control cell growth and division are distinct, albeit coupled during normal development.

Cell growth requires the accumulation of cellular mass. Apart from water, ions and small molecules (~75% of cell mass), protein is the largest component of the cell, comprising ~18% of total cell mass in animals [86]. Cell growth is therefore intimately linked to the synthesis of proteins, and perturbations of the mechanisms that regulate protein synthesis affect cell and organismal growth rate. One example of this is *Minute* mutations in *Drosophila*. These mutations were originally identified as a series of genetic factors that reduce the rate of development [87]. Further analysis indicated that they reduce cellular and organismal growth rate (and the rate of cell division) by reducing the expression of ribosomal proteins and hence reducing the rate of translation and protein synthesis [88]. Despite their name, however, *Minute* mutations do not

typically affect final body and organ size or cell size, indicating that changes in growth at a cellular level are not necessarily sufficient to affect final body size. This appears to be because the duration of growth is extended to compensate for the reduction in growth rate [87].

Apart from mutations that affect the rate of translation directly, cellular growth is also affected by factors that regulate and respond to the quantity of amino acids available for protein synthesis. As discussed above, the central regulator of cell growth with respect to cellular amino acids is the TOR signaling pathway, via TORC1 [89-93] (Figure 1-1C). Of course, cellular growth requires more than just amino acids and TOR signaling also responds to the level of cellular energy, via AMPK [94, 95]; and oxygen level, via AMPK and REDD (REgulated in Development and DNA damage responses) protein [96-98]; as well as systemic nutrient levels via the IIS and Akt, which negatively regulates TSC1/2, an inhibitor of TOR [99-101] (Figure 1-1A & B). Unlike direct perturbations of the protein synthesis machinery, changes in TOR-signaling also affect final body and organ size [15]. In the case of perturbation of S6K, these changes are through effects on cell size: *Drosophila* deficient in S6K are reduced in size through a reduction in cell size rather than cell number, while S6K-mutant mammalian cells are also reduced in size [102, 103].

IIS also regulates growth with respect to nutrition, although unlike TOR-signaling, this regulation is hormonal, through the nutrition-dependent release of insulin-like peptides [100, 104, 105]. As mentioned above, IIS influences TOR-signaling via Akt and TSC1/2, and so the effects of IIS on size are partially mediated by TOR-signaling. IIS additionally regulates the rate of cell proliferation, however, and animals in which IIS is perturbed are reduced in size both through a reduction in cell size and cell number [106]. In *Drosophila* these effects on cell number appear to be mediated by FOXO: up-regulating FOXO expression in specific organs reduces their size through a reduction in cell number but not cell size, while down-regulating FOXO expression

has the opposite effect [9-11] . Thus it appears that, in *Drosophila* at least, cell size and cell number are regulated independently, the former through TOR/S6K and the latter through insulin/FOXO. This is unlikely to be the case. A reduction in cell number but not size requires a parallel reduction in both the rate of cell growth and cell proliferation so both processes must be affected by FOXO in *Drosophila* [85]. Rather, it seems likely that signaling via S6K and FOXO have differential effects on the relative rates of cell growth and proliferation, with corresponding effects on final cell size and cell number [85]. Interestingly, moderate reductions in insulin/IGF-activity in *Drosophila* affect cell size alone, while more severe reductions also reduce cell number [107]. Thus it is possible that the rate of cell growth is more sensitive to changes in insulin/IGF-signaling than the rate of cell proliferation, at least in *Drosophila*.

Cell growth and proliferation are controlled by a conserved family of kinases called cyclin dependent kinases (CDKs) and their binding partners called cyclins. Cyclins and CDKs regulate progression through the cell cycle by regulating the G1-S and G2-M transitions. With the lone exception of yeast, all eukaryotes have multiple cyclins and CDKs and the activity of these proteins ultimately regulates the rate of cell growth and division, which in turn affects tissue and organ size. A number of cell cycle regulators have been shown to play an important role in size control. Prominent among these are myc, cyclin D, cyclin E and Cdk1. In *Drosophila,* targeted overexpression of dMyc, the homologue of the c-myc oncoprotein in mammals, has been shown to cause organ overgrowth [108], while loss of dMyc inhibits body growth [109]. Myc is a transcription factor that promotes the expression of multiple growth regulators, and positively regulates ribosome biogenesis and hence global protein synthesis [110] as well as global transcription [111, 112]. Myc therefore regulates multiple growth-regulatory processes (see below). However, in the context of cell cycle progression Myc functions, in part, by inducing the

expression of Cyclin E, Cyclin D and Cdk4. These proteins inhibit the activity of the *Drosophila* Retinoblastoma-family protein (Rbf), and thereby promote the G1-S transition [109, 113-115]. Overexpression of Cyclin D/Cdk4 promotes cellular growth in *Drosophila* tissues, although this growth is context-dependent. For example in proliferating imaginal tissue, overexpression results in hyperplasia (increase in tissue volume due to increase in cell proliferation), without any effect on cell size; however overexpression in endoreplicating salivary glands results in hypertrophy (increase in tissue volume due to increase in cell size) and concomitant cell enlargement [116]. Conversely, flies (and mice) lacking Cdk4, while viable, show a significant reduction in body size [117, 118]. Cyclin E, Cyclin B and Cyclin A are also negatively regulated by components of the Hippo pathway. For instance, in *Drosophila,* Sav, Wts and Hpo negatively regulate Cyclin E while Wts negatively regulates Cyclin A and cyclin B [47, 119, 120]. Consequently, these cell cycle regulators are elevated in Hippo pathway mutants, likely contributing to the massive tissue overgrowth of these mutants [121, 122].

There are many other genes and pathways that regulate cell growth and proliferation. However, like *Minute* mutations, changes in the expression and activity of these genes do not influence final body and organ size because of compensatory mechanisms that mitigate their effects. These compensatory mechanisms are discussed in more detail towards the end of this chapter.

Growth Duration

Changes in cellular rates of growth and proliferation while necessary to control size are not in themselves sufficient. Ultimately, variations among individuals and species in body size are due to changes in the size and number of cells, which is also regulated by the period of growth. It is not a surprise perhaps that the pathways that regulate growth rate are also involved in regulating the duration of growth. As with many aspects of size control, the regulation of growth duration is

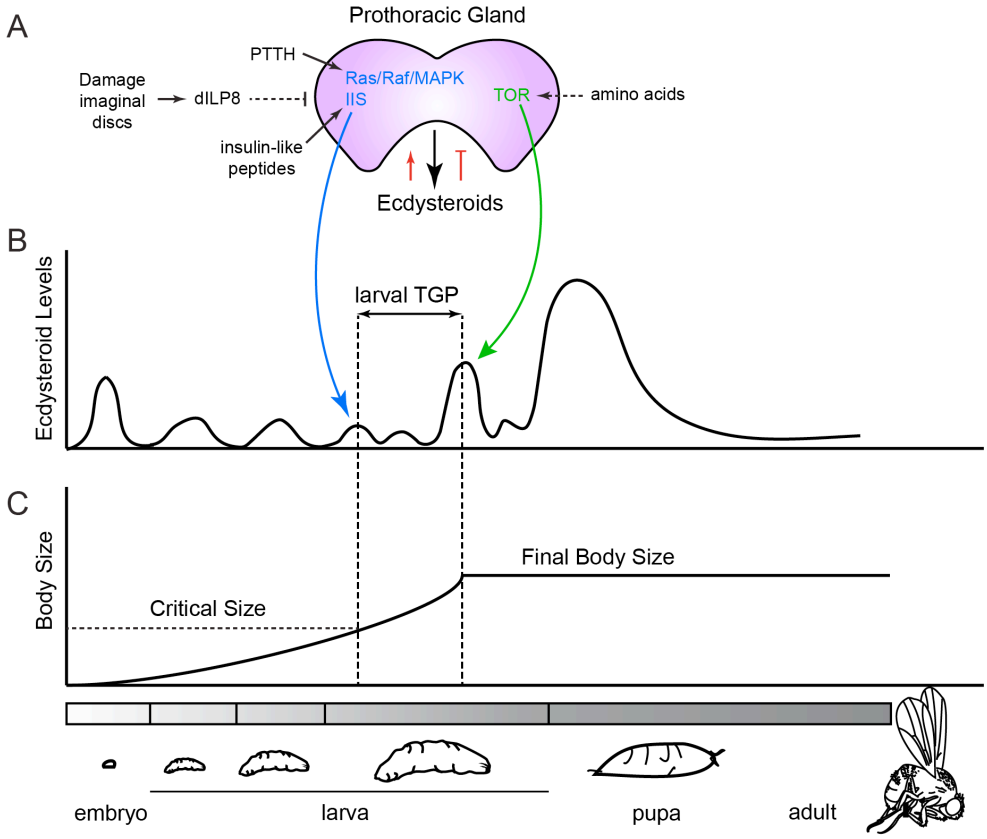
best understood in *Drosophila* and so the emphasis here will be on what is understood in this insect. Nevertheless, it is increasingly clear that similar mechanisms are employed in many animals, not just *Drosophila*.

Drosophila is a holometabolous insect that grows through three larval instars before pupating and metamorphosing into its final adult form. Because the hard exoskeleton of adult fruit flies prohibits additional growth, the final size of the adult is largely (but not entirely) determined by the size of the larva at the point at which it stops feeding and growing and begins to search for a pupation site, called larval wandering. While larval wandering occurs at the end of the larval period, the decision to pupate is made much earlier in development and is associated with the attainment of a particular body size, called *critical size*, at the beginning of the third larval instar [123-125] (Figure 1-2). Attainment of critical size is accompanied by the initiation of a hormonal cascade that ultimately causes the release of the steroid hormone ecdysone [126, 127] (Figure 1-2A). Ecdysone is synthesized in a series of ever increasing pulses, with each pulse being associated with a particular developmental transition essential to metamorphosis [126] (Figure 1-2B). For example, there is a peak in ecdysone levels at the cessation of larval feeding and the initiation of larval wandering, and a later larger peak that initiates pupation itself. Since larvae continue to grow after attainment of critical size but stop growing at larval wandering, the final size of the larvae is controlled by the critical size plus the amount of growth achieved in the period between critical size and larval wandering, called the Terminal Growth Period (TGP)[107] (Figure 1-2C). The amount of growth during the TGP is in turn regulated by the duration of the TGP and the rate of growth during the TGP. It is within this physiological context that the processes that regulate growth rate at a cellular level affect final body and organ size. For example, systemic changes in insulin/IGF-signaling do not affect critical size or (substantially) the duration of the

TGP but reduce growth rate during the TGP, thereby reducing final body and organ size [107]. In contrast, perturbations in ribosomal function also slow growth rate but delay the synthesis of ecdysone, largely eliminating any effect on final body and organ size [128].

The precise mechanism by which larvae recognize they have reached their critical size is poorly understood (although see [129]), but the signaling pathways that regulate ecdysone synthesis and respond to attainment of critical size have been well elucidated. Changes in IIS, TOR-signaling and RAS/RAF/MAPK signaling in the prothoracic gland alone cause larvae to initiate ecdysteroidogenesis at an inappropriate size (Figure 1-2A). For example, up-regulating insulin/IGF- and RAS/RAF/MAPK-signaling in the prothoracic gland causes a reduction in critical size, so that larvae metamorphose prematurely at a small size [127, 130, 131]. Down regulating these pathways in the prothoracic gland has the opposite effect [127, 130, 131]. In contrast, changes in TOR-signaling in the prothoracic gland does not appear to affect critical size itself but appear to influence the timing of the subsequent peaks in ecdysone, altering the duration of the TGP and hence final body and organ size [132] (although see [133]). An important observation as to the function of these pathways in controlling the cessation of growth is that their effects on critical size and TGP are observed when the pathways are perturbed in the prothoracic gland alone. This suggests that insulin/IGF-, TOR- and RAS/RAF/MAPK-signaling in the prothoracic gland links ecdysone synthesis to whole-body physiology so that ecdysone is synthesized at the developmentally-appropriate time. Each pathway appears to communicate different types of information to the prothoracic gland [134]. The insulin/IGF- and TOR-signaling pathways appear to communicate nutritional status, delaying metamorphosis when a larva is poorly fed and accelerating it when a larva is well fed [127, 130]. This delay is mediated by the interaction of Ultraspiracle, the binding partner of Ecdysone receptor, with FOXO, part of the IIS pathway

Figure 1-2 Physiology and the control of size in *Drosophila*



(A) The synthesis of ecdysone by the prothoracic gland is positively regulated by several signaling pathways, including IIS pathway, the RAS/RAF/MAPK-signaling pathway (blue arrow) and the TOR-signaling pathway (green arrow). Ecdysteroidogenesis is also negatively regulated by dILP8, and autoregulated by ecdysone via dynamic positive and negative feedback loops (red arrows). (B) Developmental transition are driven by peaks of ecdysone. These transitions include larval molts, attainment of critical size, the cessation of feeding (and growth) and pupariation. IIS and RAS/RAF/MAPK-signaling regulates the timing of the critical size ecdysone peak, while TOR-signaling regulates the Terminal Growth Period between the critical size peak and the ecdysone peak that stops feeding. (C) Final body size is therefore regulated by the size of the larva at critical size plus the amount of growth achieved during the TGP.

(Figure 1-1A) [135]. In contrast, the RAS/RAF/MAPK pathway (Figure 1-1B) is regulated by prothoracicotropic hormone (PTTH), which is released in a series of pulses from the larval brain and may communicate temporal information to the prothoracic gland [136, 137]. Each of these pathways appears to increase ecdysteroidogenesis to the point where ecdysone starts to regulate its own synthesis. This autoregulation involves a series of dynamic positive and negative feedback mechanisms that generate the steroid oscillations responsible for growth cessation and developmental maturation [138]. The critical size phenomenon therefore appears to represent the point at which ecdysteroidogenesis switches from being regulated by insulin/IGF- and RAS/RAF/MAPK-signaling, to being autoregulated.

Target Size

Although changes in the rate and duration of growth can lead to changes in the size of the body as a whole and of the organs within it, there is also considerable evidence that organs 'know' what size they need to be and stop growing once that size is achieved. The concept of a target organ size is supported by the observation that fetal kidneys in rats and wing imaginal discs in *Drosophila,* when cultured in the bodies of their respective adult hosts, autonomously grow to their WT size [139-141]. Similarly, when part of a liver is removed *in vivo*, the remaining tissue re-grows to its previous size [142]. Finally, slowing cell proliferation in a single developmental compartment in a *Drosophila* wing imaginal disc reduces the number of cells in the compartment but not, surprisingly, compartment size. Rather, the cells grow larger to ensure that compartment size is maintained [143].

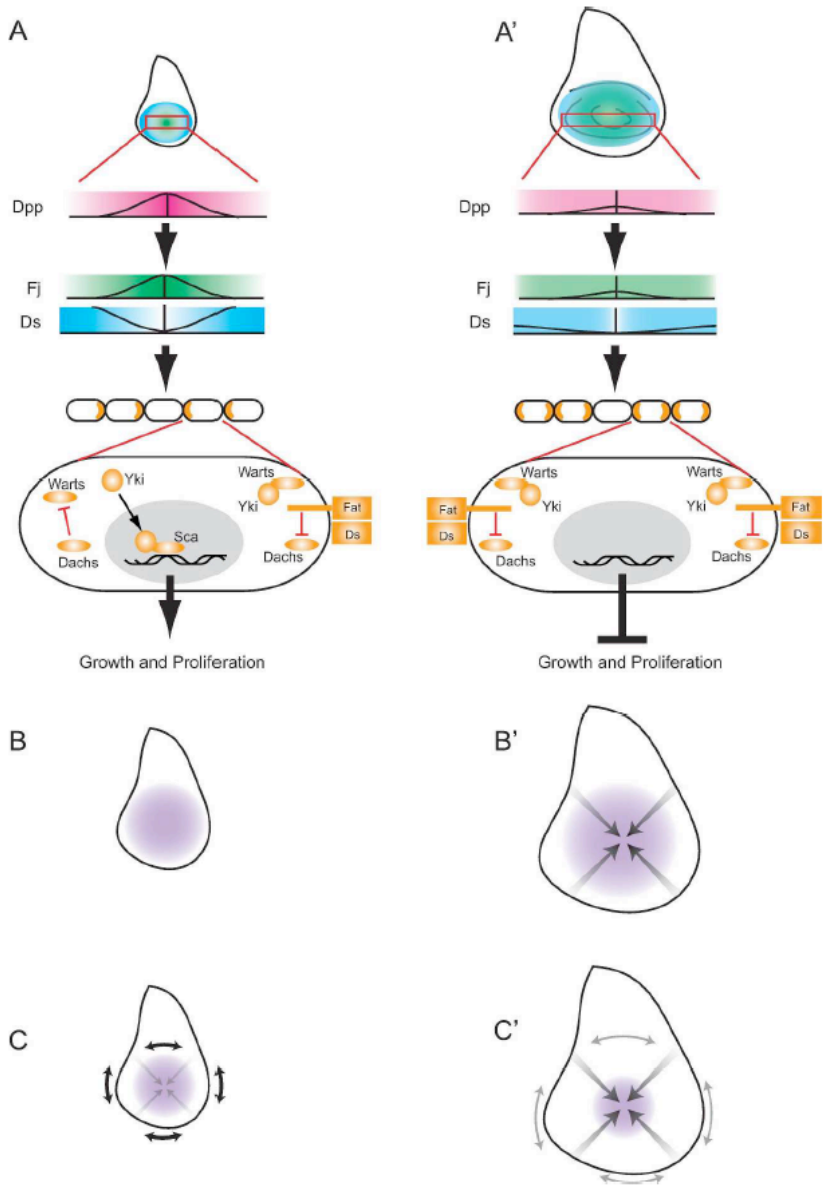
The developmental mechanisms that are hypothesized to regulate target size have fallen in to two classes: mechanisms that regulate target size by patterning genes and mechanisms that regulate target size by physical force. As with many aspects of size control, these hypotheses have been

developed to address the question of size control with respect to developing *Drosophila,* and specifically the wing imaginal disc.

Patterning genes include short-range graded paracrine signals, called morphogens, and the signaling pathways that produce and respond to them [82]. The two most important patterning genes in the wing imaginal discs are the morphogens Decapentaplagic (Dpp) and Wingless (Wg). Dpp is synthesized by cells along the anterioposterior boundary of the wing [114, 144-146] while Wg is synthesized by cells along the dorsoventral boundary of the wing [147, 148]. Both morphogens spread laterally and generate a gradient that defines the area that will be come the wing blade. Loss of either of these morphogens leads to a severe reduction in wing size [114, 149-153]. Indeed, the size difference between the wing and the haltere in *Drosophila* is due to differences in Dpp signaling [154]. The halteres are a second pair of wings that have been modified to form small club-like appendages used as gryoscopes during flight. They are defined during development by the expression of a Hox gene *Ultrabithorax* (*Ubx*) in the cells of the haltere imaginal disc [155, 156], which restricts the spread of Dpp by downregulating *Dpp* expression and upregulating expression of the Dpp receptor *thickvein* [154]. The result is a reduction in the size of the Dpp gradient, limiting its mitogenic effects and reducing the number of haltere cells to approximately one fifth the number in the wings.

The observation that wing size is regulated by the expression and spread of morphogens has led to the hypothesis that it is the dimensions of the morphogen gradient that defines target size [157]. The most basic model proposes that the concentration of Dpp is fixed at the anterioposterior boundary and at the lateral edge of the disc. Consequently, as the disc grows the Dpp gradient becomes flatter and growth stops when the Dpp gradient drops below a particular level (Figure 1-3A & A') [157]. This original model has subsequently been modified in a variety of ways to

Figure 1-3 Models of target size regulation in *Drosophila* wing imaginal discs



(A) Under the morphogen gradient model disc growth is maintained whilst a morphogen gradient is maintained. At a cellular level, there is evidence that the Dpp gradient generates opposing gradients of Fj and Ds. This in turn may lead to the asymmetrical activation of Fat, which frees Dachs to inhibit Warts, de-repressing Yki and promoting cell growth and proliferation. (A') When the gradient becomes sufficiently flat at target size, symmetrical activation of Fat inhibits

Warts, allowing Warts to deactivate Yki and suppress cell growth and proliferation. (B) Under the Shraiman model, growth in the center and the periphery of the disc is driven by the morphogen gradient, as in (A). (B') Growth stops when cells at the periphery of the disc grows beyond the morphogen gradient, inhibiting their proliferation and imposing a compressive force on the cells at the center of the disc (grey arrows), stopping their growth also. (C) Under the Aegerter-Wilmsen model, growth in the center of the disc is driven by the morphogen gradient while growth at the periphery is driven by the stretch imposed by cell proliferation at the center of the disc (black arrows). (C') Growth stops when compression at the center of the disc imposed by the peripheral cells overcomes the growth-promoting effects of the morphogen gradient, which in turn eliminates stretch at the periphery of the disc, stopping cell growth and proliferation there also.

accommodate new evidence regarding the shape of the Dpp gradient and how it changes with disc size [158-160]. There is some evidence that cells are able to detect and show a proliferative response to a gradient of Dpp, through Dpp generating opposing gradients of Dachsous (Ds) and Four-jointed (Fj) expression, with *Ds* expressed highest at the periphery of the wing disc and *Fj* expressed highest medially (Figure 1-3A & A') [55, 161]. Both Ds and Fj regulate Hippo signaling via Fat (Ds as a ligand for Fat and Fj as a Golgi kinase that modulate Ds/Fat binding) and juxtaposition of cells expressing different levels of either Ds or Fj induces elevated level of cell proliferation, as measured by BrDU incorporation [55]. Although binding of Ds to Fat inhibits proliferation by activating Warts, it is thought that, if Fat is activated asymmetrically in a cell, it leads to the asymmetric cellular localization of Dachs. This allows Dachs to locally promote the degradation and inactivation of Warts and thereby stimulate growth [55] (Figure 1-3). Consequently, as a disc grows and the Dpp gradient is lost, the resulting loss of asymmetry in both Fat activation and Dachs cellular localization leads to a cessation in cell proliferation. This is supported by the observation that uniform expression of Fj and Ds inhibit cell proliferation and growth [55]. However, additional research suggests that the juxtaposition of cells with different Dpp-signaling levels is not necessary to drive proliferation [162] and that the asymmetrical localization of Dachs is maintained even when Dpp-signaling is uniform [163]. These observations suggest that the Dpp and Ds/Fj gradients are generated independently but act together to generate uniform cell proliferation across the imaginal disc [163]. Nevertheless, what role both systems play in regulating final organ size, if any, is unclear, particularly since the temporal dynamics of the gradients have not yet been fully elucidated [160].

Models of target disc size that implicate patterning genes are based on the evidence that growth is promoted and maintained by Dpp, Wg and Vg. These proteins form a gradient of

concentration across the wing disc, yet the average growth rate across the area of the wing disc is remarkably uniform [159, 164] (although there does appear to be elevated levels of cell proliferation at the center of very young wing discs, [165]). A pair of conceptually similar models has been proposed to reconcile this apparent paradox and also account for the termination of growth and achievement of target size at the end of development [164, 166] (Figure 1-3B & C). Both of these models assume that the wing imaginal disc functions essentially as a rigid solid with little to no rearrangement of cells during development. Morphogen-promoted cell proliferation in the center of the wing disc therefore causes cells in the center to push against surrounding cells. This results in two different mechanical forces being set up in the organ. Firstly, a stretching force is exerted on the surrounding peripheral cells by the proliferating central cells. Secondly, these peripheral cells then exert a compressive force back to the cells in the center of the disc, which tends to inhibit their proliferation. Growth at the center of the wing ceases when morphogen-promoted growth can no longer overcome the inhibitory effect of compression by surrounding cells. These two models differ on what causes proliferation of the peripheral cells. The Shraiman model attributes it to the Dpp morphogen gradient experienced by the peripheral cells [166] (Figure 1-3B & B'). The Aegerter-Wilmsen model attributes growth of these cell to stretching induced by cell proliferation at the center of the disc [164] (Figure 1-3C & C'). Experimental validation of these models has not been extensive, although there is growing evidence in favor of the mechanical gradient model [167]. Both models are supported by the observation that there is a non-homogenous distribution of mechanical stress in the wing imaginal disc, with the highest compression being present in the center of the disc [168, 169]. Further, the orientation of the long axis in the peripheral cells compared to the central cells suggests that the peripheral cells are stretched tangential to the disc's radial axis [165], a hypothesis confirmed by the recoil response of the peripheral cells upon

laser-ablation of tangential versus radial cell junctions [169]. Intriguingly, cells at the periphery of the wing imaginal disc divide parallel to the lines of stress [169], supporting the hypothesis that stretch induces proliferation. More generally, there is considerable evidence to support a role for mechanical forces in regulating cell proliferation both in mammalian tissues as well as *Drosophila* imaginal discs [170, 171]. Nevertheless, fundamental questions remain. Perhaps most importantly, we do not know the molecular mechanism responsible for translating mechanical signals into changes in cell proliferation. Emerging work from mammalian and other systems seems to suggest a role for cytoskeletal proteins in transducing mechanical signaling to the cell [170]. Cell adhesion molecules are also crucial in maintaining the balance of mechanical stress between neighboring cells and the extra-cellular matrix (ECM)[170]. Further, there is growing evidence for a role for the Hippo pathway effectors YAP and TAZ in transducing mechanical signals to the cell [172-176], leading to the suggestion that YAP is a growth-regulatory sensor for mechanical force [166].

Regardless of the mechanism used to transduce physical forces into changes in the expression of growth control genes, it remains unclear the extent to which discs use a target size mechanism to regulate their final size. Presumably, any organ-autonomous size-control mechanism must also interact with the mechanisms that cause variation in final disc size, for example the IIS pathway. Further, as discussed above, the duration of organ growth is regulated and coordinated by circulating hormones, for example ecysteroids in *Drosophila*. The degree to which the cessation of organ growth is regulated by organ-autonomous versus systemic mechanisms is unknown. It is possible that target size represents a redundancy mechanism that prevents organ overgrowth when systemic regulators of growth duration fail. Alternatively, attainment of target size may

regulate the release of and/or response to circulating hormones that control developmental transitions and the duration of growth.

Negative Growth

So far, this chapter has focused on aspects of growth control that lead to increase in cell number and size, that is 'positive' growth. However, an important aspect of growth control is the removal of ostensibly superfluous tissues. This is achieved by apoptosis, in which individual cells activate a self-destruction mechanism in response to external and internal signals. Apoptosis is a response to activation of caspases and is characterized by a series of biochemical events that lead to morphological changes in the cell and eventually cell death. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation and DNA fragmentation. In contrast, necrosis, the other form of cell death, is characterized by cell lysis and the release of cellular contents. Apoptosis is a regulated process essential for correct development of adults from embryos. This has been most clearly demonstrated in *Drosophila* where the inhibition of apoptosis in the wing imaginal disc, through expression of the anti-apoptotic protein *p35*, leads to a loss of uniform disc size [108]. Further, apoptosis has been shown to be fundamental to correct development of the nervous system in *Drosophila*, where at least half of the cells are eliminated through programmed cell death during embryogenesis [177].

In *Drosophila,* multiple cell death pathways converge on the three proteins: reaper, grim and head-involution-defective (hid). In the absence of these death-domain proteins, apoptosis is virtually eliminated. Conversely, ectopic expression of any of these genes is sufficient to induce cell death [177]. Reaper, hid and grim all regulate cell death through their ability to bind to and inhibit the action of Diap1*,* one of the two Inhibitor of Apoptosis Protein (IAP) in *Drosophila.* IAPs in turn bind to and antagonize effector caspases, and therefore, negatively regulate

apoptosis. Together, the IAPs and the death domain proteins are the crucial targets by which signaling pathways such as JNK and the Hippo pathway regulate organ size.

The antagonistic relationship between the Hippo pathway and apoptosis was revealed through the observation that overexpression of Sav (Sav1) and Wts (Lats1/2) in *Drosophila* eye-antennal imaginal discs results in an increase in cell death, and ultimately in adult eyes with an irregular ommatidial pattern [121]. Conversely, loss of function mutants of *Sav* (*Sav1*) and *Wts* (*Lats1*/2) as well as their upstream regulator *Hpo* (*Mst1*/2) show massive tissue overgrowth [52, 121, 178-180]. *sav* and *hpo* (*Mst1*/2) mutant cells were shown to contain elevated Diap1 levels, thus resulting in an inhibition of cell death [121, 180]. As discussed above, Lats1/2 functions by inhibiting YAP, a transcriptional co-activator. Logically, it could therefore be expected that YAP/Yorkie overexpression would have the opposite effect of Sav, Wts or Hpo overexpression. This was exactly what was found: Yorkie-overexpressing clones in wing imaginal discs shown to cause massive overgrowth of wing imaginal discs, extending to overgrowth in the adult notum [37]. Yorkie appears to regulate organ size by regulating the transcription of multiple downstream targets including various cell cycle progression genes and inhibitors of apoptosis. A detailed description of all of these is beyond the scope of this review, however, the best-characterized mechanisms by which Yki promotes growth are as follows. Firstly, Yki inhibits cell death by up-regulating transcription of *diap1* [37]. Secondly, Yki promotes cell proliferation by up-regulating transcription of *cycE* and the microRNA *bantam,* the latter inhibiting the expression of *hid.* [47]. Lastly, Yki also upregulates the expression of the growth regulator dMyc, with loss of dMyc preventing Yki-mediated growth [49, 181]. More recent studies in *Drosophila* have now elucidated another potential target of the Hippo pathway: Dronc*,* the *Drosophila* homologue of initiator

caspase-9. *Dronc* levels are elevated in *hpo*(Mst1/2) cells and blocking *Dronc* expression is sufficient to block Hippo-induced cell death [182].

Systemic Growth Coordination

The final body or organ size achieved at the end of development depends on the four aspects that have been previously discussed in this chapter, that is growth rate, growth duration, target size and negative growth. This is not however the end of the story. The final size achieved by an organ must be proportionate to final body size in order for the organ to function correctly within its physiological context. In addition, the timing of development and maturation of the organs needs to be coordinated with that of the body such that organs achieve developmental maturity at the appropriate time. Consequently, there need to be systemic mechanisms that regulate and coordinate growth and development across the body. In this section, I will discuss our emerging understanding of how the different processes that regulate size are coordinated, both locally within organs and globally among organs.

The best understood aspects of local growth coordination are cell competition and compensatory cell proliferation. Compensatory cell proliferation is induced as a homeostatic mechanism in response to the presence of dying cells within proliferating tissues. The study of compensatory proliferation has perhaps been best studied in *Drosophila*, through the induction of apoptosis in the cells of the wing imaginal discs. The presence of apoptotic cells leads to the non-autonomous proliferation of adjacent cells caused by the JNK-dependent up-regulation of *wg* and *dpp* expression [183-185]. The result is the JNK-dependent regeneration of damaged imaginal discs [186, 187]. The precise signaling mechanisms of compensatory proliferation, however, may differ based on the specific tissue that is affected. For instance, in differentiating eye imaginal discs, apoptotic cells induce compensatory proliferation by inducing Hedgehog signaling rather than

Wg/Dpp signaling [188]. Further, while mitogenic signaling involving Wg and Dpp is likely one of the key pathways involved in compensatory cell proliferation, other signals also appear to be involved. This is because compensatory proliferation induced by gamma-irradiation still occurs in *dpp* and *wg*-mutant wing discs [185]. Recent studies have uncovered a likely candidate: the Hippo signaling pathway. These studies establish a role for Yki in mediating compensatory cell proliferation. On induction of apoptosis in the wing imaginal disc, Yki activity increases in the adjacent cells stimulating a proliferative response [189, 190]. This increase in Yki activity is JNK-dependent, thus establishing a novel link between the JNK- and the Hippo signaling pathway [189].

A related phenomenon to compensatory proliferation is cell competition, which is induced when proliferating tissue contains two differentially growing cell types. Cell competition was first observed in *Drosophila* that carried *Minute* mutations. As discussed above *Minute* mutations are dominant ribosomal protein mutations, which are homozygous lethal but when heterozygous cause a cell-autonomous reduction in growth rate. When patches of *Minute* +/- cells (called *clones)* are generated in an otherwise WT fly, the slow-growing cells are eliminated through JNK-dependent apoptosis, and the resulting adult tissue is composed entire of WT cells [191, 192]. Such cell competition appears to be a mechanism to ensure that organs grow to their correct final size despite localized cell-autonomous perturbations in proliferation. The intensity of cell competition is likely to be further heightened by the apoptotic-induction of compensatory proliferation in the surrounding cells, increasing the differential growth rates between fast and slow growing cells, and further increasing the effects of cell competition [193]. Since its discovery, numerous regulators of cell competition have been discovered. The first of these was Myc, through the observation that cell competition can be induced by differential expression of *Myc* in neighboring cells, with cells expressing higher levels of *Myc* eliminating cells expressing lower

levels of *Myc* [108] [194] Conversely, clones lacking expression of *Myc* are outcompeted by their wild type counterparts when induced in the wing imaginal disc [109]. Subsequently, numerous other regulators of cell competition have been identified in *Drosophila*, including JAK-STAT and Hippo-mediated cell competition [195-198]. It is important to note, however, that while cell competition has perhaps been best studied in *Drosophila,* there are several notable mammalian examples. Three recent studies reveal a conserved role for c-Myc in inducing cell competition in mouse Embryonic Stem Cells (ESCs), mouse embryonic epiblast and mouse cardiomyocytes [199-201]. All these studies demonstrate that establishment of differential c-Myc levels in a population of cells is sufficient to induce cell competition, and the cells expressing lower levels of c-Myc are outcompeted by those expressing higher levels (For further details see [202, 203]).

While local growth coordination ensures that different parts of an organ are of the correct size relative to one another, global or systemic growth coordination ensures that different organs are the correct size relative to one another. This can, in principle be achieved by common exposure of growing organs to external and internal growth-regulating factors. For example, in *Drosophila* raised at higher temperatures there is a coordinated decrease in adult organ and body size [204, 205]. Similarly, systemic overexpression of an insulin-like peptide (dILP2), a secreted molecule whose levels are correlated with nutrition, results in a coordinated increase in size of the organs and the body [2]. Not all organs, however, respond in the same way to systemic regulators of body size. For example, in *Drosophila* changes in nutrition have less of an effect on the size of the genitalia [204, 206] and the CNS [207] than on other organs: Under conditions of low nutrition, when dILP levels are low, both of these organs maintain their size even when the rest of the body shows a decrease in size. They therefore appear to disproportionately larger in malnourished flies when compared to well-fed individuals. This is achieved using two different mechanisms. In the case of the CNS

the activation of an alternative kinase, the Anaplastic Lymphoma Kinase (ALK), results in the activation of the IIS pathway even in the absence of nutrition [207]. The genital discs achieve the same result by suppressing expression of FOXO, which in other tissues negatively regulates growth when nutrition and insulin signaling is low, but its relative scarcity in the genitalia ensures that genital growth is not inhibited under the same conditions [206].

Although common exposure to growth-regulating factors is sufficient to coordinate growth among organs, such unidirectional systemic regulation is not robust to perturbations in the growth of an individual organ. How is coordinated growth maintained when the growth of one organ is slowed or accelerated? To achieve this organs must be able to recognize their own growth and development and communicate this information to the rest of the body. This seems to be the case in *Drosophila*. In *Drosophila,* perturbing the growth of individual imaginal discs in developing larvae causes a delay in the attainment of critical size and a suppression of ecdysteroidogenesis [125, 208, 209] (Figure 1-2A). This appears to be a mechanism to give the damaged discs additional time to regenerate to their correct size. What is interesting, however is that the other undamaged imaginal discs do not overgrow despite the additional developmental time [210].This is because the undamaged discs slow their growth to match with the growth perturbed discs [210, 211,212]. How this growth coordination occurs between imaginal discs is not entirely clear, although pieces of the puzzle are beginning to emerge.

Damaged imaginal discs have been shown to secrete an insulin-like peptide, dILP8, which appears to regulate developmental timing by inhibiting the production of ecdysone from the protharacic gland by upregulation of nitric oxide synthase (NOS) [213-215] (Figure 1-2A). Damaged imaginal discs have also been shown to signal through a retinoid-dependent manner to repress PTTH production, which also suppresses ecdysone production [209]. Flies mutant for dILP8 or the

members of the retinoid-signaling pathway do not retard development when the growth of one disc is retarded [209, 215]. The reduced level of circulating ecdysone in larvae with damaged discs also appears to be limiting for growth in the remaining undamaged discs. Ecdysone signaling is known to promote growth of imaginal discs [216-218] and application of ecdysone to larvae with a growth-perturbed disc rescues the growth rate of the other discs [210]. Ecdysone signaling therefore, seems to be one of the key mechanisms involved in growth coordination.

Natural variation in body size

Body size shows a tremendous variation both within and between species. This natural variation can be attributed to genetic variation and environmental variation. Amongst the environmental factors that have the most significant impact on size, nutrition, temperature and oxygen concentration have received the most attention. Body size variation within and between species can be explained by distinct biological phenomena. Size variation *within* species can be attributed to two aspects: genetic variation between individuals and phenotypic plasticity in response to the environment. Phenotypic plasticity refers to the ability of one genotype to produce more than one phenotype when exposed to different environments. Thus, two different individuals of the same species may have vastly different body sizes depending on the quality of developmental nutrition that they were exposed to. However, in terms of size variation *between* species, the predominant contributor is genetic variation resulting in genetic divergence of one species from another. Genetic divergence refers to the process by which two or more populations of an ancestral species accumulate independent mutations through time, often after the populations have become reproductive isolated over time. For example, one likely mechanism to explain size variation in two different species of *Drosophila* may be an accumulation of single nucleotide polymorphisms or indels in cis-regulatory loci of a particular gene. Natural variation

in body size among different populations of animals can thus be explained by their underlying genetic variation and phenotypic plasticity. In this section, I discuss briefly how natural body size variation in response to changes in nutrition, temperature and oxygen concentration can be explained by phenotypic plasticity and the underlying intra- and inter-species genetic variation.

Nutrition

Developmental nutrition is one of the most crucial factors in regulating growth rate and final adult size. The relationship between nutrition and growth is rather intuitive: cellular growth takes place through the conversion of nutrients to new tissue, and therefore, increased nutrition would result in an increased growth rate and consequently larger body size. What is surprising, however, is that in almost all animals, this is mediated through common nutrient-sensitive signaling pathways: the IIS/TOR signaling pathways. The molecular mechanisms by which these pathways regulate cellular growth and body size have already been discussed earlier in this chapter.

Apart from the myriad molecular studies implicating the IIS pathway in body size regulation, there is considerable evidence from population studies of naturally occurring *Drosophila* populations suggesting a strong correlation between genetic variation in the IIS pathway and size variation across latitudinal clines. For example, the chromosomal inversion *In(3R)Payne* which contains, among others, genes of the IIS pathway, has been shown to be strongly associated with body size and wing area[219-222] . Similarly, the frequencies of the inversion *In(2L)t* containing the IIS genes *chico, Pten* and *Tor,* decreases with increase in latitude and is associated with a low body weight [222]. Additionally, naturally segregating alleles of the *InR* have been associated with body weight in *Drosophila* [223].

Apart from population-level variations in IIS being associated with body size, intra-individual variation in IIS pathway activity among organs within an individual has been implicated in organ-specific patterns of phenotypic plasticity. For example, in rhinoceros beetles, the male horns are more sensitive to changes in nutrition, possibly due to higher expression of *InR* and consequently are more responsive to changes in environmental perturbation[224]. These findings corroborate evidence from *Drosophila* and the horned beetle *Onthophagus nigriventris,* which suggest that male genitalia are less responsive to changes in nutrition due to low expression of the IIS gene *foxo* [206, 225] thus providing further support to the role of IIS in mediating phenotypic plasticity.

Temperature

Temperature is an important regulator of body size, particularly for ectothermic animals. In almost all ectotherms, an increase in environmental temperature during development results in a corresponding decrease in body size. This observation is captured in the 'temperature-size rule' (TSR) and is nearly universally applicable with well-documented examples from bacteria, protozoa, plants and animals [226-229]. Canonically, this has been attributed to the effect of temperature on biochemical kinetics [230]. Nevertheless, a definite mechanistic explanation of TSR still remains elusive. There have been three main hypothesis proposed to explain the effect of temperature on body size. The van der Have and de Jong model of TSR [231] proposes that higher temperatures decrease developmental time much more than they increase growth rates, ultimately resulting in adults with smaller body sizes. The von Bertalanffny/Perrin model proposes that temperature directly regulates final body size and growth rate but not duration [232, 233]. Under this hypothesis, temperature affects the rate of catabolism more than the rate of anabolism, and the cessation of growth occurs when these rates are equal to each other. With

increasing temperature therefore, this balance is achieved at smaller body sizes resulting in individuals with a smaller final body size. Lastly, several models [229, 234-236] propose that rather than being a biophysical constraint, the TSR is an adaptive response. Metabolic rates increase much more with temperature than diffusion rates of oxygen, and the authors suggest that this would lead to oxygen limitation to larger individuals at higher temperatures. Consequently, smaller body sizes at higher temperatures has been suggested to be an adaptive response by reducing both the diffusion distance and requirement of oxygen.

Remarkably, natural populations of both endotherms and ectotherms show the same trend as TSR. This observation called Bergman's Rule states that populations and species found in colder environments have a relatively larger size than those found in warmer environments. Bergman's rule captures to a great degree variations in body size as observed along latitudinal clines in populations of a wide variety of animals. Yet, the proximate mechanisms that might explain both TSR and Bergman's rule remain poorly understood. Evidence from *Drosophila* suggests that the control of the size response to changes in temperature appears to be regulated at the level of individual of organs, with wings showing a higher degree of phenotypic plasticity than other organs [204]. Interestingly, recent research from *Manduca* and *Drosophila* suggests that there is likely no common developmental mechanism that might explain the TSR in different animals. For instance, in *Manduca*, an increase in environmental temperature decreases developmental time much more than it increases growth rate, thus resulting in individuals with a smaller body size [237, 238]. On the other hand, in *Drosophila*, temperature regulates final body size primarily by regulating critical size, TGP as well as the growth rate [205].

Oxygen

The relationship between oxygen levels and body size of animals was first proposed by the

discovery of giant insect fossils from the late Paleozoic era—a time in which the atmosphere was significantly more hyperoxic than the present [239]. This led to the general observation that hyperoxic environments result in an increase in body size, while a hypoxic environments cause a decrease in body size. Hypoxia and hyperoxia are usually defined with reference to the environmental partial pressure (PO2) of oxygen, which is 21kPa; therefore hyperoxia refers to conditions in which PO2 is greater than 21kPa while hypoxia refers to a decrease below 21kPa. In *Drosophila*, body size decreases linearly with a decrease in PO2 from 21kPa to 7kPa, to about 60% of the body size of flies raised under normoxic conditions[240]. Hypoxia increases developmental time and decreases growth rate [241], resulting in a decrease in cell size as well as cell number in the *Drosophila* wing [240]. However, despite this overall effect of decrease in growth rates and body sizes by hypoxia, the effect on the tracheal system—the oxygen carrying respiratory system in insects—is quite the reverse. Indeed, evidence from several insect species suggests that hypoxia causes in an increase in growth of tracheoles and branching of the tracheal system [242-245]. The molecular mechanisms responsible for sensing oxygen involve the Hypoxia Inducible Factor 1 (HIF), first isolated as a master regulator of the transcriptional response to hypoxia [246]. Subsequent work in *Drosophila* revealed that similar to mammals, the oxygen sensing machinery involves HIF1, called *sima* in *Drosophila*[247]. Under hypoxic conditions, sima is no longer targeted for degradation[248] but instead activates gene expression in concert with its partner protein tango. Sima accumulates in trachea under hypoxia, where it induces the expression of the *Fibroblast Growth Factor* (FGF) receptor *breathless*[249]. Breathless together with its receptor Branchless drives tracheal branching and sprouting[250, 251]. However in somatic tissues apart from the tracheal system where growth is inhibited in response to hypoxia, sima reduces cellular growth rates and cell and body size by blocking protein synthesis through the IIS

and TOR pathways [252, 253]. Thus the cellular pathways regulating oxygen-dependent growth appear to converge on those regulating nutrient dependent growth.

Summary

What is clear from the above review is that enormous progress has been made in understanding the developmental genetic and physiological processes that regulate growth of bodies and the organs within them. To a large extent, the role of individual signaling pathways in organ-autonomous size control has been extremely well elucidated. However, how these pathways interact with systemic factors such as hormones and nutrition to achieve correct final size is only beginning to be elucidated.

In particular in *Drosophila,* two aspects of size control remain poorly understood. Firstly, how is growth coordinated within and between organs to ensure that the size of different parts of an organ are correct relative to one another, and that each organ is the correct size relative to the body as a whole? Prior research in *Drosophila* suggests that systemic coordination of growth across the whole individual is mediated through hormonal signaling such as ecdysone signaling. An outstanding question that remains is how does systemic ecdysone signaling interacts with organ-autonomous pathways to regulate final imaginal disc size. Secondly, given that changes in expression of the IIS pathway genes alter the sensitivity of the pathway to changes in nutrition, what are the mechanisms of transcriptional regulation for these genes? Regulation of organ size in response to changes in nutrition must ensure that the final organ size is proportionate to the body size while at the same time maintain functionality of the organ. In this context, what role do subtle transcriptional changes of genes in the IIS pathway have in regulating organ size ?

My primary motivation for this thesis was to understand how size control in *Drosophila* is achieved with respect to these questions. In the following chapters I describe some of my

findings and how these address our current gaps in understanding. In Chapter II I present and discuss my findings pertaining to the interaction between the organ-autonomous IIS pathway and systemic ecdysone signaling and how this is essential to regulate compartment and organ size in *Drosophila* wing imaginal discs. In Chapter III I present my findings focusing on how the *InR* gene in the IIS pathway is regulated transcriptionally and discuss how these preliminary findings can be used to address the role of IIS in organ-specific nutritional phenotypic plasticity. Lastly, in Chapter IV discuss the implications of this work and how this work can be further extended upon by future experiments.

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CHAPTER II: INTRA-ORGAN GROWTH COORDINATION IN DROSOPHILA IS MEDIATED BY SYSTEMIC ECDYSONE SIGNALING

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Abstract

Regulation of final organ size is a complex developmental process that involves the integration of systemic and organ-specific processes. Previously, we have shown that in developing *Drosophila,* perturbing the growth of one imaginal disc – the parts of a holometabolous larva that become the external adult organs – retards growth of other discs and delays development, resulting in tight inter-organ growth coordination and the generation of a correctly proportioned adult. Whether different parts of the same imaginal disc similarly coordinate their growth to generate a functioning adult organ is, however, unclear. In this study, we use the wing imaginal disc in *Drosophila* to study and identify mechanisms of intra-organ growth coordination. We generate larvae in which the two compartments of the wing imaginal disc have ostensibly different growth rates (WT or growth-perturbed). We find that there is tightly coordinated growth between the WT and growth-perturbed compartments, where growth of the WT compartment is retarded to match that of the growth-perturbed compartment. Crucially, this coordination is disrupted by application of exogenous 20-hydroxyecdysone (20E), which accelerates growth of the WT compartment. We further elucidate the role of 20E signaling in growth coordination by showing that in WT discs, compartment-autonomous up-regulation of 20E signaling accelerates compartment growth and disrupts coordination. Interestingly, growth acceleration through exogenous application of 20E is inhibited with suppression of the Insulin/Insulin-like Growth Factor Signaling (IIS) pathway. This suggests that an active IIS pathway is necessary for ecdysone to accelerate compartment growth. Collectively, our data indicate that discs utilize systemic mechanisms, specifically ecdysone signaling, to coordinate intra-organ growth.

Introduction

In multicellular organisms, organ growth is a tightly regulated developmental process, involving coordination with growth of other organs in the body, and with overall body size, to generate a correctly proportioned individual. Research in *Drosophila* over the last two decades has elucidated the mechanistic details of a number of growth regulatory pathways that are known to be essential for organ growth. Nevertheless, how these pathways are regulated systemically during development to result in the generation of correctly proportioned organs and individuals is not well understood.

The *Drosophila* wing imaginal disc is one of the most well studied models of organ growth and patterning during development. Over the course of larval development, the wing imaginal disc undergoes rapid growth, growing from ~50 cells to ~50000 cells over four days to generate the tissue that metamorphoses into final adult wing and much of the thorax [1]. Growth and patterning in the wing disc is regulated organ autonomously by morphogens such as Dpp and Wg [2, 3] and systemically by circulating growth factors such as insulin-like peptides [4, 5]. Any growth perturbation to the wing disc during development results in an increase in the developmental time [6-8]—presumably to allow the growth perturbed disc to grow to its correct size. This developmental delay is mediated by the damaged wing disc suppressing the synthesis of the molting hormone ecdysone in the prothoracic gland. Nevertheless, despite this increase in developmental time, other undamaged imaginal discs do not overgrow but rather slow their growth to match that of the damaged wing disc [6, 8-10] This indicates that there is some systemic growth coordination mechanism that coordinates growth between organs.

Data from several recent studies are beginning to elucidate the mechanisms by which discs regulate ecdysone synthesis and developmental timing. First, growth-perturbed discs secrete an

insulin-like peptide, dILP8 [11, 12] which appears to inhibit the production of ecdysone in the prothoracic gland by up-regulating the expression of nitric oxide synthase [10]. Second, damaged imaginal discs signal through a retinoid-dependent manner to repress prothoacicotropic hormone (PTTH) production [8]. PTTH is a positive regulator of ecdysteroidogenesis and so suppression of PTTH synthesis also suppresses release of ecdysone. Whilst the mechanisms by which damaged discs delay development are increasingly well understood, how damaged discs suppress growth in undamaged discs is less clear. Intriguingly, there is increasing evidence that ecdysone is also a positive regulator of imaginal disc growth [13]. Therefore, the slow growth of undamaged imaginal discs may well be because of low levels of circulating ecdysone. Indeed, there is strong evidence that this is the case – in larvae with damaged discs the growth of the undamaged discs can be rescued by feeding growth-perturbed larvae with 20 hydroxy ecdysone (20E) [9]. Thus, exogenous application of 20E disrupts inter-organ growth coordination.

Whilst we might expect systemic mechanisms to coordinate growth between organs, it is unknown whether similar mechanisms exist to coordinate growth between different parts within an organ. There is increasing evidence that organs have autonomous mechanisms to regulate growth within tissues. These mechanisms appear to rely on the interaction between morphogens and physical forces within a growing organ to control the rate and cessation of cell proliferation and ensure uniform growth across a WT organ [14-16]. However, it is unclear whether such organ-autonomous mechanisms are sufficient to ensure uniform growth when one part of the organ is growth perturbed.

Efforts to address the existence of intra-organ growth coordination have been made using the developmental compartments of the wing imaginal disc. The wing imaginal disc consists of distinct anterior (A) and posterior (P) developmental compartments, that reflect shared cell

lineage. The cell population in each compartment is specified early in development, and cells within one compartment ostensibly grow relatively independently of cells in another compartment. Martin and Morata generated wing imaginal discs in which the anterior and posterior compartment had different rates of growth: the anterior compartment was wild type [17] and 'fast growing', while the posterior compartment was heterozygous for a *Minute* mutation and 'slow growing' [18]. The authors found that the 'fast growing' anterior compartment was relatively large early in development, but autonomously reduced its growth rate toward the end of development such that by pupation both anterior and posterior compartments were the same size as in mature wing imaginal discs. Based on these data, the authors proposed that individual compartments function as independent developmental units, and that the growth and development of one compartment is not linked to that of the other [18]. On the other hand, recent evidence suggests that perturbing the growth of the posterior compartment in the wing disc non-autonomously reduces the growth rate of the adjacent anterior compartment [19]. More recently, autonomously increasing the growth rate of one compartment was shown to cause a corresponding reduction in size of the adjacent compartment, a process shown to be dependent on the proteoglycan Dally [20]. These data suggest that growth rate of one compartment is, in fact, dependent on the growth rate of its adjacent compartment. An open question, therefore, is whether growth among the developmental compartments is indeed coordinated, and if it is, the mechanism by which this is achieved.

Here we manipulate growth independently in different developmental compartments of the wing imaginal disc in *Drosophila,* and show that there are indeed mechanisms that coordinate growth among different parts of an organ. Surprisingly, however, this growth coordination is mediated systemically, via ecdysone signaling: up-regulating ecdysone signaling in an individual

developmental compartment accelerates its growth and disrupts coordination. The ecdysone-stimulation of growth is dependent on Insulin/Insulin-like Growth Factor Signaling (IIS), contrary to previous findings that suggest that ecdysone suppresses growth by inhibiting IIS. Collectively, our data support a model of growth that utilizes ecdysone and IIS to coordinate growth both between and with organs.

Materials and Methods

Drosophila stocks

The fly stocks used in the study are as follows (full genotype and stock numbers are in parentheses): *Rps3^{plac92}* (*w^{1118}; P{neoFRT}82B P{Ubi-GFP(S65T)nls}3R P{A92}RpS3^{Plac92}/TM6C, Sb^{1}*, 5627), *InR.CA* (*y^{1} w^{1118}; P{UAS-InR.del}2*, 8248), *InR.DN* (*y^{1} w^{1118}; P{UAS-InR.K1409A}2*, 8252), *FRT 82B* (*w*; P{neoFRT}82B P{arm-lacZ.V}83B/TM6C, Sb^{1} Tb^{1}*, 7369), *EcR.RNAi* (*w^{1118}; P{UAS-EcR-RNAi}104*, 9327), *en-RFP* (*w^{1118}; P{en2.4-GAL4}e16E, P{UAS-RFP.W}2/CyO*, 30557*), tub-Gal80ts* (*w[*]; sna[Sco]/CyO; P{w[+mC]=tubP-GAL80[ts]}7,* 7018) were obtained from the Bloomington *Drosophila* Stock Center. *RpS3.RNAi* (*w^{1118}; P{GD4577}v37741,* 37741), and its control *60000* (*y,w[1118];P{attP,y[+],w[3`]}*) were obtained from the Vienna *Drosophila* Stock Center. *UAS-FLP; mwh, jv, FRT2A/SM6a-TM6B*, *en-GAL4/CyO; RpS17^{4}, ubi-GFP, FRT2A* were the kind gift of Francisco Martin. *ci-Gal4, UAS-FLP;* was generated by Takashi Hayashi by recombination on the X chromosome.

Generation of mitotic clones

We used the anterior imaginal disc driver *cubitus interruptus* (*ci*) or the posterior imaginal disc driver *engrailed* (*en*) to drive the expression of a FLP recombinase in an FRT *Minute*

background. All flies were raised at low density on standard cornmeal/molasses medium, and maintained at 25˚C unless otherwise stated.

Measurement of wing imaginal disc growth in growth-perturbed larvae

Larvae were raised on standard cornmeal/molasses medium supplemented with yeast in 60 x 15mm Petri dishes in constant light at 25°C. *ci>EcR.RNAi,* and corresponding control larvae were raised at 17°C. Third instar larvae across a range of body sizes were collected on day 7 after egg lay (AEL). For the conditional knockdown using *tub-Gal80ts*, larvae were raised until L1/L2 ecdysis at 17°C. Larvae were then collected in six hour cohorts and transferred to 29°C to allow conditional expression of the *RpS3.RNAi* transgene and dissected at varying time points. Wing imaginal discs were dissected in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 20 minutes, followed by washing with PBS containing 0.1% Triton-X-100 (PBT). Tissues were blocked in PBT containing 0.2% BSA and 2% NGS for 1h. Primary antibody (mouse anti-En 4D9 (Hybridoma Center), 1:100) was incubated overnight at 4°C. Secondary antibody (goat anti-mouse IgG Alexa Fluor 594 (Invitrogen), 1:1000) was incubated for 2h at RT. Discs were mounted either in Vectashield (Vectorlabs) containing Hoechst 33258 (Sigma) (1:500) or in Vectashield containing DAPI. Discs were imaged on either a Leica DM6000B microscope and measured using Image-Pro (Media Cybernetics) or on an Olympus BX51 microscope and measured using ImageJ. We measured total disc area and the size of the engrailed-stained posterior compartment. We calculated the size of the anterior compartment as total disc size minus posterior compartment size.

20E treatment of larvae

Flies were allowed to lay for 24h on standard corn meal agar supplemented with yeast paste in 60X15mm Petri dishes and second instar larvae were collected 80h after egg deposition. Third

instar larvae were collected after second-to-third instar ecdysis in 4h cohorts. Either 0.75 mg of 20-hydroxyecdysone (20E) (A.G.Scientific) dissolved in 100% ethanol or an equal volume of 100% ethanol was added to 5ml of Instant *Drosophila* Medium (Ward's Natural Science). Larvae were transferred to food with or without 20E 12h after ecdysis to third instar and imaginal wing discs were dissected 24h later.

Statistical Analysis

All disc and compartment size data were log transformed prior to analysis, and the line-of-best-fit was estimated using the major axis (MA) regression. The slope of ontogenetic allometry between posterior and anterior compartment size was then calculated, and compared between genotypes by the common slope test using the *smatr* package in *R* [9, 21]. The common slope test tests whether the slope of the MA regression varies among groups. The test assumes that there is a linear relationship between covariates, that each group of observations is independently sampled, and that the residual error is normally distributed. When conducting multiple common slope tests involving the same control, we applied a Bonferroni correction to ostensibly significant *P*-values by multiplying by the number of comparisons involving the control. We did not apply the correction to non-significant *P*-values, since the correction does not render these *P*-values significant. We used the *ssnanova* function in the *gss* package in *R* to fit a cubic spline to non-linear growth trajectories, with a 95% confidence interval. Throughout, significance is set at $P < 0.05$. All data and an annotated R-script for the analyses are provided on Dryad.

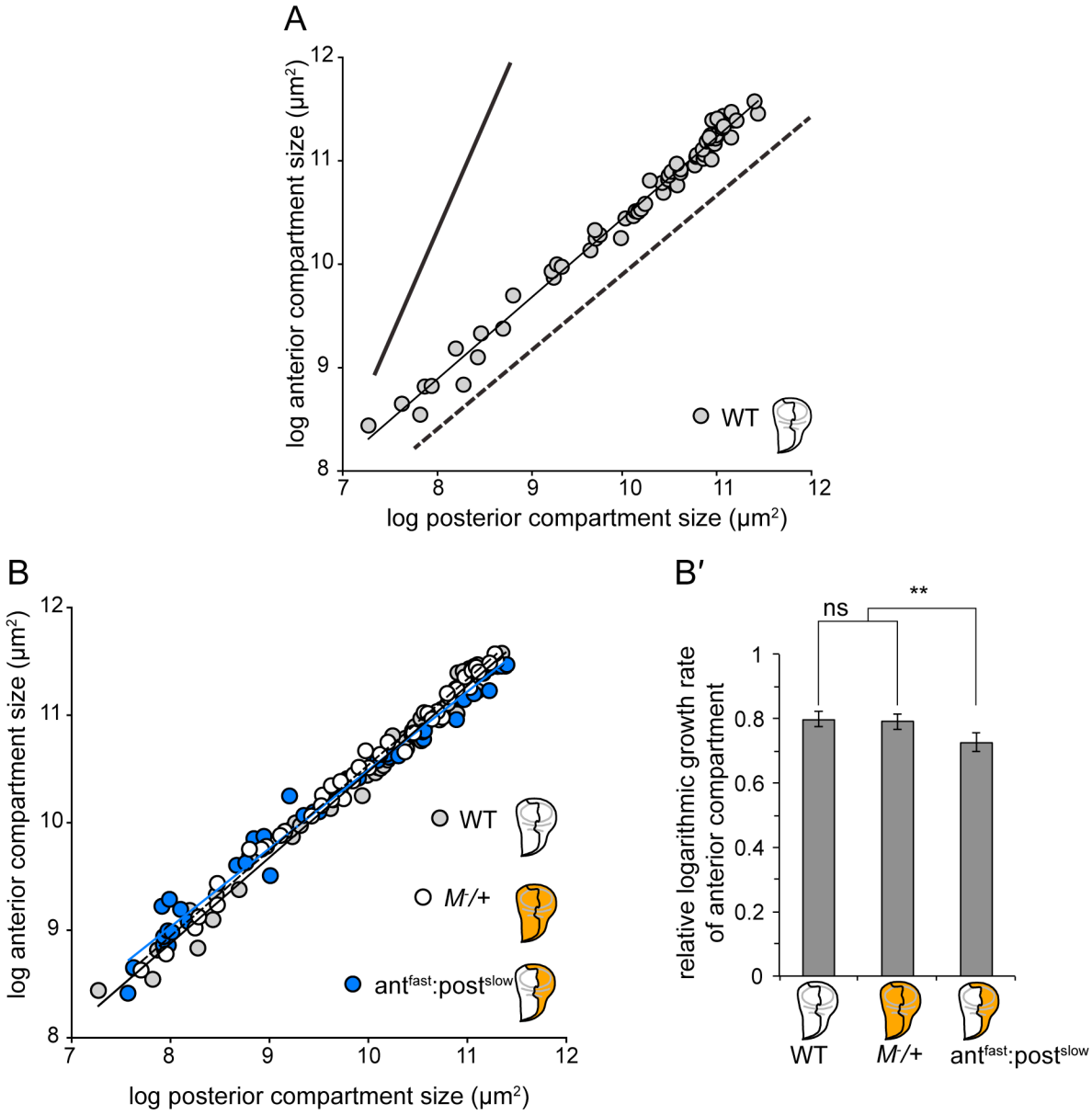
Results

Growth is coordinated between compartments within an organ

To examine the coordination of growth between compartments throughout development, we plotted the ontogenetic allometry of the anterior against the posterior developmental

compartment of the wing imaginal disc in *Drosophila* (Fig. 2-1A). The ontogenetic allometry is the scaling relationship between the size of two morphological traits plotted through development. When plotted on a log-log scale, the slope of the ontogenetic allometry is the ratio of the logarithmic growth rates of the two traits [9]. If compartments grew autonomously to a target size, then slowing the growth of one compartment would change the ratio of its logarithmic growth rate with respect to the other, and consequently, change the slope of the ontogenetic allometry (solid line, Fig. 2-1A). On the other hand, if compartment growth were mediated non-autonomously, then slow growth in one compartment would result in a concomitant reduction in growth rate of the other compartment and maintain the ratio of their logarithmic growth rates throughout development (broken line, Fig. 2-1A). Irrespective of a change in intercept, a change in the slope of the ontogenetic allometry represents a change in the relative logarithmic growth rate of one compartment relative to the other. We first determined the ontogenetic allometry between compartments in ostensibly uniformly-growing wing imaginal discs and asked whether this relationship depended on the growth rate of the disc as a whole. We examined the relationship between the anterior and posterior compartments in WT and slow growing heterozygous *Minute* larvae (*M/+)*. We used a previously described *Minute* allele, *P{A92}RpS3*^{Plac92} , which in heterozygous flies delays development by 51h [22]. In WT and (*M/+)* discs, the ontogenetic allometry is linear throughout growth of the third larval instar (Fig. 2-1B). Further, the slope is the same in both WT and slow-growing *M/+* larvae (common slope test, $P = 0.8486$) indicating that relative growth rates of the anterior and posterior compartments are constant and independent of the growth rate of the disc as a whole (Fig. 2-1B′). Having established that the ontogenetic allometry is the same in both fast growing and slow growing

Figure 2-1. Growth is coordinated among compartments within imaginal discs.



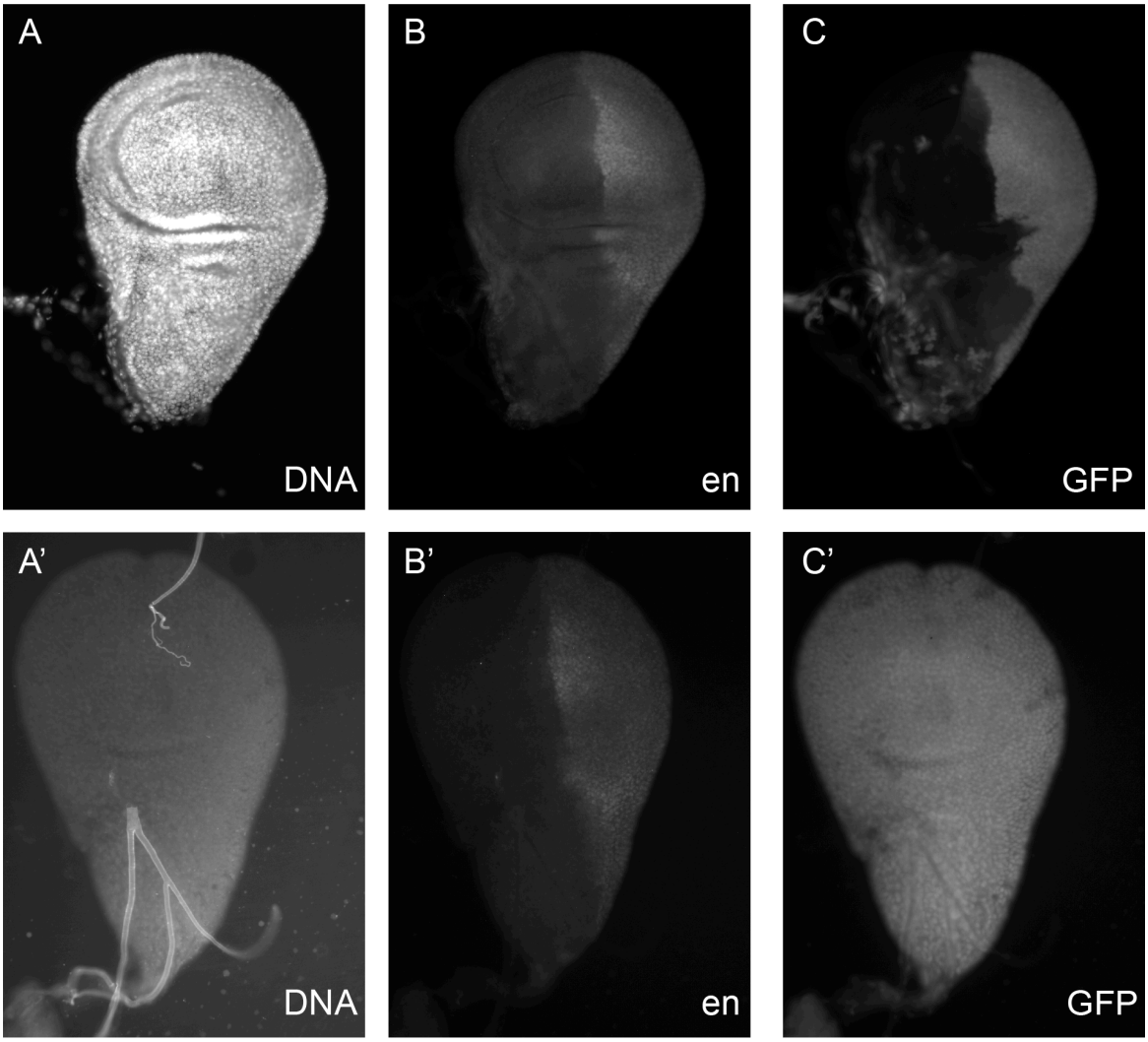
(A) The ontogenetic allometry between the anterior and posterior compartments in the third instar wing imaginal disc in WT larvae (n = 63). Predicted ontogenetic allometries between the anterior and posterior compartments in the third instar wing imaginal disc if compartment growth is mediated compartment autonomously (heavy solid line) or compartment non-autonomously (heavy broken line) (B) The ontogenetic allometry between anterior and posterior compartment size in WT (gray circles) (n = 63) and *M-/+* (open circles) (n = 50) imaginal discs are the same,

Figure 2-1 (cont'd)

indicating that the growth rate of the anterior relative to the posterior compartment is the same in both fast- and slow-growing discs (common slope test, $P = 0.8486$) (B′). In contrast, the ontogenetic allometry between anterior and posterior compartment size in ant^{fast}:$post^{slow}$ imaginal discs (blue circles) (n = 46) is significantly different than in both WT and *M-/+* discs (common slope test, Bonferroni corrected $P < 0.0074$ for both), indicating that the anterior compartment grows 8% more slowly relative to the posterior compartment when the posterior compartment is growth perturbed (B′). All error bars are 95% confidence intervals. WT : *ci-Gal4, UAS-FLP/+; FRT82B arm-lacZ/+*. *M-/+: ci-Gal4, UAS-FLP; FRT82B, ubi-GFP, P{A92}RpS3^{Plac92} /+*. ant^{fast}:$post^{slow}$: *ci-Gal4, UAS-FLP; FRT82B, ubi-GFP, P{A92}RpS3^{Plac92} / FRT82B arm-lacZ*.

wing discs, we generated larvae in which the anterior and posterior compartments ostensibly have different growth rates.. This was achieved by generating larvae with the genotype *ci-Gal4, UAS-FLP; FRT82B, ubi-GFP, P{A92}RpS3*^{Plac92} */ FRT82B arm-lacZ.* These larvae are heterozygous for the *Minute* allele *P{A92}RpS3*^{Plac92} and are slow growing. However, in the anterior compartment *ci-GAL4* drives expression of the recombinase FLP, which induces mitotic recombination to generate clones that are either homozygous for *P{A92}RpS3*^{Plac92} (*M⁻/M⁻*) or homozygous for the WT allele (+/+). The *M⁻/M⁻* clones die leaving the +/+ clones to proliferate and fill the entire compartment, evident by a loss of GFP (Fig. 2-2C). Consequently, the anterior compartment is WT and ostensibly fast-growing relative to the posterior *M⁻/+* compartment. Therefore, we refer to these discs as ant^{fast}:post^{slow} discs. It is important to note that anterior clone generation is essentially complete by the end of the second instar (L2), and therefore, from the beginning of the third instar (L3), the anterior compartment is almost entirely composed of +/+ cells left over after elimination of *M⁻/M⁻* cells. The initial cell doubling time of WT imaginal disc cells is 5-6hrs early in L3 +/+ discs and increases to 30hrs at the end of L3, while that of *M⁻/+* cells is ~11hrs in early L3 discs and increases to 34hrs at the end of L3 [23]. Thus, if compartments do grow autonomously, the slope of the ontogenetic allometry between anterior (y-axis) and posterior (x-axis) compartments should be significantly steeper in ant^{fast}:post^{slow} discs, particularly in smaller discs, when the difference in doubling time between of WT and *M⁻/M⁻* cells is expected to be greatest. However, upon plotting the ontogenetic allometry of the anterior (y-axis) and posterior (x-axis) compartment in ant^{fast}:post^{slow} and WT larvae, we find that the ostensibly 'fast growing' anterior compartment did not show an increase in growth rate (Fig. 2-1B). Rather, the slope of the ontogenetic allometry in ant^{fast}:post^{slow} discs

Figure 2-2. Generation of mitotic clones in the anterior compartment



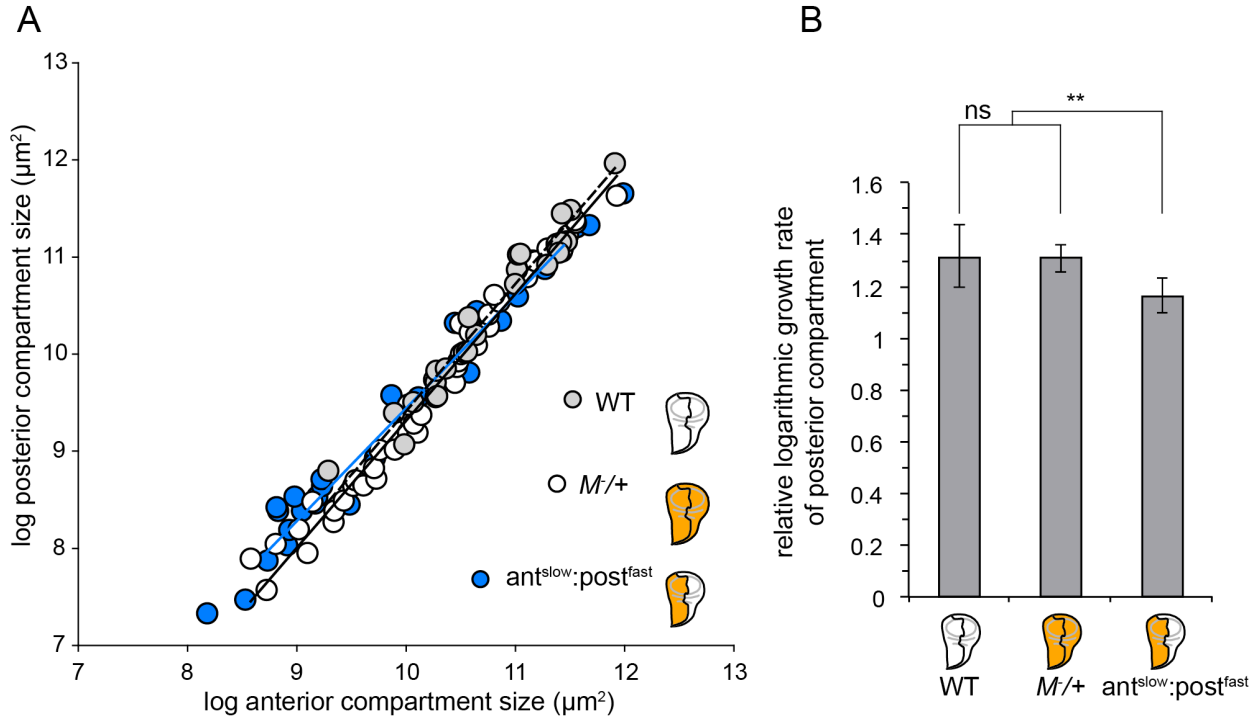
Mid-third instar wing imaginal discs from *ci-Gal4, UAS-FLP; FRT82B, ubi-GFP, P{A92}RpS3*^{Plac92} */ FRT82B arm-lacZ* discs imaged for DNA (A), *Engrailed* (B) and GFP (C). Mitotic clones in the anterior compartment are marked by loss of the GFP marker and occupy the entire anterior compartment. (A′-C′) Mid-third instar wing imaginal discs from *M*$^{-}$*/+* discs imaged for DNA (A′), *Engrailed* (B′) and GFP (C′).

was slightly but significantly shallower than that in both WT and *M*/+ control discs (common slope test, Bonferroni corrected $P < 0.0074$ for both) indicating a ~8% reduction in the relative growth rate of the anterior compartment relative to the posterior compartment in the third larval instar (Fig. 2-1B'). This is the opposite of what is expected if the anterior compartment were able to grow at a WT rate. It is possible that the reduced growth rate of the anterior compartment is due to ongoing death of *M*/*M* cells, compensating for the increased rate of proliferation of +/+ cells. However, this seems unlikely given that the anterior compartment is essentially entirely composed of +/+ cells by the beginning of L3. Nevertheless, reduced growth rate of the compartment may reflect changes in the rate of cell growth rather than cell proliferation.

We repeated the experiment using *en-Gal4/UAS-FLP; RpS17*^{4}*, ubi-GFP, FRT2A/FRT2A* larvae. In these larvae, the posterior compartment is ostensibly fast growing, while the anterior compartment is slow growing. We refer to these discs as ant^{slow}:post^{fast} discs. In this case the slope of the ontogenetic allometry of the posterior (y-axis) and anterior (x-axis) compartment is slightly lower than in WT and *M*/+ control disc (common slope test, $P = 0.$ 0.0034), indicating an ~11% reduction in the relative growth rate of the posterior compartment relative to the anterior compartment (Fig. 2-3) Again this is contrary to what is expected if the posterior compartment were able to grow at a WT rate.

To ensure that any differences in relative compartment growth rate between genotypes was not due to experimental error, and to determine the precision of our methodology, we repeated our experiment to determine the ontogenetic allometry of compartments in both WT and ant^{fast}:post^{slow} wing imaginal discs. We found that the slopes of the replicate ontogenetic allometries differed by, on average, 0.14%, and were statistically indistinguishable (Fig. 2-4).

Figure 2-3. Growth is coordinated in ant^{slow}:post^{fast} discs



(A) The ontogenetic allometry between anterior and posterior compartment size in WT (gray circles) (n = 23), *M-/+* (open circles) (n = 49) and ant^{slow}:post^{fast} (blue circles) (n = 33) imaginal discs are significantly different (common slope test, $P = 0.0034$), indicating that the anterior compartment grows 11% more slowly relative to the posterior compartment when the latter is ostensibly growth perturbed (B). All error bars are 95% confidence intervals. (WT: *en-Gal4, UAS-RFP*/+. ant^{slow}:post^{post}: *en-Gal4/UAS-FLP; RpS17^{4}, ubi-GFP, FRT2A/ FRT2A ri mwh*, *M-/+* : *CyO/UAS-FLP; RpS17^{4}, ubi-GFP, FRT2A /FRT2A ri mwh*)

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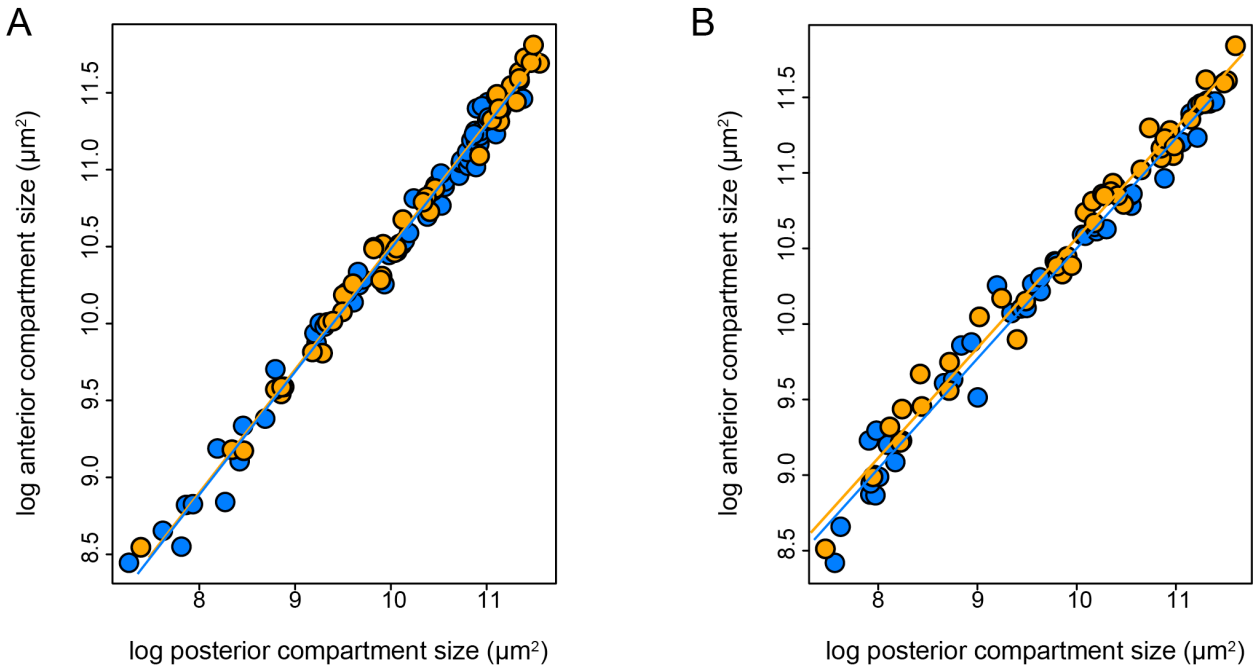
Figure 2-4: Repeated determination of ontogenetic allometry in WT and ant^{fast}:post^{slow} discs.

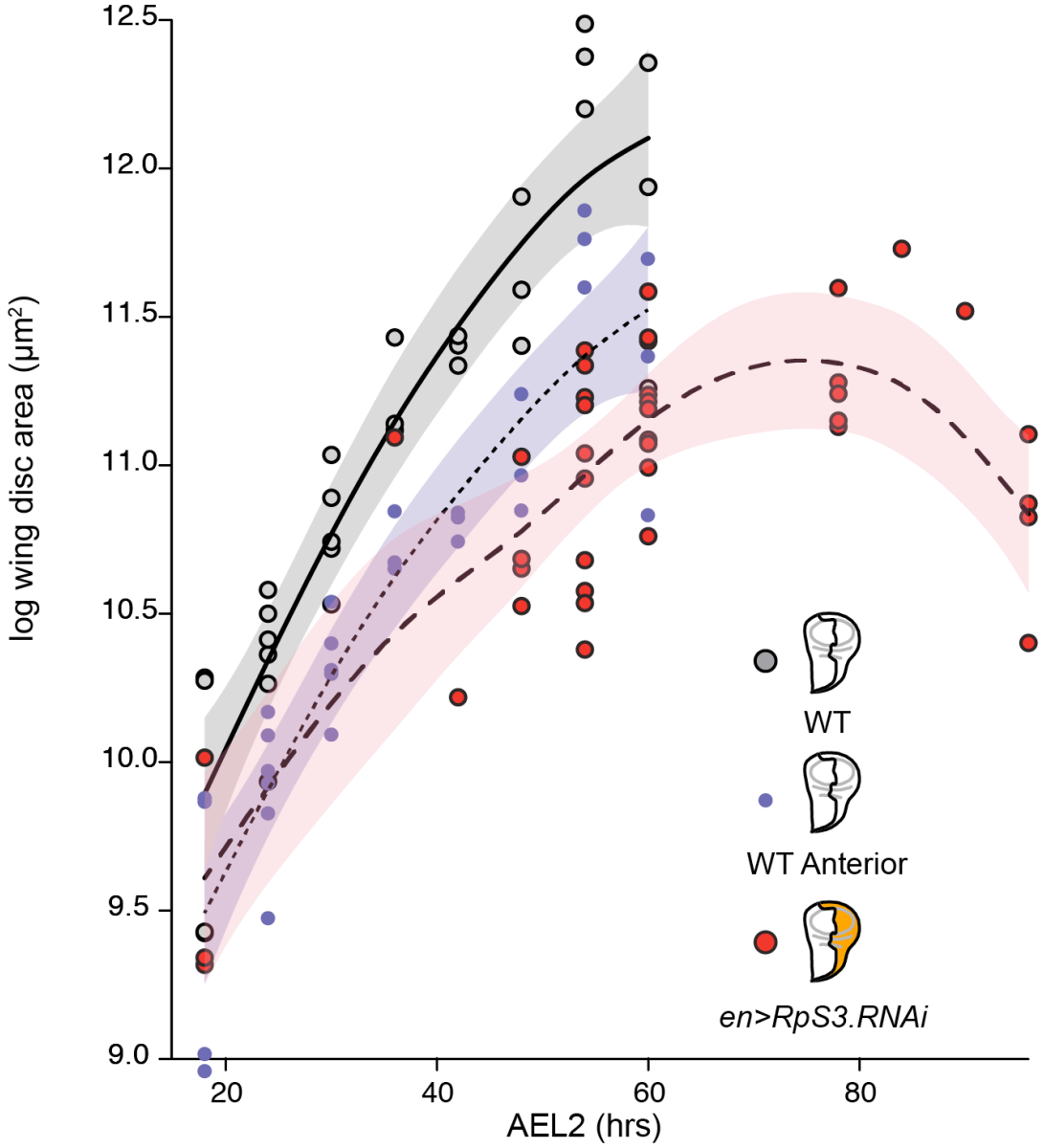


(A) The slope of the ontogenetic allometry between anterior and posterior compartment size in WT discs is the same when measured in two independent biological samples ($n_1 = 63$; $n_2 = 42$) (common slope test, P=0.9508). (B) The slope of the ontogenetic allometry between anterior and posterior compartment size in ant^{fast}:post^{slow} discs is the same when measured in two independent biological samples ($n_1 = 46$; $n_2 = 44$) (common slope test, P=0.9619). For both charts, data used in Fig. 2-1 and 4 are blue, data for repeated measure are orange. Average difference in slope between repeated measures is 0.14% of the steeper slope. (WT : *ci-Gal4, UAS-FLP/+*; *FRT82B arm-lacZ/+*; ant^{fast}:post^{slow}: *ci-Gal4, UAS-FLP; FRT82B, ubi-GFP, P{A92}RpS3^{Plac92} / FRT82B arm-lacZ*.)

Collectively, these data indicate that, as for growth between imaginal discs [9], there are mechanisms that prevent the overgrowth of ostensibly WT compartments in larvae that are otherwise growth-perturbed. We refer to these mechanisms as 'growth coordination mechanisms'. This coupling of growth rates is observed regardless of which compartment is growth perturbed.

Since larvae of the genotype ant^{fast}:$post^{slow}$ are genotypically identical to *M*/+ larvae in all tissues except the anterior compartment, it is not clear whether it is the growth-perturbed posterior compartment that is mediating a reduction in growth rate of the anterior compartment. Therefore, in order to identify whether growth coordination between compartments can be mediated by a compartment-autonomous growth perturbation, we generated larvae in which one compartment was slow-growing due to a ribosomal protein knockdown similar to our previous study [9]. Knocking down *RpS3* in the anterior or posterior compartment results in larval lethality at the L1 stage. We then used a posterior compartment temperature-regulated conditional knockdown of *RpS3* starting at L1/L2 ecdysis to generate growth perturbed discs of the genotype *en-Gal4, UAS-RFP/RpS3.RNAi; tub-Gal80ts/+*. In these larvae, only the posterior compartments of imaginal discs have a growth perturbation, whereas other larval organs are un-growth perturbed. Surprisingly, in these discs there is a severe loss of compartment identity and cells expressing *Engrailed* are almost, but not completely, eliminated during L3. Nevertheless, there is a strong reduction in growth rate of the wing disc during L3 relative to the discs of control larvae (Fig. 2-5). Further, if one consider the discs of *en-Gal4, UAS-RFP/RpS3.RNAi; tub-Gal80ts/+* larvae to essentially comprise only the anterior compartment, they also grow slower than the anterior compartment of WT discs (Fig. 2-5).

Figure 2-5: Imaginal disc growth is retarded in *en>Rps3.RNAi* larvae



Growth trajectory of wing imaginal discs from *en>Rps3.RNAi* (red circles) (n = 41) and corresponding controls (grey circles) (n = 30) after ecdysis to L2 (AEL2) indicates that *en>Rps3.RNAi* have a severe reduction in growth rate and size as compared to controls. Under the assumption that *en>RpS3.RNAi* consist of only anterior cells, a comparison between the

Figure 2-5 (cont'd)

growth trajectory of the anterior compartment in WT wing imaginal discs (purple circles) (n = 30) and *en>RpS3.RNAi* wing imaginal discs indicates that *en>RpS3.RNAi* are still growth retarded as compared to controls. Shading indicates 95% confidence intervals. (WT: *en-Gal4, UAS-RFP/60,000; tub-Gal80ts/+*. *en>RpS3.RNAi*: *en-Gal4, UAS-RFP/UAS-RpS3.RNAi; tub-Gal80ts/+*)

Ecdysone treatment results in a disruption of growth coordination between compartments

We have previously shown that low levels of ecdysone are necessary for inter-disc growth coordination. In larvae with growth-perturbed wing discs and low levels of ecdysone synthesis, feeding 20E rescues the growth rate of other non-growth perturbed imaginal discs to a near-WT growth rate [9]. We reasoned therefore, that ecdysone signaling may also be involved in mediating coordination between compartments within discs. To test this hypothesis, we fed 20E to larvae with ant^{fast}:$post^{slow}$ discs and measured the ontogenetic allometry of the anterior and posterior compartments in the wing imaginal disc. Larvae were staged at the L2-L3 transition and collected in 4h cohorts, and then 12h later transferred to food containing 20E or ethanol (EtOH). Wing imaginal discs were dissected 24h after transfer to 20E or EtOH containing food, and compartment sizes were measured as previously described. We found that, as for inter-organ growth coordination, ecdysone is involved in mediating intra-organ growth coordination and this can be disrupted by exogenous application of 20E. In 20E-fed larvae with ant^{fast}:$post^{slow}$ discs, the slope of the ontogenetic allometry of the anterior compartment size against the posterior compartment size is significantly higher than that of the EtOH-treated control (common slope test, P = 0.0044) (Fig. 2-6A). As previously described, an increase in the slope of the ontogenetic allometry indicates that the anterior compartment has a greater relative growth rate compared to the posterior compartment. Analysis of the slopes indicates that there is a ~40% growth acceleration in the anterior compartment relative to the posterior in 20E-fed ant^{fast}:$post^{slow}$ larvae (Fig. 2-6A′). This is not because the anterior compartment is particularly responsive to ecdysone. In *M*/+ larvae, where growth of the whole disc is uniformly slowed, 20E treatment does not significantly affect the relative growth rate of the anterior compartment against the posterior compartment (common slope test, P = 0. 9850) (Fig. 2-6B, 2-6B′). These results

Figure 2-6. 20E treatment disrupts growth coordination between compartments in ant^{fast}:post^{slow} larvae.

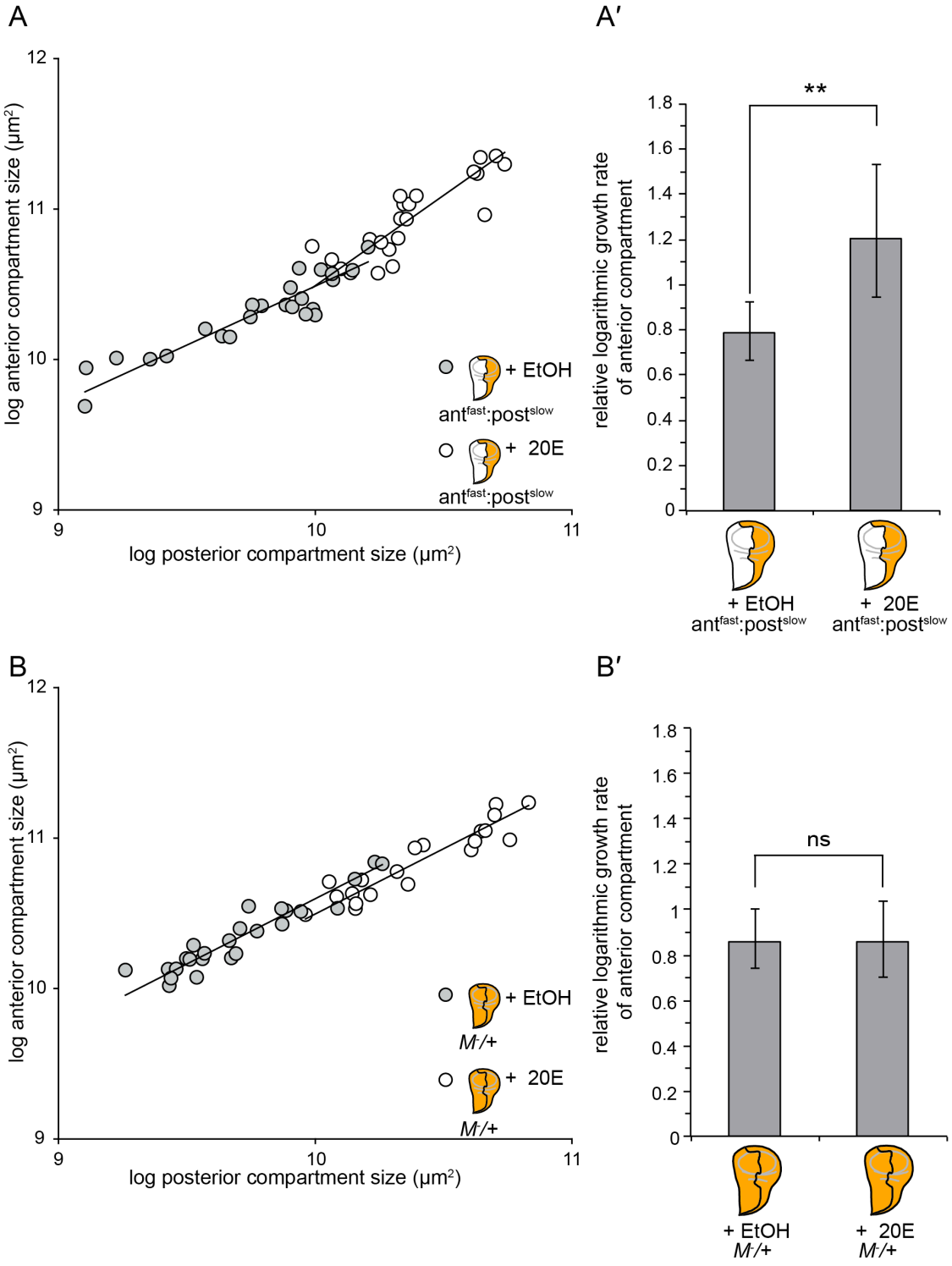


Figure 2-6 (cont'd)

(A) The ontogenetic allometry between anterior and posterior compartment size in ant^{fast}:post^{slow} imaginal discs from larvae fed EtOH (gray circles) (n = 24) or 20E (open circles) (n = 22) are significantly different (common slope test, $P = 0.0044$), indicating that 20E application increases the anterior compartment's relative growth rate by 40% (A′). (B) The ontogenetic allometry between anterior and posterior compartment size in *M͞/+* imaginal discs from larvae fed EtOH-fed *M͞/+* (gray circles) (n = 25) or 20E (open circles) (n = 20) are the same (common slope test, $P = 0.9850$), indicating that 20E application does not affect the relative growth rate of the compartments in wholly slow-growing wing imaginal discs (B′). All error bars are 95% confidence intervals. ant^{fast}:post^{slow}: *ci-Gal4, UAS-FLP; FRT82B, ubi-GFP, P{A92}RpS3^{Plac92} / FRT82B arm-lacZ.*

indicate that in 20E-fed ant^{fast}:post^{slow} discs, there is an acceleration of growth in the anterior compartment relative to the posterior compartment, indicating a disruption of the intra-organ growth coordination.

Growth coordination between compartments is disrupted by changes in EcR signaling

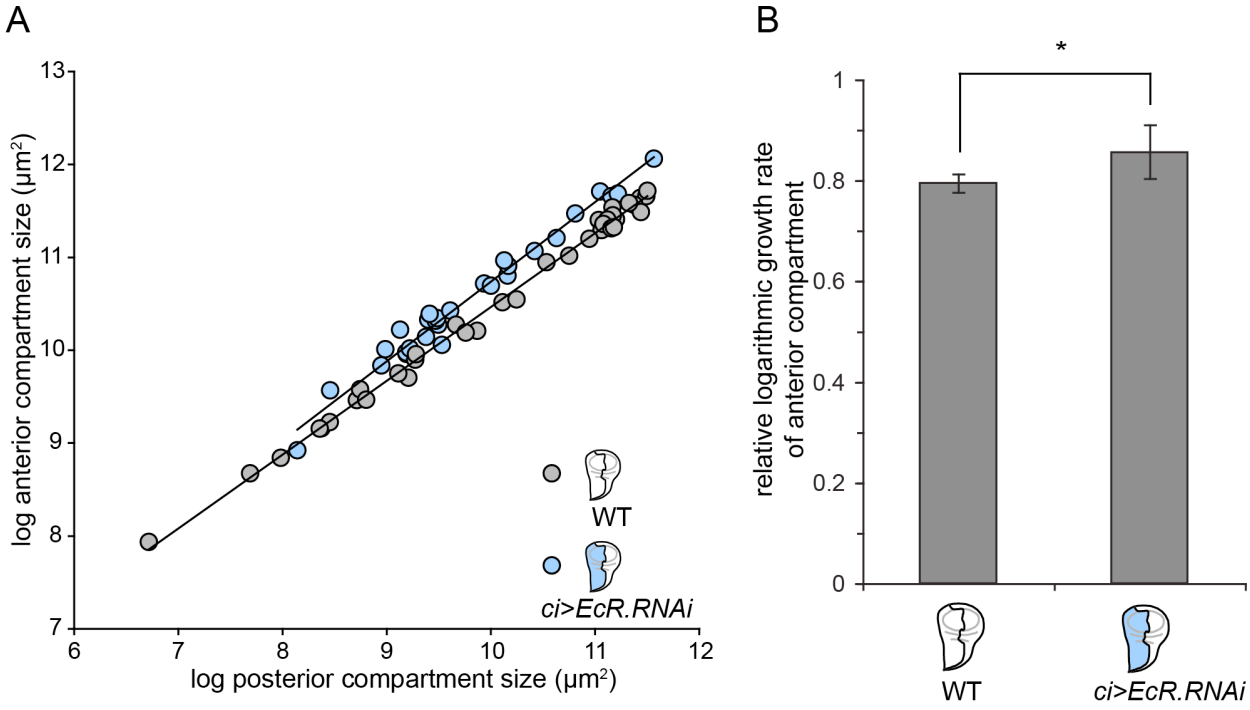
If exogenous application of 20E to ant^{fast}:post^{slow} larvae results in acceleration in the growth rate of the anterior compartment, then organ autonomous up-regulation of ecdysone signaling should also alter WT inter-compartmental coordination. In order to test this, we exploited the fact that the unliganded EcR protein in complex with Ultraspiracle (Usp) represses target genes by binding to gene promoters [24-26]. On binding of EcR to 20E, the EcR-Usp complex undergoes a conformational change, and bound genes become transcriptionally active both through de-repression and activation of gene expression through recruitment of additional co-factors [27, 28]. Thus, suppression of *EcR* expression by RNAi-knockdown eliminates repression of the EcR-Usp complex on target genes, and, for some targets, would be equivalent to 20E-dependent increase in gene expression. Evidence for this comes from the observation that knocking down *EcR* expression in the wing disc promotes precocious expression of patterning genes such as Senseless and Cut and differentiation genes such as Broad [29]. If knocking down *EcR* can drive patterning we reasoned it might also drive growth and proliferation of the wing imaginal disc. Therefore, to test whether compartment autonomous upregulation of ecdysone signaling could also disrupt coordination between compartments, we knocked down EcR expression in the anterior compartment in both ant^{fast}:post^{slow} and WT larvae. In WT discs, knocking down *EcR* in the anterior compartment alters coordination between compartments and increases the growth rate of the anterior compartment relative to the posterior compartments and increases the growth rate of the anterior compartment relative to the posterior by ~8% (common slope test, P =

0.0243) (Fig. 2-7A, 2-7B). This modest acceleration in growth likely reflects the fact that ecdysone synthesis is not retarded in WT larvae and so our manipulation only moderately increased ecdysone signaling above endogenous levels. To test whether knockdown of *EcR* in the anterior compartment had a more marked effect on growth coordination when endogenous ecdysone levels are low we repeated the experiment in ant^{fast}:$post^{slow}$ discs. We failed, however, to recover L3 larvae. Nevertheless, our data support a role for 20E in promoting growth of imaginal discs and in growth coordination across a disc.

Growth coordination between compartments is disrupted by changes in Insulin-signaling

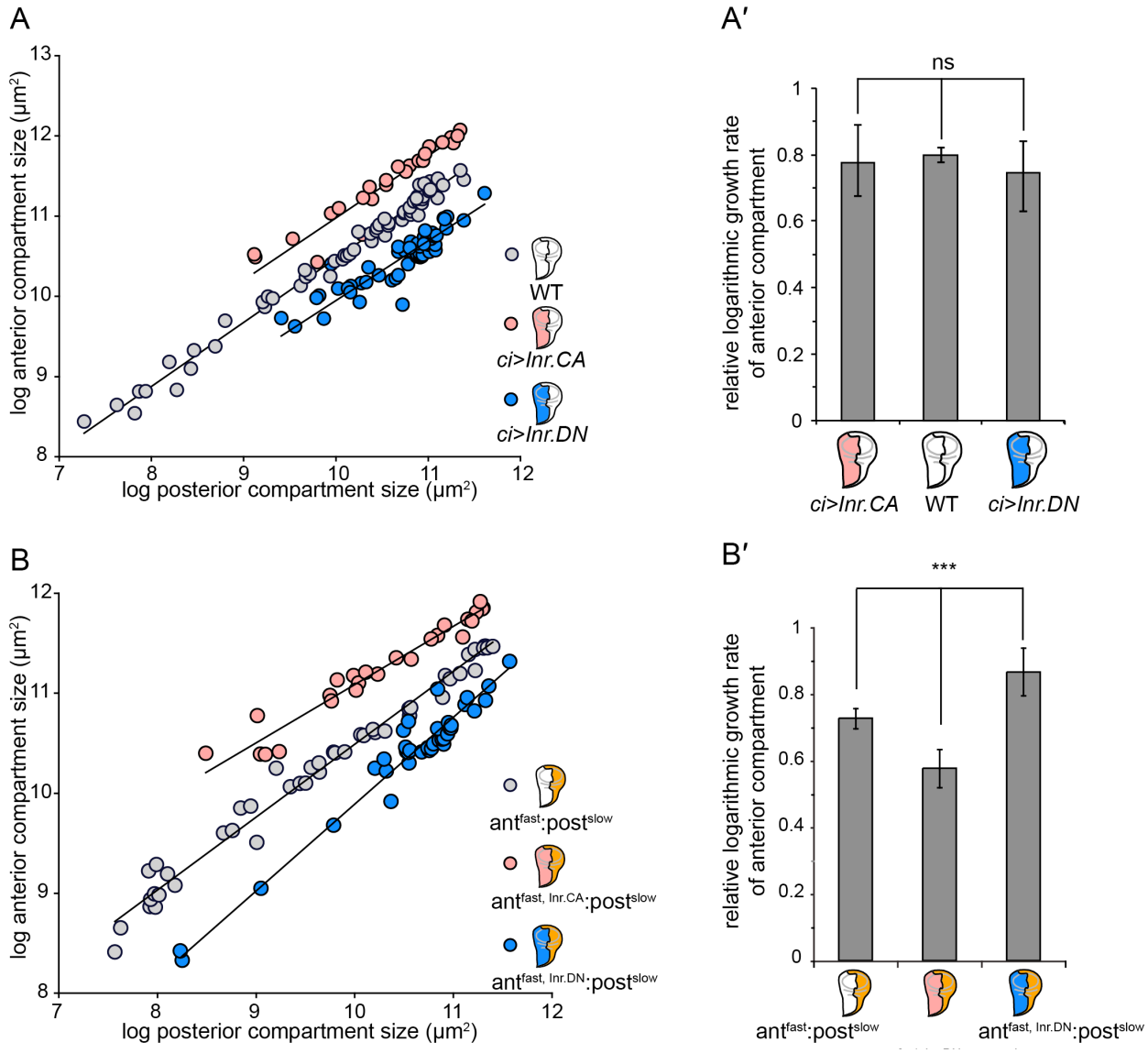
Collectively, our data suggest that in the context of coordinated growth within and between imaginal discs, ecdysone functions as a growth promoter. This role for ecdysone as a growth promoter is supported by recent findings that show that imaginal disc growth is slowed when ecdysone synthesis is inhibited or absent, and rescued when ecdysone levels are restored [9, 10, 13]. However, this is contrary to the known role of ecdysone in suppressing the growth of larval tissues by suppressing the IIS pathway [30-32]. Together, these data suggest that ecdysone may have different effects on different tissues. If ecdysone suppresses growth by suppressing the IIS pathway in larval tissues, it is possible that it activates growth in imaginal discs through the same pathway. We therefore explored whether, like ecdysone signaling, IIS/TOR signaling is sufficient to alter coordination between developmental compartments within discs. We first up- and down-regulated IIS in the anterior compartment of WT wing imaginal disc, by expressing a constitutively active (InR.CA) and dominant negative (InR.DN) form of the insulin receptor, respectively. We found that, whilst the anterior compartment was proportionally larger (InR.CA) or smaller (InR.DN) relative to the posterior compartment than in controls, the relative growth rate of the two compartments was unchanged in either manipulation (Fig. 2-8A, 2-8A′). Thus,

Figure 2-7. EcR knockdown in the anterior compartment disrupts growth coordination in WT larvae



(A) The ontogenetic allometry between anterior and posterior compartment size in WT(gray circles) (n = 38) and *ci>EcR.RNAi* (blue circles) (n = 28) imaginal discs, both reared at 17˚C, are significantly different (common slope test, $P = 0.0243$), indicating that partially up-regulating ecdysone signaling by knockdown of *EcR* increases the relative growth rate of the anterior compartment by 8% (B). All error bars are 95% confidence intervals. WT: *ci-Gal4, UAS-FLP/+* raised at 17°C. *ci>EcR.RNAi*: *ci-Gal4, UAS-FLP/+; UAS-EcR.RNAi/+* raised at 17°C.

Figure 2-8. InR expression is necessary but not sufficient to disrupt intra-organ growth coordination.

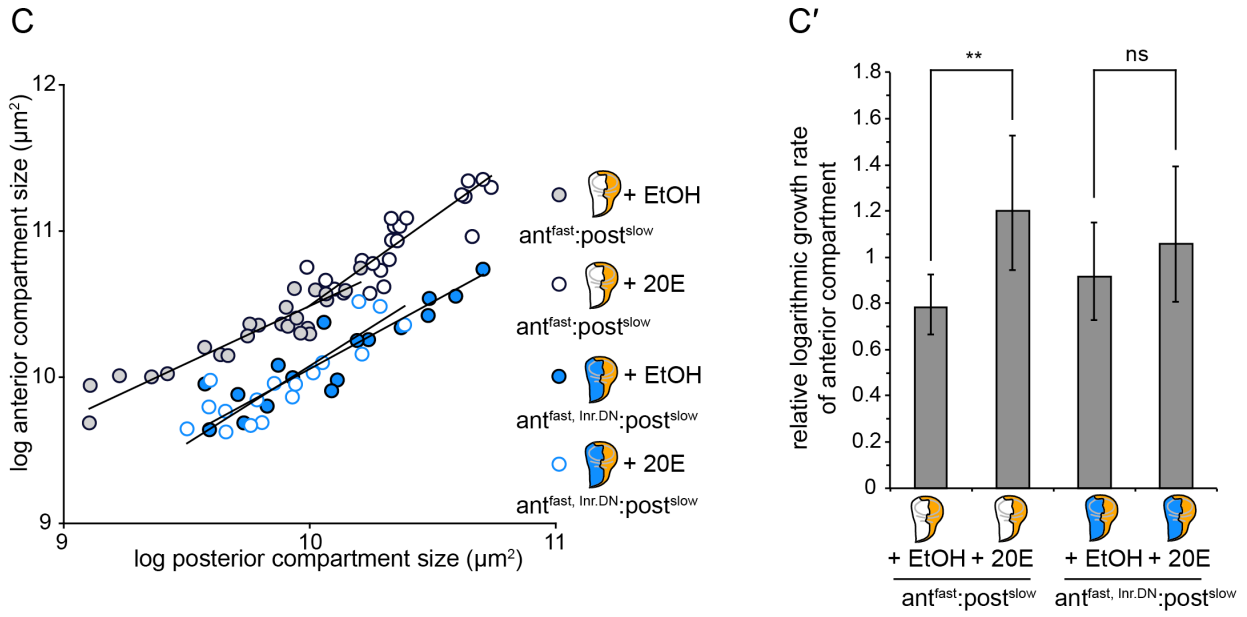


(A) The slope of the ontogenetic allometry between anterior and posterior compartment size in WT (gray circles) (n = 63), *ci-Gal4>InR.CA* (pink circles) (n = 25) , and *ci-Gal4>InR.DN* (blue circles) (n = 54) imaginal discs are the same (common slope test, *P*=0.4899), indicating that up- or down-regulating IIS in the anterior compartment alone does not affect its growth rate relative to the posterior compartment (A′). (B) The slope of the ontogenetic allometry between anterior and posterior compartment size in ant^{fast}:post^{slow} (gray circles) (n = 46), ant$^{fast, InR.CA}$:post^{slow} (pink

Figure 2-8 (cont'd)

circles) (n = 25) and $ant^{fast,\ InR.DN}$:$post^{slow}$ (blue circles) (n = 37) imaginal discs are significantly different (Common slope test, Bonferroni corrected $P<0.0001$), indicating that changes IIS in the anterior compartment in ant^{fast}:$post^{slow}$ discs is sufficient to affect its growth rate relative to the posterior compartment (B′).

Figure 2-8 (cont'd)



(C) The slope of the ontogenetic allometry between the anterior and posterior compartment in ant$^{fast,\ InR.DN}$:post^{slow} imaginal discs is the same in 20E-fed (blue open circles) (n = 17) and EtOH-fed larvae (blue filled circles) (n = 17) (common slope test, P= 0.3942), indicating that the elevated relative growth rate of the anterior compartment in response to 20E (Fig 2A) is inhibited when IIS is suppressed in the anterior compartment (C′). The ontogenetic allometries of ant^{fast}:post^{slow} fed either 20E or EtOH (Fig 2A) are presented for reference. WT: *ci-Gal4, UAS-FLP/+; FRT82B arm-lacZ/+*. *ci>InR.DN*: *ci-Gal4, UAS-FLP/+; UAS-InR.K1409A* ^{33}*/+*. *ci>InR.CA*: *ci-Gal4, UAS-FLP/+; UAS-InR.del* ^{33}*/+*, ant^{fast}:post^{slow}. *ci-Gal4, UAS-FLP; FRT82B, ubi-GFP, P{A92}RpS3*^{Plac92} */ FRT82B arm-lacZ* (Control). ant$^{fast,\ InR.DN}$:post^{slow}: *ci-Gal4, UAS-FLP; UAS-InR.K1409A* ^{33}*/+; FRT82B, ubi-GFP, P{A92}RpS3*^{Plac92} */ FRT82B arm-lacZ.* ant$^{fast,\ InR.CA}$:post^{slow}: *ci-Gal4, UAS-FLP; UAS-InR.del* ^{33}*)/+ FRT82B, ubi-GFP, P{A92}RpS3*^{Plac92} */ FRT82B arm-lacZ.* Note that the WT and ant^{fast}:post^{slow} data are the same as those in Fig. 2-1A, and 1B since these experiments were conducted in parallel and the data split between Fig. 2-1 and Fig. 4.

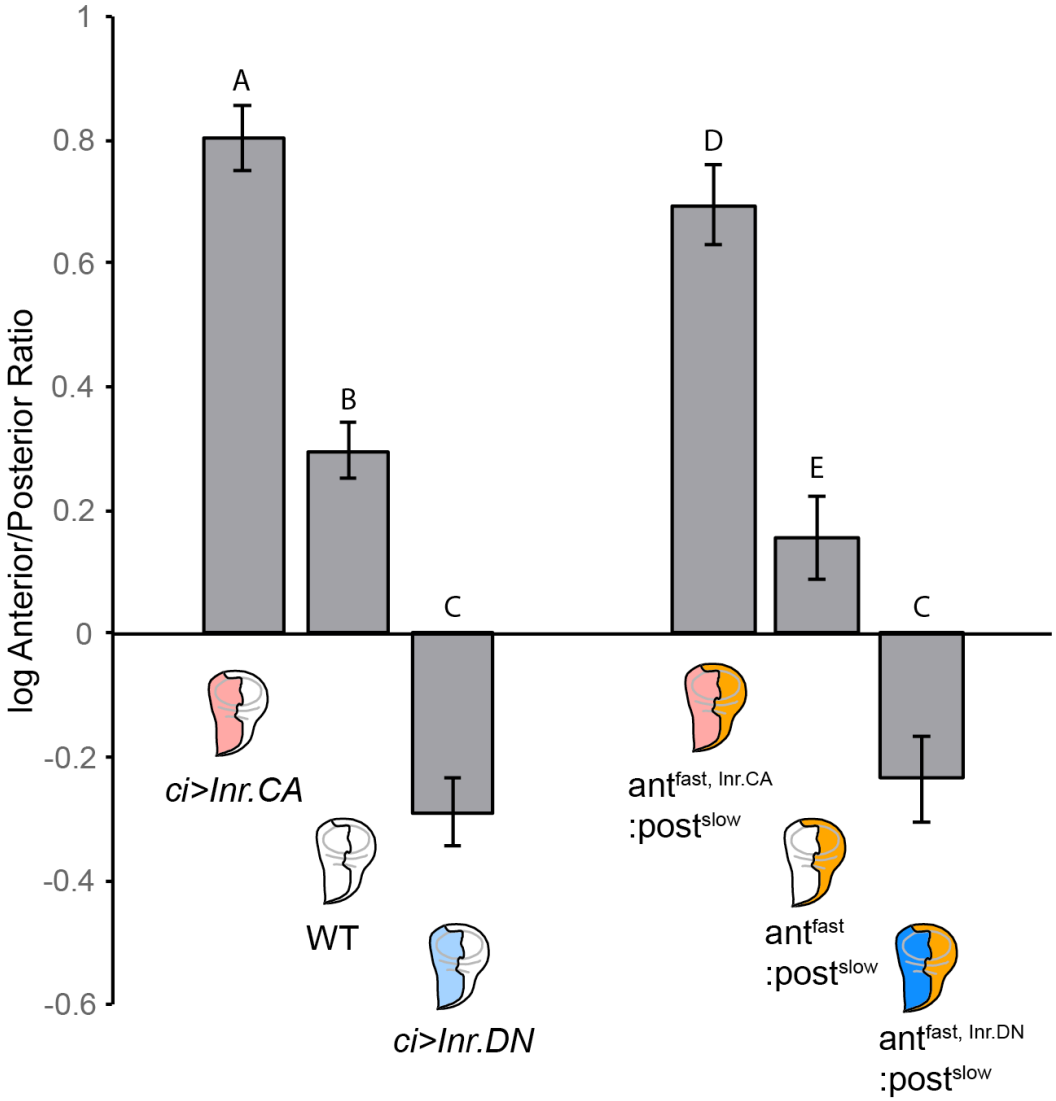
upregulation or downregulation of *InR* in otherwise WT imaginal discs affects compartment size, but not their relative growth rates in L3 larvae.

Counterintuitively, expression of InR.DN in the anterior compartment increases its growth rate relative to the posterior compartment, whilst expression of InR.CA has the opposite effect (Fig. 2-8B, 2-8B′). The result is that compartment autonomous changes in insulin-signaling have less of an effect on final anterior-to-posterior size ratio in ant^{fast}:$post^{slow}$ discs than in otherwise WT discs (Fig. 2-9).

We then asked whether IIS is necessary for the ecdysone-mediated alteration of growth coordination in ant^{fast}:$post^{slow}$ wing imaginal discs, by expressing a dominant negative allele of InR (InR.DN) in the anterior compartment when larvae were fed 20E. Analysis of the ontogenetic allometry between anterior and posterior compartment-sizes in the 20E fed larvae reveals that the slope of this ontogenetic allometry is not significantly different than that in the EtOH treated larvae (Fig. 2-8C, 2-8C′). Thus, the 20E treatment fails to disrupt growth coordination in larvae with autonomous down-regulation of IIS in the anterior compartment, supporting the hypothesis that IIS in the anterior compartment is necessary for 20E stimulated growth.

Note that in the EtOH-treated larvae (Fig 2-8C, 2-8C′) the slope of the ontogenetic allometry is not significantly different between ant^{fast}:$post^{slow}$ and $ant^{fast,\ InR.DN}$:$post^{slow}$ discs (common slope test, $P = 0.2632$). It is possible that ethanol bypasses the effects of compartment-autonomous down-regulation of IIS on growth coordination (Fig. 2-8B, 2-8B′). A more likely explanation, however, is that our inability to detect a difference reflects reduced statistical power due to a smaller sample size. This is supported by the observation that in both ant^{fast}:$post^{slow}$ and $ant^{fast,}$

Figure 2-9 Compartment autonomous changes in IIS have reduced effect on final anterior:posterior ratio in ant^{slow}:post^{fast} discs



The effects of increased InR activity in the anterior compartment has significantly less effect on final anterior:posterior ratio in ant$^{fast,\ InR.CA}$:post^{fast} discs (log (A/P) = 0.69) than in *ci>InR.CA* discs (log (A/P) = 0.8). Similarly, decreased InR activity in the anterior compartment has less effect, albeit not significantly, on final anterior:posterior ratio in ant$^{fast,\ InR.DN}$:post^{fast} discs (log (A/P) = -0.23) than in *ci>InR.DN* discs (log (A/P) = -0.28) (n = 10-12 for each group). Columns with different letters are significantly different (Tukey post-hoc test, $P < 0.05$)

$^{InR.DN}$:*post*^{slow}discs, relative growth rate of the anterior and posterior compartments is the same with and without EtOH treatment (common slope test, $P > 0.2307$ for both)..

Discussion

Our results reveal that growth among developmental compartments in an organ is tightly coordinated, such that even if the growth of one compartment is perturbed, both compartments grow at more-or-less the same relative rate as observed in WT flies. This growth coordination between compartments is disrupted by exogenously feeding 20E to growth-perturbed larvae, resulting in an acceleration in the growth rate of the unperturbed compartment. This growth acceleration upon feeding 20E is dependent on IIS in the unperturbed compartment. Collectively these data support a model of imaginal disc growth regulation whereby growth perturbation in one compartment causes a systemic reduction in circulating ecdysteroids, which results in reduction in growth rate of the adjacent compartment.

These data are surprising in light of previous studies that suggest that imaginal discs and individual compartments within imaginal discs can autonomously grow to their target size. Bryant and Levinson cultured WT imaginal discs in the abdomen of adults hosts and found that these discs grow autonomously to their normal size [34]. More recently, Martin and Morata generated 'fast' discs and compartments in *M*$^{-}$/+ larvae and demonstrated that these compartments have higher growth rates relative to the body as a whole and to adjacent compartments [18, 23]. These authors further demonstrate that the 'fast' compartments and discs are developmentally advanced as compared to *M*$^{-}$/+ controls. Collectively, these data support the hypothesis that imaginal disc possesses an autonomous mechanism for arresting growth once they reach a target size, and that this mechanism operates at the level of developmental compartments. Whilst compartments may possess a target size, our data suggest that they do not

grow independently to this size, at least *in vivo.* Rather growth between developmental compartments is coordinated even when one compartment is growth perturbed**,** and this growth coordination appears to be regulated by systemic rather than disc autonomous mechanisms.

Our conclusions are supported by data from Mesquita et. al [19], who also looked at inter-compartmental growth in the *Drosophila* wing imaginal disc. They observed that slowing the growth of one compartment non-autonomously slowed the growth of the adjacent compartment. They further demonstrate that the signal from the growth-perturbed compartment is dependent on *Drosophila* p53. However, they do not elucidate what the signal is. Our results suggest that the signal involves ecdysone. This is surprising given our current understanding of wing imaginal disc growth. Recent models of disc growth suggest that growth of the wing imaginal disc is driven mainly by morphogen gradients formed by the patterning genes Wg, Dpp, and Vg, which drive cellular proliferation within the disc [14, 35-39]. More recent studies further implicate disc-autonomous mechanisms in regulating the relative size of different compartments within the wing[20]. Our data show that systemic signaling, mediated by ecdysone, is also critical for regulating growth rates among different parts of the disc.

The involvement of ecdysone in intra-organ growth coordination echoes its known role in inter-organ growth coordination. As noted above, growth among organs is tightly coordinated when one organ is growth perturbed—a consequence of the growth-perturbed organ suppressing ecdysone synthesis [8, 9]. Addition of ecdysone to these growth-perturbed larvae is able to rescue the growth rate of undamaged imaginal discs. Ecydosne is however not able to rescue the growth rate of the growth perturbed tissues [9], most likely because the inherent growth perturbation of these tissues prevents them from responding to ecdysone. Similar to these studies on inter-organ growth coordination, our data suggest ecdysone is able to rescue the growth rate of WT

compartments in *M⁻*/+ larvae, and this is mediated by compartment-autonomous ecdysone signaling.

Whilst our data indicate that ecdysone is an important growth-coordinating signal among developmental compartments, it is unclear precisely which tissue is influencing ecdysone synthesis. It is possible that in larvae with ant^{fast}:post^{slow} discs, since the whole of the rest of the larva is *Minute*, the limitation on ecdysone might be an autonomous effect of the *Minute* mutation on the prothoracic gland. However, our data demonstrate that knock-down of *RpS3* using *engrailed*-GAL4, which is not expressed in the prothoracic gland, still retards disc growth. This suggests that the growth coordination mechanism is regulated by a signal from the compartments themselves. As discussed above, in studies where systemic growth is retarded through localized tissue damage, including knock-down of ribosomal proteins, it is the damaged/growth-pertrubed tissue itself that inhibits ecdysone synthesis by signaling via dILP8 [10-12]. Therefore, in larvae with ant^{fast}:post^{slow} discs, ecdysteroidgenesis could be limited via a dILP8-dependent mechanism. Which compartment is generating a putative dILP8 signal is, however, unclear. dILP8 levels are highest at the L2-L3 transition and decline during L3, before increasing somewhat before pupariation (Colombani et al. 2012). It is possible, therefore, that in larvae with ant^{fast}:post^{slow} discs, it is the immature slow-growing posterior compartment that is secreting dILP8. Conversely, the residual generation and death of *M⁻*$^{/-}$ cells in the anterior compartment through mitotic recombination early in L3 may also drive dILP8 synthesis. Further experiments exploring the role of dILP8 in intra-organ growth coordination are clearly necessary.

A key feature of growth coordination is that ecdysone acts as a promoter of growth for imaginal discs. This appears contrary to previous findings that show that ecdysone inhibits larval body

growth by inhibiting IIS or Myc in the fat body [31, 40]. However, evidence from other insect species suggests that ecdysone can function as either a growth promoter or inhibitor, depending on its concentration [41]. Specifically, *in vitro* evidence from *Manduca* shows that low concentrations of ecdysone can promote growth of imaginal tissues, while higher concentrations stimulate differentiation, and stop cell proliferation [42]. Further evidence from *Manduca* suggests that ecdysone promotes mitosis by regulating the cell cycle, and thus acts as a mitogen[43, 44]. These data echo data from *Drosophila* that suggests that ecdysone regulates cell cycle progression and promotes imaginal disc growth via the ecdysone inducible gene *crooked legs* [44]. Collectively, it is apparent, therefore, that ecdysone is a central regulator of larval and imaginal tissue growth, although the tissue-specific effects and molecular mechanisms involved have not yet been completely elucidated. Research from our and other labs support the hypothesis that imaginal discs reduce their growth rates in response to low levels of ecdysone [9, 13]. At the same time, low levels of ecdysone increase body growth rate and final adult body size [31]. Together these data suggest that ecdysone suppresses the growth of larval tissue (which comprises the majority of the larva) but promotes growth of imaginal tissues. This hypothesis has intuitive appeal in that a key function of ecdysone is to 'prepare' the larva for pupariation and metamorphosis, a process that involves breakdown and autophagy of the larval tissues to provide nutrients for final growth and differentiation of the imaginal discs.

Research over the past decade has elucidated mechanisms by which ecdysone functions as a suppressor of larval growth. These studies demonstrate a role for IIS in ecdysone-mediated suppression of larval growth. Specifically, ecdysone signaling in the fat body suppresses IIS, which in turn inhibits systemic IIS and larval growth through repression of dILP2 release from the brain and promotes fat body autophagy [30-32, 45, 46]. How ecdysone promotes imaginal disc

growth is less clear, however. A recent paper by Herboso et al, indicated that ecdysone promotes growth by suppressing *Thor* signaling in the imaginal discs. Discs from larvae with reduced ecdysone synthesis have elevated levels of Thor, a repressor of growth that is a target of the IIS pathway. The hypothesis that ecdysone regulates and coordinates growth via IIS/TOR signaling is further supported by our observation that down-regulation of InR activity prevents the WT compartment of ant^{fast}:post^{slow} discs from increasing its relative growth rate in response to ecdysone.

However, additional data suggest a more nuanced role for IIS in coordinating growth among developmental compartments. In particular, changes in InR activity in the anterior compartment do not affect relative compartment growth rate in larvae that are otherwise wholly WT. Rather, changes in InR activity increase or decrease relative compartment size, presumably due to changes in compartment growth earlier in development. This is surprising, given that mutations of *InR* reduce the growth and proliferation of clones in the wing imaginal disc during L3[47]. In ant^{fast}:post^{slow} discs, however, changes in InR activity does alter growth coordination during L3, but in a counterintuitive way: reduced InR activity increases relative growth rate, whilst increased InR activity decreases relative growth rate. This is the opposite of what we would predict if ecdysone promotes growth by directly upregulating IIS. One interpretation of these data is that the anterior compartments of the ant^{fast}:post^{slow} disc adjust their relative growth rate to rescue the final anterior:posterior size ratio, presumably using a mechanism independent of ecdysone. Why this rescue is not evident in WT larvae is unclear, but suggest that the rescue mechanism is able to override the ecdysone-regulated mechanism that coordinates growth rates between compartments with different potential growth rates.

From our study and those of others, it seems unlikely that ecdysone promotes imaginal disc growth only through its effects on IIS. In particular, the role of ecdysone in the regulation of differentiation and patterning genes such as Broad, Senseless and Cut has been well elucidated [24, 29]. Patterning genes are known to regulate cell proliferation [48]. It is therefore possible that ecdysone also regulates imaginal disc growth by regulating the expression of patterning genes in the imaginal disc. One of the challenges in elucidating the role of ecdysone signaling in imaginal disc development is that manipulating ecdysone-signaling organ-autonomously in imaginal discs is technically difficult. We likely only subtly up-regulated ecdysone signaling by knocking down *EcR* compartment-autonomously and found that this mild knockdown accelerated compartment growth. It is seems likely that this effect is related to the degree of the knockdown, however, for two reasons. First, complete knockdown of EcR will ultimately block ecdysone signaling, even if it de-represses the expression of certain genes. Second, ecdysone levels can both promote and inhibit insect growth and development depending on its level. As discussed above, moderate level of ecdysone are sufficient to stimulate imaginal disc growth *in vitro*, while high levels suppress cell proliferation [42]. More precise methods of manipulating ecdysone signaling at a cellular and tissue level are therefore needed.

In summary, our study provides evidence for an ecdysone-dependent mechanism that coordinates growth between compartments in the wing imaginal disc of *Drosophila*. Our data suggest that the control of cell proliferation across the imaginal disc is not an entirely autonomous process, but is coordinated through humoral signaling. Our research also highlights the crosstalk between different systemic signaling mechanisms – insulin/IGF- and ecdysone-signaling – in the generation of correctly proportioned organs. The developmental mechanisms regulating organ size, while best studied in *Drosophila*, are conserved across all animals. It is likely, therefore,

that the mechanisms coordinating growth between and within organs would also be conserved. There is considerable evidence that localized growth perturbation causes systemic growth retardation in humans. For example, children suffering from chronic inflammatory diseases such as Crohn's disease have systemic growth hormone insensitivity and experience severe growth retardation as a complication of the disease [49]. The utilization of systemic signaling mechanisms to coordinate growth within and between organs may thus be a conserved mechanism across all animals.

APPENDIX

Materials and Methods

Drosophila stocks

The fly stocks used in the study are as follows (full genotype and stock numbers are in parentheses): *Foxo21 (yw; Foxo21/TM3, Sb,* Lab Stock), *Foxo.RNAi (w*; UAS-Foxo.RNAi,* VDRC 30556), *Thor.RNAi (w[*]; P{w[+mC]=UAS-Thor.wt}2,* BDSC 9147), *Thor. RNAi (y[1] sc[*] v[1]; P{y[+t7.7] v[+t1.8]=TRiP.HMS01555}attP40,* BDSC 36667)

Generation of mitotic clones and measurement of wing imaginal disc

Wing imaginal discs were dissected and measured as previously earlier in the chapter. Mitotic clones in the anterior compartment were generated as previously described.

Results and Discussion

In order to identify the targets of EcR and/or ecdysone signaling in the imaginal disc which may be involved in the coordinated growth of compartments, we explored the role of two effectors downstream of the IIS/TOR pathway. As previously stated, ecdysone has been recently shown to promote imaginal disc growth by negatively regulating the expression of *Thor,* a repressor of growth that is a target of the IIS/TOR pathway. Since compartment-autonomous increases in ecdysone signaling can break intra-organ growth coordination we reasoned that knock down of *Thor* expression may do the same by accelerating the growth rate. In order to test this hypothesis, we measured the ontogenetic allometry between compartments in larvae with an anterior compartment knockdown of *Thor*. We find that there is no difference between the growth rates between control and knockdown discs (Fig. 2-10A, A′). Simultaneously, we also tested whether overexpression of *Thor* disrupted growth coordination between compartments (Fig. 2-10B, B′). Once again we find that overexpression of *Thor* had no effect on the ontogenetic allometry between compartments.

Figure 2-10 Compartment autonomous modulation of Thor expression does not disrupt intra-organ growth coordination

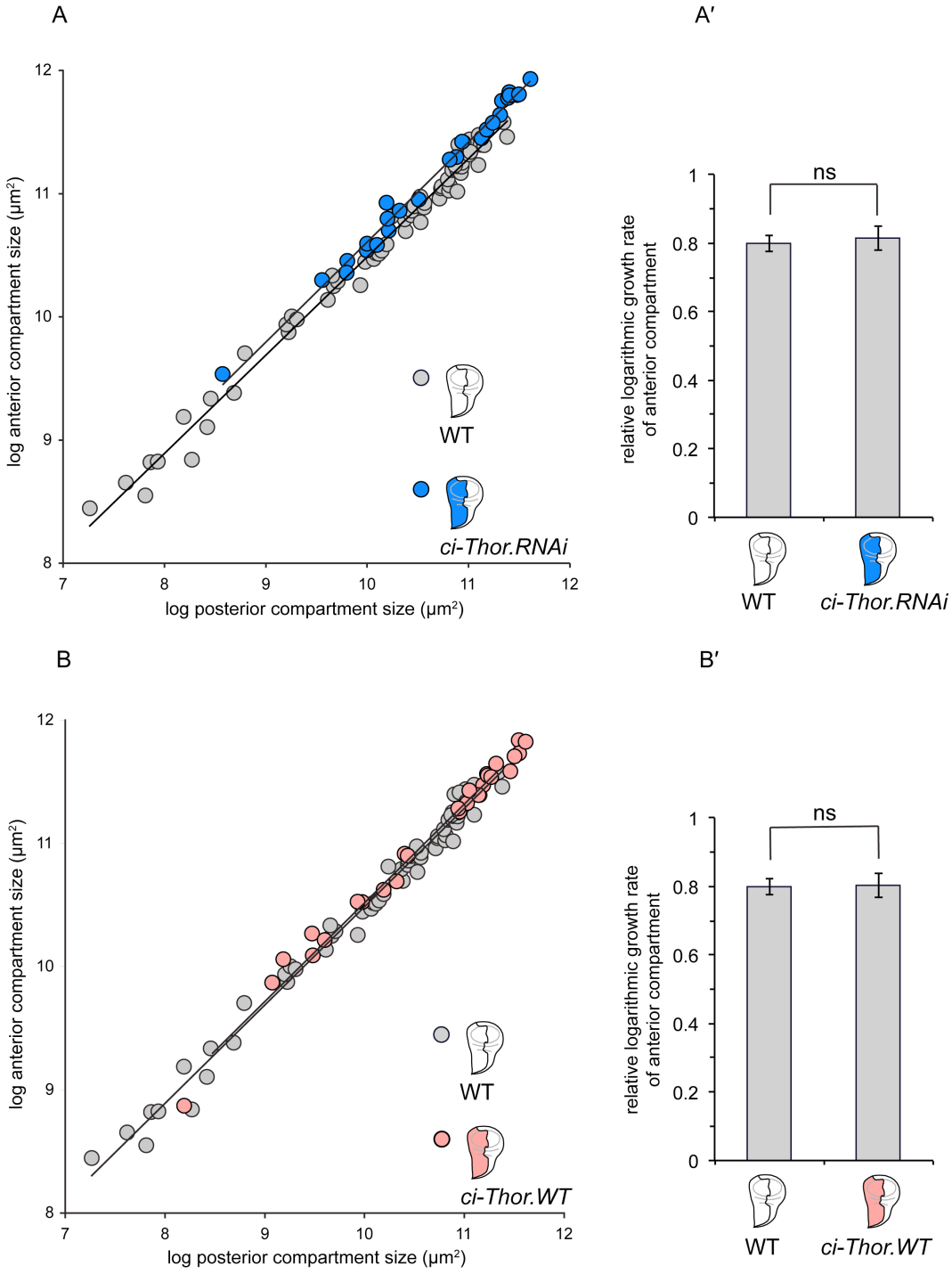


Figure 2-10 (cont'd)

(A) The slope of the ontogenetic allometry between anterior and posterior compartment size in WT (gray circles) (n = 63) and *ci>Thor.RNAi* (blue circles) (n = 26) imaginal discs are not significantly different (common slope test, $P > 0.05$) indicating that *Thor* knockdown in the anterior compartment does not affect its growth rate relative to the posterior compartment (A′) (B) The slope of the ontogenetic allometry between anterior and posterior compartment size in WT (gray circles) (n = 63) and *ci>Thor.WT* (pink circles) (n = 27) imaginal discs are not significantly different (common slope test, $P > 0.05$) indicating that overexpression of *Thor* in the anterior compartment does not affect its growth rate relative to the posterior compartment (B′). All error bars are 95% confidence intervals. WT: *ci-Gal4, UAS-FLP/+*. *ci>Thor.RNAi*: *ci-Gal4, UAS-FLP/+; UAS-Thor.RNAi/+*. *ci>Thor.WT*: *ci-Gal4, UAS-FLP/+; UAS-Thor.WT/+*;

Lastly, we tried to identify whether the disruption in growth coordination between compartments in ant$^{fast,\ InR.CA}$:post^{slow} discs could be recapitulated by manipulating expression of dFoxo, a downstream target of the IIS pathway. In order to test this, we generated ant$^{fast,\ foxo21}$:post^{slow} larvae in which the anterior compartment was dFoxo null. In these larvae, the anterior compartment is homozygous for the dFoxo null allele *foxo21* and is therefore expected to have an increased growth rate relative to the posterior. However, on analyzing the ontogenetic allometry between compartments in ant$^{fast,\ foxo21}$:post^{slow} and control, we find that compartment-autonomous manipulation of *foxo* does not increase the relative growth rate of the anterior compartment (Fig. 2-11A, A′). We also assessed the effect of manipulation *dFoxo* expression in WT larvae, by measuring the ontogenetic allometry in larvae with an anterior compartment knockdown of *dFoxo*. In these larvae as well, *dFoxo* knockdown has no effect on the ontogenetic allometry between anterior and posterior compartments as compared to WT (Fig. 2-11B, B′).

These results suggest an intriguing role for IIS in mediating intra-organ growth coordination. While compartment autonomous modulation of *dFoxo* with a null allele does not disrupt coordination between compartments, up-regulating or down-regulating IIS by modulating activity of *InR* has a significant effect in perturbing intra-organ growth coordination. Additionally, preliminary data suggests that up-regulating IIS in the anterior compartment by *dFoxo* knockdown in ant^{fast}:post^{slow} disrupts intra-organ growth coordination in a manner similar to that in ant$^{fast,\ InR.CA}$:post^{slow} imaginal discs (data not shown). Moreover, in larvae systemically null for *dFoxo,* there is a disruption of growth coordination between imaginal discs (A. Shingleton, personal communication); presumably due to systemic upregulation of ecdysone signaling. Lastly, in the context of growth coordination, it appears unlikely that *Thor* mediates the effects of 20E in promoting imaginal disc control, contrary to a recent study [13]. Therefore,

ecdysone presumably promotes imaginal disc growth by acting via multiple downstream effectors.

Figure 2-11 Compartment autonomous modulation of dFoxo expression does not disrupt intra-organ growth coordination

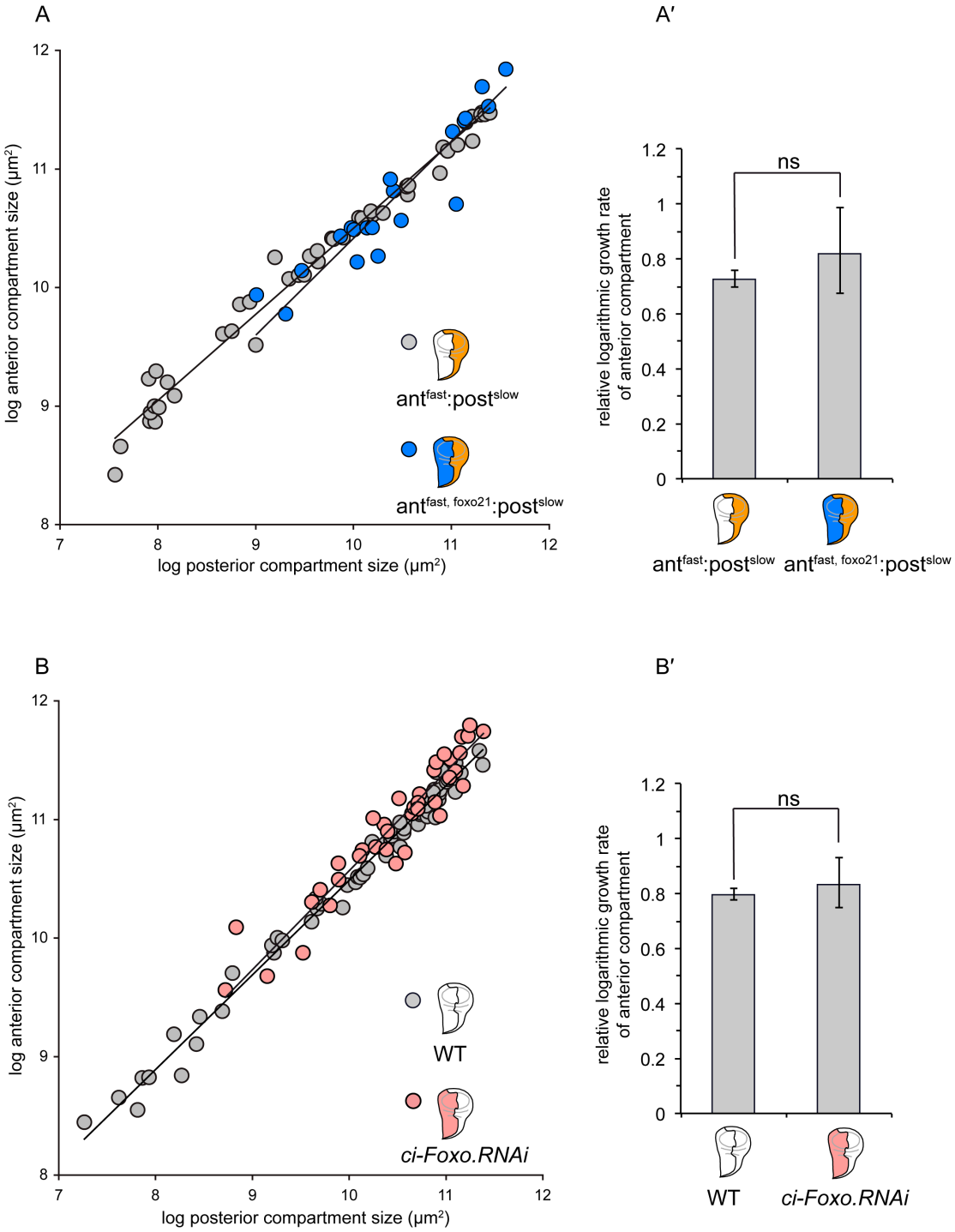


Figure 2-11 (cont'd)

(A) The slope of the ontogenetic allometry between anterior and posterior compartment size in in ant^{fast}:$post^{slow}$ (gray circles) (n = 46) and $ant^{fast,\ foxo21}$:$post^{slow}$ (blue circles) (n = 20) imaginal discs are not significantly different (common slope test, $P > 0.05$), indicating that up-regulating IIS in the anterior compartment with a compartment autonomous *dFoxo* null does not affect the relative growth rate of the anterior compartment(A′). (B) The slope of the ontogenetic allometry between anterior and posterior compartment size in WT (gray circles) (n = 63) and *ci>Foxo.RNAi* (pink circles) (n = 38) imaginal discs are not significantly different (common slope test, $P > 0.05$) indicating that *Foxo* knockdown in the anterior compartment does not affect its growth rate relative to the posterior compartment (A′). All error bars are 95% confidence intervals. WT: *ci-Gal4, UAS-FLP/+*. *ci>Foxo.RNAi*: *ci-Gal4, UAS-FLP/+; UAS-Foxo.RNAi/+*. ant^{fast}:$post^{slow}$: *ci-Gal4, UAS-FLP; FRT82B, ubi-GFP, P{A92}RpS3^{Plac92} / FRT82B arm-lacZ.* $ant^{fast,\ foxo21}$:$post^{slow}$: *ci-Gal4, UAS-FLP; FRT82B, ubi-GFP, P{A92}RpS3^{Plac92} / FRT82B Foxo^{21}*

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CHAPTER III: IN-VIVO CHARACTERIZATION OF THE *INR* REGULATORY LOCUS IN *DROSOPHILA*

[1]Part of the work described in this chapter was published in the following manuscript: Wei, Y., **R.H. Gokhale**, A. Sonnenschein, K. Montgomery, A. Ingersoll and D.N. Arnosti (2016) "Complex cis-regulatory landscape of the insulin receptor gene underlies the broad expression of a central signaling regulator" *Development.* In press.

Introduction

The Insulin/Insulin Growth Factor Signaling (IIS) pathway is a conserved signaling pathway involved in development, growth and metabolism in all metazoans. It is involved in a multitude of functions in animal physiology--regulating animal lifespan, maintaining glucose homeostasis, long-term memory formation, and germline proliferation to name a few. However, perhaps the best-studied aspect of this pathway is its role in promoting nutrient-dependent growth. In *D. melanogaster*, the input to this pathway is circulating insulin-like peptides (dILPs), whose levels depend on the nutritional status of the individual[1]. Circulating dILPs bind to and activate the Insulin Receptor (InR) which subsequently activates the downstream phosphorylation cascade mediated by the kinases PI3K and Akt [2]. One of the targets of Akt is the transcription factor dFoxo. Phosphorylation of dFoxo leads to its translocation to the cytoplasm, inhibiting its transcriptional activity [3-5]. dFoxo targets the expression of a number of growth inhibitors [6-8], and therefore, the *inhibition* of dFoxo under high IIS activity results in the nutrient-dependent stimulation of growth.

In *Drosophila melanogaster,* loss of function mutations of *InR* exhibit pleiotropic recessive phenotypes, leading to embryonic lethality[9]. *InR* expression is crucial to embryonic development [9, 10], as well as development and growth of the nervous system [9, 11]. However, the most important function of *InR* is in the regulation of growth by controlling cell growth and proliferation. In *D.melanogaster, InR* expression regulates final body and organ size of the individual by regulating cell number and cell size in a cell-autonomous manner [12]. At the level of individual organs, for example, upregulation of *InR* levels in the eye resulted in an organ autonomous increase in size[12]. Individuals homozygous for hypomorphic mutations in *InR* have a significantly smaller body size than wild type individuals [12]. This finding echoes observations in

mice, in which *InR* and *Insulin-like Growth Factor Receptor* (*IGFR*) knockouts cause a developmental delay and a reduction in body size[13]. Thus, organ-autonomous increase or decrease in *InR* expression can result in an increased final body and/or organ size. However, we still lack an understanding of the *cis* regulatory mechanisms contributing to *InR* expression *in vivo*.

In order to address this outstanding question, we carried out a transgenic rescue assay to determine the minimal regulatory sequences needed for InR expression in vivo. We further characterized the *InR cis* regulatory locus by analyzing in vivo reporter gene expression and comparing it to regulatory information gleaned from genome wide regulatory data sets in *Drosophila.* We find that while individual reporter gene fragments have distinct tissue-specific expression, there is no single characteristic set of chromatin-level properties that distinguishes these putative enhancer fragments. Together, this study suggests a far more complex picture of regulation than previously suggested for the *InR* gene regulatory locus.

Materials and Methods

Fly Stocks

The following fly strains were obtained from the Bloomington Stock Center (stock numbers in parentheses): pBac{attp-3B}VK00001 (9722), InR^{GC25}(9554), InR^{E19}(9646), Putative *InR* enhancers: GMR27H05-Gal4(47519), GMR28A11-Gal4(45164), GMR28D03-Gal4(47521), GMR28E02-Gal4(49458), GMR38E09-Gal4(48080), GMR28G01-Gal4 (45547), GMR28G04-Gal4(45548), GMR28H01-Gal4(45947), GMR29A02-Gal4(45175), GMR37B05-Gal4(47564), UAS-GFP (1521). Each putative enhancer line was crossed to the UAS-GFP line. Larval tissues and adult flies were imaged on an Olympus BX-41 microscope.

Generation of transgenic flies

BAC construct CH321-24D17 containing the entire InR locus was obtained from the BacPac Resources Center (Oakland, CA). BACs were grown overnight for 16-20h and high copy number was induced using Epicentre BAC autoinduction solution (Illumina). DNA was prepared using the HiPure Midiprep kit following the manufacturer's instructions (Invitrogen). DNA was diluted to a final concentration of ~1μg/μL and 400 embryos were injected by Rainbow Transgenics Inc (Camarillo, CA). Landing site line VK00001, containing an attp site at location 59D3 on chromosome 2, was used for injection and integration of the BAC.

qPCR analysis of mRNA from transgenic flies

Three-day old adult males and virgin females were collected and flash frozen at -80^{o}C. Total RNA was extracted using Trizol Reagent (Invitrogen) and subjected to DNaseI treatment (Ambion DNaseI Kit) at room temperature for 15 minutes to eliminate genomic DNA contamination. Reverse transcription for first strand synthesis was carried out using random primers and Multiscribe Reverse Transcriptase (ABI Biosystems). Real-time PCR was performed using POWER SYBR Green Master Mix (ABI Biosystems) and analyzed on Eppendorf Mastercycler Realplex. Gene expression was assayed in 3-5 biological replicates of 8-10 flies each and normalized against expression of 28S rRNA. Primers used for assaying expression are listed in Appendix I. Standard curves were generated using six serial dilutions of total RNA extracted from two individuals of Samarkand WT first, second and third instar larvae, pupae (male) and adult flies (male). Gene expression fold changes were calculated by normalizing to WT.

Results

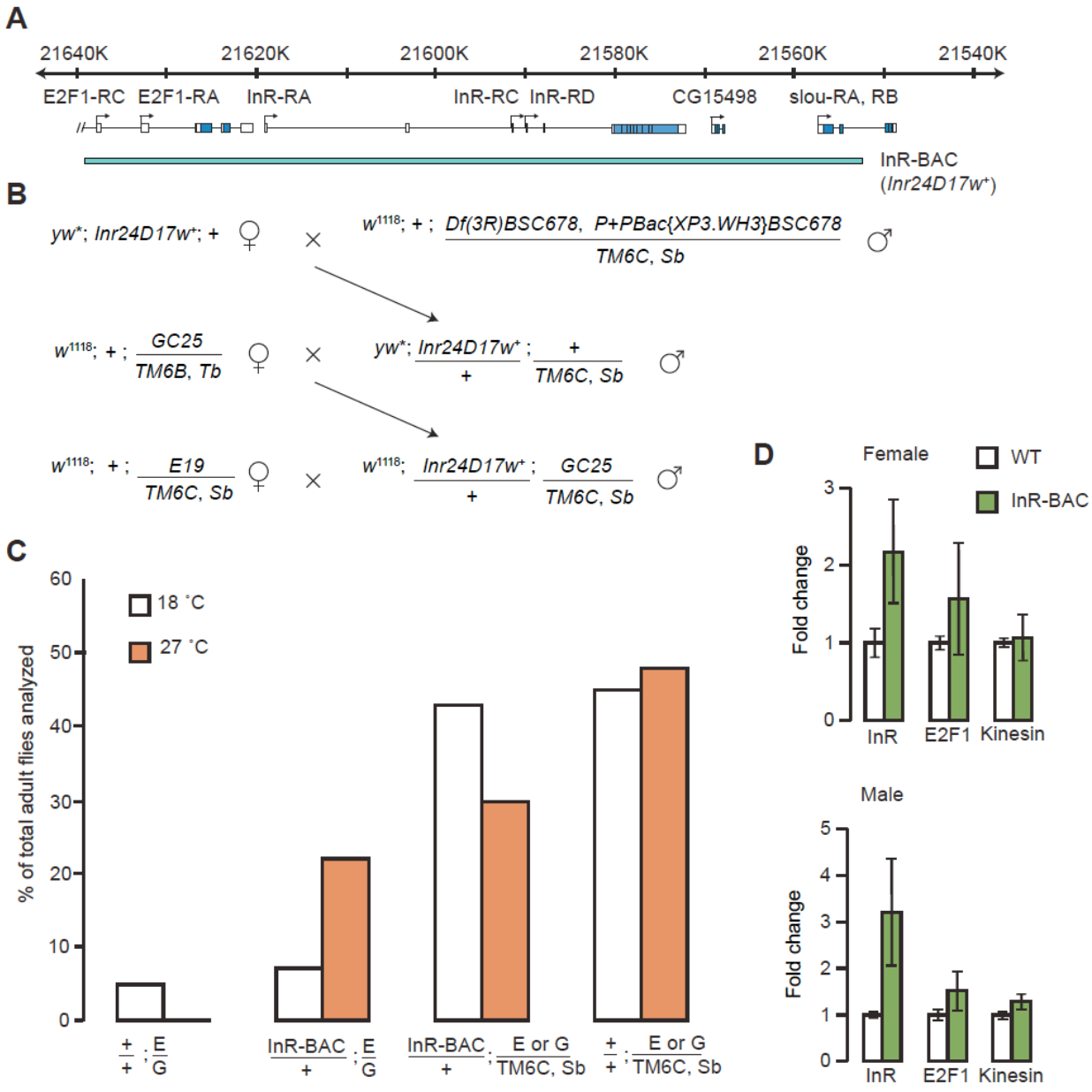
Genomic rescue construct identifies regulatory regions of InR

The Drosophila *InR* gene occupies a 50 kbp region on the 3R chromosome including ~ 40 kbp of introns (Figure 3-1A). To identify the genomic region that is responsible for *InR* expression, which may include sequences outside of the transcription unit, we generated an 80 kbp BAC construct (InR-BAC) that spans the *InR* locus, and tested its ability to rescue the lethality of an InR mutant. The BAC construct includes the entire *InR* transcription unit as well as the 3' CG15498 gene, and portions of the 5'E2F1 and 3' slou genes (Figure 3-1A). We crossed this construct into a background containing the temperature-dependent conditional lethal trans-heterozygous alleles (*InRGC25*/*InRE19*) 14. *InRGC25*/*InRE19* flies are not viable when raised at 27 °C. The BAC was able to rescue this lethality and *InR*-BAC/+ ; InRGC25/InRE19 survivors were obtained (Figure 3-1C). The presence of two copies of the BAC increases *InR* gene expression in these flies about two to three fold (Figure 3-1D). The relevant cis regulatory sequences for *InR* expression are thus located within this region; we therefore investigated the short 5' intergenic sequence and the sizeable introns of *InR* to uncover relevant cis regulatory elements.

Evidence for tissue-specific enhancers of InR

The *InR* gene is flanked by only short intergenic regions, thus important regulatory sequences may be located within its sizable introns, although *cis* regulatory elements may reside anywhere within the 80 kbp region defined by the BAC. Data from cell-type specific enhancer analysis using STARR-seq technology, as well as DNase hypersensitivity data and measurement of open chromatin using FAIRE-seq support the notion that *InR* introns are likely to harbor relevant *cis*-regulatory elements [15-19]. To evaluate the regulatory potential of intronic regions in the whole fly,

Figure 3-1 An 80kbp *cis* regulatory region is sufficient to rescue *InR* mutants



(A) The *Drosophila InR* gene spans ~50 kbp and contains multiple large introns. The entire gene along with its 5' and 3' regions are contained in an *InR*-BAC transgene (indicated by green line) inserted into chromosome 2. (B) Crosses used for the rescue assay. Females heterozygous for InR^{E19} were crossed to males heterozygous for InR^{GC25} and progeny were assessed at permissive and nonpermissive temperatures. (C) *InR* BAC rescue experiment showing fractions of progeny

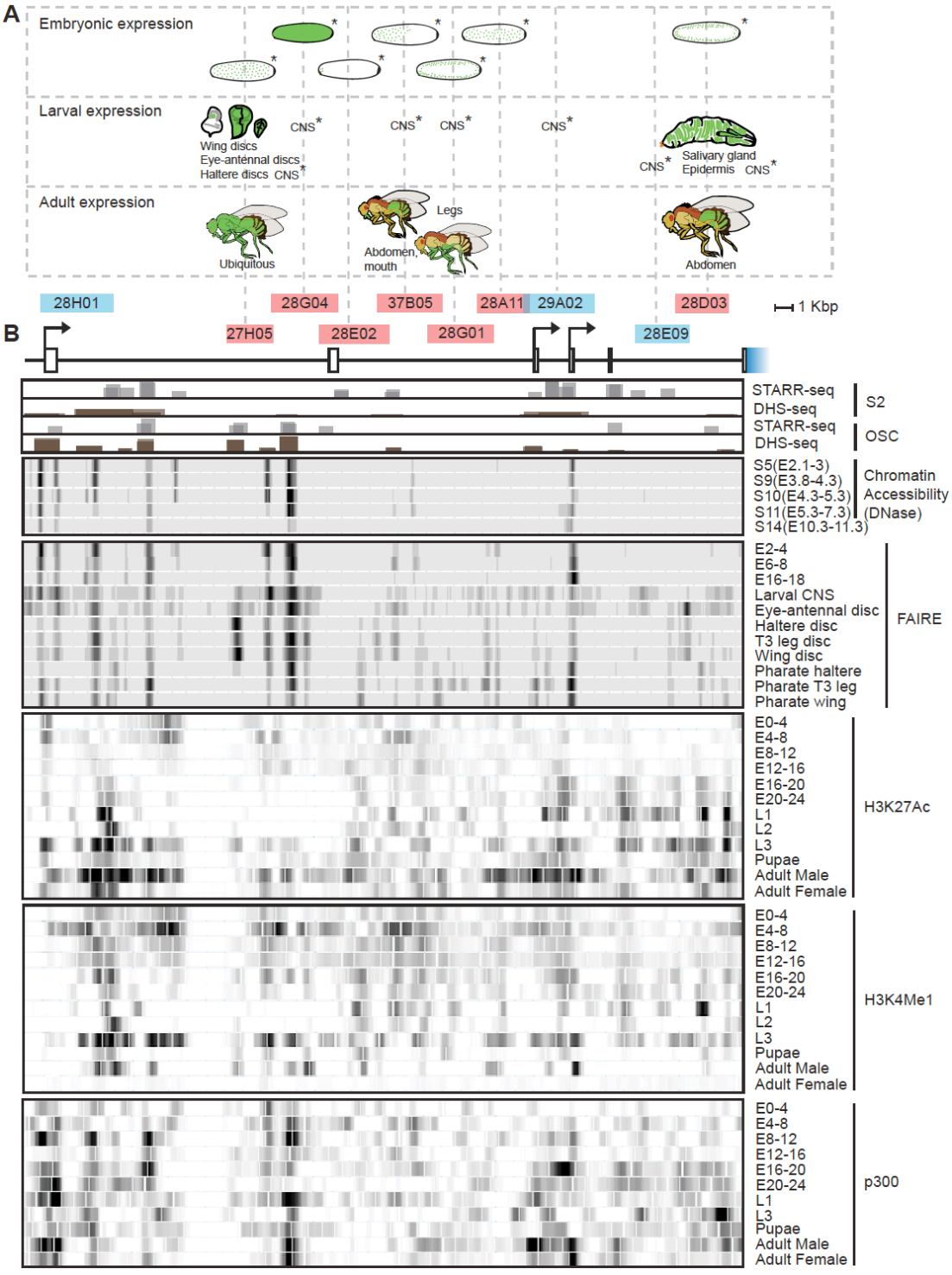
with and without *InR* transgene at permissive (18°C) and nonpermissive (27°C) temperatures. The transheterozygous InR^{E19} / InR^{GC25} allele combination is lethal (represented here showing second and third chromosome genotypes as *InR*-BAC/+; E/G, where E represents InR^{E19}, and G represents InR^{GC2_5} when raised at the nonpermissive temperature of 27°C. 97 Individuals were analyzed for crosses at 27°C, and 107 individuals for crosses at 18°C. (D) Transcript levels of *InR* and *E2F1* measured from 3-day adult homozygous *InR*-BAC females and males. Both females and males showed 2~3 fold increase in transcripts of *InR* and ~1.5 fold for *E2F1*. No significant change was observed for *kinesin* (*Kinesin heavy chain*, also *khc*) as a negative control. All transcripts were normalized to 28S transcript levels.

we tested ten GAL4 lines bearing genomic fragments derived from the *InR* gene (Figure 3-2) (Pfeiffer *et al*, 2008). Previous measurements in the embryo indicated that some of these elements drive GFP expression in dynamic and cell-type specific patterns [21-24]. We found that three of the fragments also express GFP in larvae and adults, in either ubiquitous or tissue-specific patterns (Figure 3-2, Figure 3-3). To obtain more insight on possible *cis* regulatory elements, we surveyed extant datasets for information about chromatin accessibility and ChIP-seq information that may reveal active regulatory regions in this locus. To identify possible correlated features, we plotted the results of genome-wide enhancer surveys (from S2 and ovarian stem cells (OSC)), chromatin accessibility in different developmental stages and tissues as measured by FAIRE-seq, and enhancer-associated histone modifications H3K27Ac,H3K4Me1, and the p300 coactivator [15, 16, 18-20, 25, 26], The resultant patterns do not provide a consistent, easily interpretable set of correlations across different developmental times. Enhancers found using STARR-seq do point to apparently redundantly-acting enhancers in *InR* introns with either shared or cell-type specific patterns (Figure 3-2) [18]. These enhancers overlap some of the fragments tested as GAL4 drivers, but there was not a complete agreement between these different methods. The two types of assays relied on distinct basal promoters, which may have biased detection because of enhancer-promoter specificity [27, 28].

Discussion

Our preliminary analysis of the *InR* cis-regulatory locus reveals a complex regulation in which multiple enhancers control spatial and temporal expression of the gene. Together with the detailed analysis of the transcriptional response of these enhancers to individual transcription factors, this analysis gives us valuable insight into how to design a synthetic gene that may be uniquely rewired to a particular transcriptional input (See Appendix II).

Figure 3-2 Regulatory landscape of the InR locus

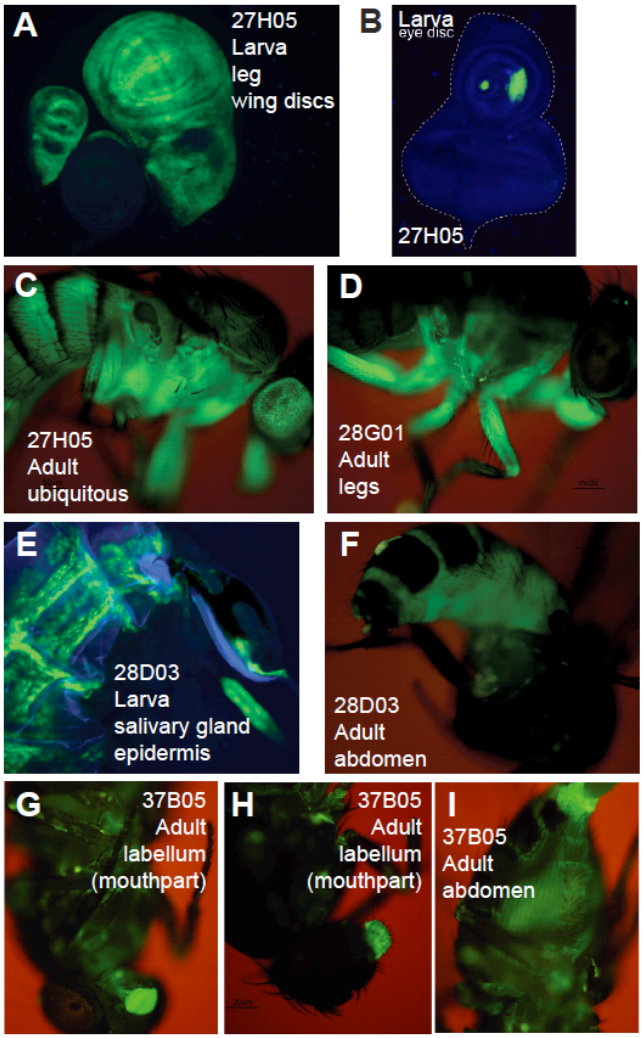


(A) Transcriptional output of genomic fragments associated with the *InR* locus. Janelia GAL4 lines that contain genomic fragments in the *InR* locus were crossed to a UAS-GFP line. Extant information collected from FlyLight database for embryonic and larval activity is also shown (indicated by *). Fragments labeled in red showed GFP signal in embryos, larval, or adult flies. Fragments labeled in blue showed limited or no expression. Representative images in larvae and adult flies from this study are shown in Fig. 3-3. The details for each fragment are shown in Data Table 3-1. (B) Alignment of the *InR* gene locus with previously identified features. STARR-seq elements are from S2 and OSC cells [18], chromatin accessibility identified by DNase-seq in cell lines [18], developing embryos [15-17, 20], FAIRE in different developmental stages and tissues [19], and enhancer-associated modifications H3K27Ac, H3K4Me1 and p300 binding in different developmental stages (modENCODE). For STARR-seq and DHS-seq, the data from two experiments are shown, where darker areas indicate reproducible results, and lighter areas found in only one experiment. Bar height indicates enhancer activities or reads for the peaks. Width of STARR-seq signals set at 600 bp, the average length of the fragments [18]. Chromatin accessibility data for different developmental stages indicated at right (S developmental stage, E for hours of embryonic development). FAIRE, H3K27Ac, H3K4Me1, and p300 data are presented as peak intensities, with darker shades indicating higher peaks. All data scales are normalized to local maximum. Genome version is *Drosophila* genome dm3/R5.

Table 3-1 Expression details of reporter fragments tested in this study

Janelia fragments	Coordinates	Larvae (CNS)	Larvae (Imaginal Disc)	Larvae (Other)	Adult
28H01	No Data	No Expression	No Expression	No Expression	No Expression
27H05	3R:21,606,484..21,609,042	No Expression	Wing, Haltere	No Expression	Ubiquitous
28G04	3R:21,603,204..21,607,084	No Data	No Data	No Data	No Data
28E02	3R:21,600,134..21,603,731	No Data	No Data	No Data	No Data
37B05	3R:21,597,120..21,601,014	No Expression	No Expression	No Expression	Abdomen, Mouth Parts
28G01	3R:21,594,305..21,598,173	No Expression	No Expression	No Expression	Legs
28A11	3R:21,592,500..21,595,413	No Expression	No Data	No Data	No Data
29A02	3R:21,588,539..21,593,033	No Expression	No Expression	No Expression	No Expression
28E09	3R:21,583,277..21,586,976	No Expression	No Expression	No Expression	No Expression
28D03	3R:21,580,937..21,584,313	Brain (?)	No Expression	salivary gland, body wall	Abdomen

Figure 3-3 Adult and larval expression of UAS-GFP reporters



(A) GFP expression in larval leg and wing discs with Gal4 driver 27H05. (B) GFP expression in larval eye disc with Gal4 driver 27H05. (C) Ubiquitous GFP 131 expression in adult with Gal4 driver 27H05. (D) GFP expression in adult legs with Gal4 driver 28G01. (E) Larval salivary gland and epidermis expression with Gal4 driver 28D03 (F) Adult abdominal expression with Gal4 driver 28D03. (G) GFP expression in adult mouthparts with Gal4 driver 37B05. (H) GFP expression in adult mouthparts with Gal4 driver 37B05. (I) GFP expression in adult abdomen with Gal4 driver 37B05.

Our rescue assay to identify the minimal regulatory region of *InR* identified an 80kb region that rescues the lethality of a temperature-sensitive *InR* transheterozygote. The conditional lethal transheterozygous allele combination InR^{GC25}/InR^{E19} is inviable at 27^{o}C but is viable 18^{o}C. However, even at the permissive temperature this allele combination exhibits a number of physiological defects such as decreased cell number, increased developmental time and a smaller body size[14]. Our genomic rescue with the *InR*-BAC allele rescued the lethality of this transheterozygote at 27^{o}C, but it is not immediately clear whether this is a complete rescue to a WT phenotype in terms of expression of the gene. We found no observable differences in body size, wing size or developmental time between the rescue flies and the corresponding controls when raised on regular food at 27^{o}C. However, we do find that when we subject these flies to a high sugar diet, there is an appreciable difference between developmental times between the *InR*-BAC rescue flies and the corresponding WT controls. These flies also exhibit a lower fecundity as compared to controls. (See Appendix I). qPCR analysis to determine the expression of *InR* in the rescue flies suggests an increase in mRNA transcripts levels almost identical to what would be predicted by increase in gene dosage. My qPCR analysis was limited to that from adult flies, so it is unclear whether gene expression is restored to WT levels across all tissues and developmental contexts. However, given our preliminary physiological analysis of the *InR*-BAC flies, this appears to be unlikely. The qPCR analysis also revealed that along with increased expression *InR, E2F* expression is also elevated in *InR*-BAC flies. Therefore it is not immediately clear whether these altered phenotypes are a result of increased *InR* expression or increased *E2F* expression.

Our analysis of the *InR* regulatory locus by using Gal4 reporter analysis reveals a distinct set of enhancers distributed across the large introns of the gene. What is most interesting is that there

appears to be distinct enhancer fragments driving expression in a tissue-specific manner. It is not immediately clear whether each of these enhancers are functional in vivo and whether they drive endogenous gene expression. Further bioinformatic analysis coupled with cell-based luciferase reporter assays suggests a far more complex regulation than previously thought for the *InR* regulatory locus. For instance, a number of enhancer fragments which showed reporter gene expression in the Gal4 based assays showed only minimal activity in cell-based luciferase assays (Wei et al., 2016). One way to determine which enhancers are actually employed in vivo would be to use a CRISPR/Cas9 mediated genome engineering approach to create transgenic flies lacking one or more of these enhancers. Alternatively, a simpler transgenic approach (similar to the one outlined in Appendix II) can be utilized to determine the relative contribution of individual enhancer fragments to total *InR* gene expression in vivo.

Research on signal transduction pathways has canonically focused more on interactions at the protein level, with only a limited understanding of how transcriptional regulation of different components affects signal transduction and output through the pathway. Increasingly, however, it is becoming evident that different signal transduction pathways that had thus far been thought of as relatively independent are interconnected via transcriptional regulatory inputs. For example, the Cagan lab recently demonstrated that in *D. melanogaster* the Wnt pathway interacts with the IIS pathway—with the Wg protein upregulating *InR* [29]. Such regulatory mechanisms are thought to provide a means of long-term regulation to the pathway. With the availability of genome wide transcription factor binding data sets, we now have a wealth of information of the relative enrichment of different transcription factors across different genes. These studies are crucial to provide a complete picture of the transcriptional inputs that are necessary for normal *in vivo* expression of any gene. However, whether transcription factor binding actually results in a

significant contribution to total expression, and whether this regulation is essential for a particular developmental context is not clear from these studies. In order to address this question, it is necessary to focus on individual transcriptional inputs to a particular gene and look at the effect of removing or altering transcriptional regulation on gene expression and phenotype.

APPENDICES

APPENDIX I

CHARACTERIZATION OF *INR*-BAC FLIES

Methods

Measurement of developmental delay

Flies were allowed to lay for 24h on either HSD or LSD containing food vials. HSD and LSD food was prepared based on the Bloomington semi-defined medium recipe, but contained either 342g/L or 51.3g/L of sucrose, respectively. The number of pupariating individuals was recorded every 24 hrs.

Measurement of fecundity

InR-BAC and control flies were allowed to lay for 24 hours or 4 hrs respectively, on apple juice agar plates supplemented with dry yeast. Embryos were then counted and transferred to vials. Flies arising from these embryos were then counted and percent egg fertility (number of eclosed adults/number of eggs laid*100) was calculated. Lay rate was also calculated by as the number of eggs laid per female per hour. Data was analyzed from three independent biological replicates.

Results and Discussion

Developmental delay on HSD is partially rescued in InR-BAC flies

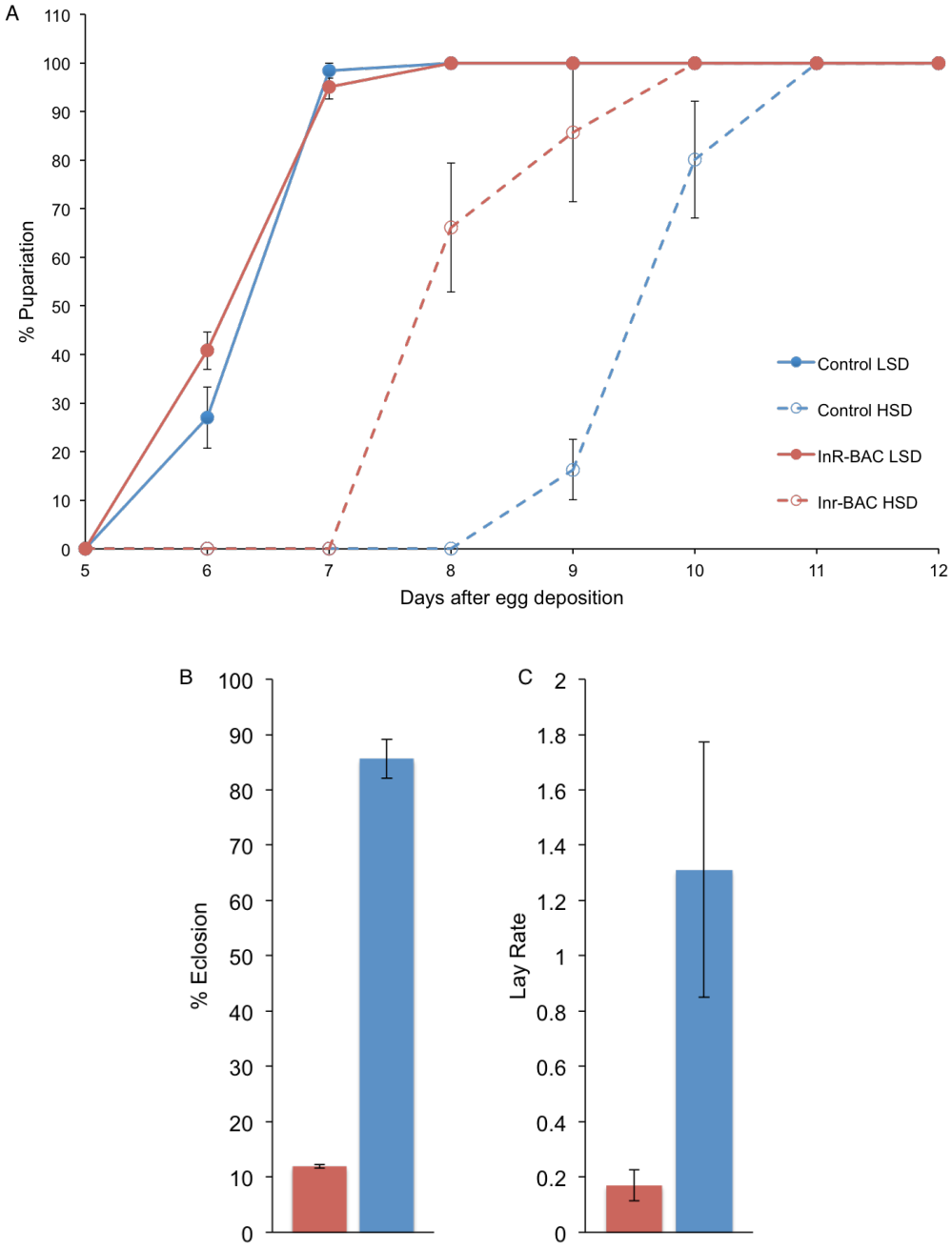
In order to identify whether transgenic increased dosage of *InR* had an altered phenotype in terms of insulin signaling we measured the developmental delay in *InR*-BAC flies raised on a High Sugar Diet (HSD) or a Low Sugar Diet (LSD) as compared to controls. Flies raised on a high sugar diet have been previously shown to have developmental delay of ~96 hrs [30]. Consistent with these data, WT controls raised on a HSD (Fig. 3-4, dashed blue line) had a developmental delay of ~72 hrs as compared to those raised on a LSD (Fig. 3-4, solid blue line). Interestingly, this delay is partially rescued in *InR*-BAC flies, which have a developmental delay of ~30hrs (Fig. 3-4, dashed red line). HSD has been previously demonstrated to cause a

reduction in expression of *InR* in eye-discs of larvae[29]. Therefore, it is likely that in *InR*-BAC flies, the increased dosage of *InR* compensates a decrease in expression caused by the HSD and this contributes to the partial rescue of the developmental delay. There is, however, no difference in the developmental time in control and *InR*-BAC larvae raised on the LSD.

InR-BAC flies exhibit a diminished fecundity

The IIS pathway has been known to influence a number of life-history traits such as fecundity, life span and aging. Specifically, ablation of dILP secreting cells in the brain has been show to result in decreased female fecundity as measured by egg laying [31]. In addition, the IIS/TOR pathway is essential for the growth, proliferation and surivival of germ line as well as somatic stem cells in the ovary [32-34]. Therefore, we decided to investigate whether subtle alterations in the transcription of *InR* have any effect of fecundity. Our preliminary data indicates that, increased dosage of *InR* and therefore increased activity through the IIS seems to result in female flies with lower fecundity, as evidenced by stark reductions in egg viability (Fig. 3-4B) and female egg laying rate (Fig. 3-4C). Mutations in various IIS components as well as *InR* mutants have been previously demonstrated to cause female sterility [10]. Similarly, a point mutant E2F1 allele was shown to affect female fertility with mutant mothers laying fewer eggs [35]. However, increasing expression of either gene has not been previously shown to be associated with decreased fertility. However, overexpression of E2F1 has been shown to increase apoptosis both in vivo and in vitro [36] and it is possible that in this case as well, increased apoptosis in the ovary or germ line cells causes impaired egg laying and/or development.

Figure 3-4 Phenotypic Analysis of *InR-BAC* transgenic flies



(A) Developmental delays in *InR*-BAC (red lines) and control flies (blue lines) raised on HSD (dashed lines) and LSD (solid lines). Fecundity of *InR*-BAC (red) and control flies (blue) as measured by (B) egg viability and (C) Egg Lay rate of adult female flies. Error bars represent SEM (Standard Error of Mean).

APPENDIX II

TRANSGENIC FLY SYSTEM TO ASSESS THE EFFECT OF dFOXO FEEDBACK TO InR

Introduction

The Insulin/Insulin-like growth factor Signaling (IIS) pathway transduces environmental cues of nutrient availability to affect intracellular cellular processes via a transmembrane receptor and a phosphorylation cascade resulting in inhibition of the transcription factor dFoxo. Since the primary function of a signaling pathway is to relay environmental information to the cell, in order for it to adjust to fluctuating environments, a signaling pathway must have two essential qualities: robustness and sensitivity. Robustness is the ability to withstand perturbations in signaling without significantly altering the output of the pathway; sensitivity on the other hand refers to the ability of discriminating between small changes in the input to the pathway[37, 38]. One way to ensure robustness is the presence of feedback loops which are known to confer robustness and reduce noise through the pathway[38]. In the IIS pathway, feedback is achieved via dFoxo, which is a downstream target of the IIS pathway[39]. Under conditions of adequate nutrition, the phosphorylation cascade results in the inactivation of dFoxo; however, under limited nutrition, dFoxo is active and unphosphorylated and exerts feedback control on the levels of the Insulin Receptor (InR). qPCR analysis of mRNA transcripts suggests that *InR* expression is upregulated approximately 2-3 fold [39].

It has been demonstrated previously that organ-autonomously altering the levels of dFoxo in *D. melanogater* alters the sensitivity of the pathway to nutrition [40]. However, it is not immediately clear whether this is due to the transcriptional feedback of dFoxo to *InR*. Additionally, it is not clear whether the dFoxo feedback to *InR* is physiologically relevant, and if it is, how much it contributes to total gene expression of *InR*. Moreover, there are no studies demonstrating a direct effect on modified dFoxo feedback on larval development or final body and organ size in

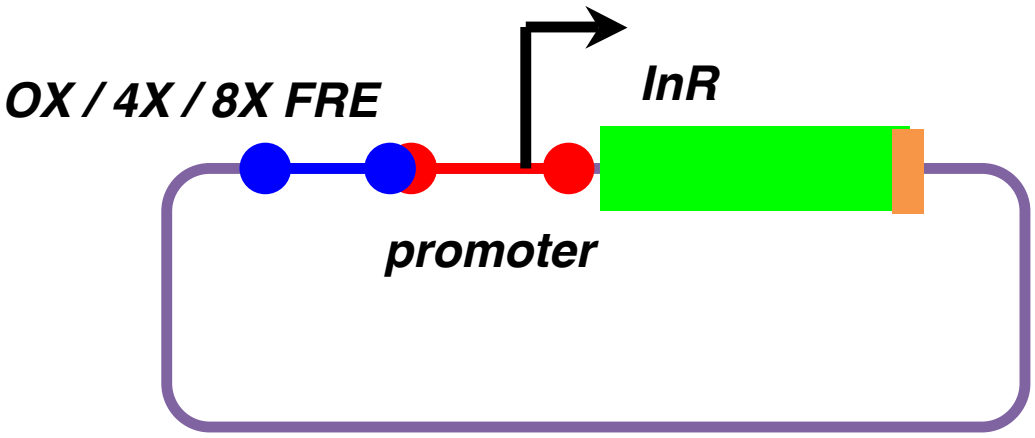
D.melanogaster. In order to test this, I propose below a transgenic approach which can be utilized to address these questions.

Transgenic system to test the role of dFoxo feedback

Vector Construction

For construction of a dFoxo inducible *InR* gene, 4XFRE and 8XFRE DNA fragments were synthesized from overlapping fragments and annealed *in vitro* to yield two fragments of 68nts and 126nts respectively (Fig. 3-5). The 4XFRE sequence contains four predicted Foxo4 binding sites and had been previously demonstrated to activate transcription of a synthetic promoter *in vitro*[3]. 0XFRE sequence was generated as a random 22nt sequence and verified to not contain any predicted Foxo binding sites. pHonda is a phiC31 based vector containing a *twist* basal promoter, and 3' alpha tubulin UTR and has been previously demonstrated to be an effective vector in which potential regulator sequences can be tested in [41]. The 340bp spacer sequence in pHonda1 was removed and the 4XFRE/8XFRE fragments were cloned into the *Age*I and *Bgl*II sites in the MCS of pHonda1 to generate the vector 4XFRE-pHonda1 and 8XFRE-pHonda1. The *lacZ* gene in pHonda1 cloned between *Xho*I and *Kpn*I was replaced with a 78nt polylinker (*Xho*I-*Kpn*I polylinker) containing unique restriction sites (*Asc*I, *Avr*II, *EcoR*I, *Hind*III, *Pac*I, *Stu*I, *Not*I) and in frame and out of frame STOP sites to generate the vectors 0XFRE/4XFRE/8XFRE-pHonda1-polylinker. *InR* cDNA was obtained as the construct pMT-*InR*-FLAG [42] and was a kind gift from Dr. Stephen Cohen. This construct was modified to introduce and additional FLAG sites by Quickchange mutagenesis to generate the construct pMT-InR-2XFLAG containing a double FLAG-tag at the C-terminus of the InR cDNA. InR cDNA was subcloned from pMT-*InR*-2XFLAG into 4XFRE-pHonda1-polylinker and 8XFRE-pHonda-polylinker using the *EcoR*I and *Not*I sites to generate the constructs 0XFRE/4XFRE/8XFRE-InR-2XFLAG-pHonda1.

Figure 3-5 Transgenic system to test the role of dFoxo feedback



In this transgenic system, *InR* cDNA (green) expression driven by a basal promoter (red) and a DNA sequence containing either 0, 4 or 8 additional dFoxo binding sites (blue) cDNA expression can be tracked by a 2XFLAG-Tag(yellow).

Subsequently, to generate constructs containing endogenous regulatory regions responsive to dFoxo overexpression, Fragment 2 and Fragment 4 from the reporter library of Wei et al., were cloned upstream of InR-2X-FLAG in the vector 0X-FRE-InR-2X-FLAG-pHonda1. Additional restriction sites were introduced in the vector by cloning a 60nt polylinker containing unique restriction sites (*Age*I, *Avr*II, *Asc*I, *AsiS*I, *Fse*I, *Eag*I) in the *Age*I restriction site upstream of *InR*. A 30nt polylinker containing unique restriction sites (*Sbf*I, *Fsp*I) was cloned in the *Asc*I site previously cloned in the *Xho*I-*Kpn*I polylinker thereby destroying the *Asc*I site. Fragment 2 and Fragment 4 were amplified from genomic DNA by PCR and cloned into the *Avr*II and *Asc*I restriction sites to generate the constructs Fragment2-InR-2X-FLAG-pHonda1*, Fragment4-InR-2X-FLAG-pHonda1* All constructs were validated by sequencing.

Testing constructs in S2 cells

Drosophila S2 cells and Kc cells (Kc167) and were cultured in Schneider medium (Gibco) supplied with 10% FBS (Gibco) and penicillin-streptomycin (100 Unit/ml Penicillin, 100 ug/ml Streptomycin, Gibco). To assess expression of InR protein driven by the metallothionine promoter, 1000ng of pMT-*InR*-2XFLAG was transfected into 2 x 10^6 *Drosophila* S2 cells or Kc cells in 6-well plates using Transfectene reagent (Qiagen). 24h later a final concentration of 100uM $CuSO_4$ was added and cells were incubated at 25°C for an additional 72hrs. To assess expression of InR protein from vectors in which *InR* expression is driven by FOXO sites, 1000ng of 0X-*InR*-2XFLAG-pHonda1, 4XFRE-*InR*-2XFLAG-pHonda1, 8XFRE-*InR*-2XFLAG-pHonda1, Fragment2-InR-2X-FLAG-pHonda1* or Fragment4-InR-2X-FLAG-pHonda1* were transfected into 2 x 10^6 *Drosophila* S2 cells and incubated at 25°C for 72hrs. To test whether these constructs are responsive to dFoxo overexpression, cells were also co-transfected with 250ng pAX-dFoxo-FLAG vector; 250ng pAX empty vector was used as a control. Cells were

harvested three days after transfection by transferring to a 1.7 ml microfuge tube, centrifugation at 10,000 rpm in an Eppendorf centrifuge 5415C for one min at room temperature, and lysed by freezing at -80°C for five min and thawing at 37°C for one min a total of three times, after addition of 50 ul of lysis buffer (50mM Tris-Cl pH8.0, 150mM NaCl, 1% Triton X-100). Total protein levels were measured by Bradford assays. 10-20ug of cell lysates were run on 4-20% precast polyacrylamide gels (Biorad), transferred to PVDF membrane and probed with M2 anti-Flag antibody (mouse monoclonal, 1:10,000, Sigma, F3165). Antibody incubation was performed for overnight at 4°C for the primary antibody and one hr at room temperature for the secondary antibody in TBST (20 mM Tris-Cl, pH 7.5, 120 mM NaCl, 0.1% Tween-20) with 5% non-fat dry milk, washed three times, five min each, after primary and secondary antibody incubation. Blots were developed using HRP-conjugated secondary antibodies (Pierce) and Clarity Western ECL substrate (Biorad)

Preliminary Results

Robust expression of pMT-InR-2XFLAG was induced in S2 cells and was detected by a strong FLAG signal in immunoblotting experiments. However, repeated experiments to detect expression of 0XFRE-*InR*-2XFLAG-pHonda1, 4XFRE-*InR*-2XFLAG-pHonda1 and 8XFRE-*InR*-2XFLAG-pHonda1 failed to detect consistent protein expression patterns with these constructs with or without co-expression of dFoxo. Since the co-expressed dFoxo is tagged with a FLAG-Tag, this served as a useful internal control, and I was able to detect transfected dFoxo in every experiment. Nevertheless, out of many repeated experiments, I was able to detect protein expression only once from 0XFRE-*InR*-2XFLAG-pHonda1.

I also tested the expression of Fragment2-InR-2X-FLAG-pHonda1* and Fragment4-InR-2X-FLAG-pHonda1*—which contain enhancers previously shown to be activated by co-transfected dFoxo in S2 cell based luciferase reporter assays (Wei et al., 2016) in multiple independent experiments. However, with these constructs too, I did not detect any protein expression either with or without dFoxo overexpression.

Table 3-2 Primers and oligos used in vector construction

Name	SEQUENCE
0X-FRE	ACCGGTCAGGATGAGGTCGCCCTAAAAGATCT
4X-FRE	ACCGGTAGTTTGTTTGTCGATTAAATAAACATGTAAACACTTTGTTTTGTTGATACAAAAAAAAGATCT
8X-FRE	ACCGGTAGTTTGTTTGTCGATTAAATAAACTGTAAACACTTTGTTTTGTTGATACAAACAAAAAGTTTGTTTGTCGATTAAATAAACATGTAAACACTTTGTTTTGTTGATACAAACAAAAAGATCT
XhoI-KpnI polylinker	CTCGAGGGCGCGCCCCTAGGGAATTCAAGCTTTTAATTAAAGGCCTGACGCGGCCGCGTAATAGATGAGCCCGGTACC
AscI-EcoRI polylinker	CGCGTATCCTGCAGGTATGCGCATAGAATT
AgeI-polylinker	ACCGGTATGCCTAGGTAGGGCGCGCCATGCGATCGCATTGGCCGGCCTACGGCCGTTCCGGTCAGGATGAGGTCGCCCTAAAAGATCT
Fragment 2 FWD	AATTAACCTAGGACAACAGGCAAAACCGAAGT
Fragment 2 REV	AATTAAGGCGCGCCTCGCATCGCTTCTTGGAACA
Fragment 4 FWD	AATTAACCTAGGTGTGTTGTTGCCTATTTTTCACTGT
Fragment 4 REV	AATTAAGGCGCGCCAGGTGACAACGTGCGAGATT

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CHAPTER IV: CONCLUSIONS AND FUTURE PERSPECTIVES

In my research described in the preceding chapters, I have focused on signaling mechanisms that play essential roles in development and determining the final size of the individual. In this chapter I will discuss my research in context of the emerging knowledge in the field, and future experiments that may be carried out to extend this research.

Integration of systemic and organ-autonomous mechanisms in organ size regulation

In chapter 2, I described my research focusing on imaginal disc growth regulation. Each compartment within an imaginal disc had been previously proposed to have an autonomous size regulatory mechanism, and thus, each compartment would essentially function as independent units. My research shows that this is not the case—the growth rate of each compartment is tightly linked to the growth rate of other compartments within the same organ. This coordination appears to be hormonally regulated because when one compartment is growth perturbed, the retarded growth rate of the unperturbed compartment can be rescued by systemic signaling mediated by the hormone 20E. My study also shows that active organ autonomous IIS pathway is necessary to mediate growth acceleration by 20E. Collectively, my research therefore highlights the importance of crosstalk between organ-autonomous signaling mechanisms and systemic signaling.

There are however a number of additional questions that my research raises. First, what is the organ-autonomous mechanism that responds to changes in systemic ecdysone signaling? Ecdysone has been canonically thought to function by its interaction with the EcR-Usp complex, a steroid-activated nuclear-hormone receptor [1-3]. However, the specific targets that EcR-Usp upregulates in imaginal discs are still unclear. A recent study reported a role for *Thor* in ecdysone-mediated imaginal disc growth [4]; however, we do not find a role for *Thor* in the context of intra-organ growth coordination. This finding raises the question of what other

pathways and downstream effectors may be acting downstream of EcR signaling to crosstalk with IIS and/or other signaling pathways in the imaginal disc. So far there have been only a few systematic efforts to identify unknown targets responsive to ecdysone and/or EcR-Usp signaling [5-7]. Most of these studies have been conducted by examining whole organisms in different developmental stages, making identification of specific targets in each organ is difficult. Studies focusing on transcriptional changes in imaginal discs in response to changes in ecdysone signaling are therefore needed.

Second, what is the nature of the signal by which the growth perturbed compartment signals to the rest of the body? Previous research indicated that growth perturbed imaginal discs secrete an insulin-like peptide, dILP8, which regulates developmental timing by inhibiting ecdysone synthesis in the prothoracic gland [8, 9]. More recently, Lgr3 was identified as the receptor of dILP8 in the prothoracic gland and the CNS [10-12]. It is likely that dILP8 and its corresponding receptor Lgr3 are also involved in intra-organ organ growth coordination. One way to test this would be to generate ant^{fast}:post^{slow} imaginal discs in *dILP8* null larvae. If the damage signal originates from the growth perturbed tissue, in these mutant larvae there should be no reduction in systemic ecdysone signaling and consequently no coordination of growth between the two compartments of the imaginal disc. However, my study does not distinguish whether the growth perturbed or the unperturbed compartment communicates the damage signal in ant^{fast}:post^{slow} larvae. Therefore, to test whether the unperturbed compartment secretes the damage signal, one can use an RNAi mediated approach to eliminate dILP8 expression from the unperturbed compartment. If the signal originates there, one would expect a lack of coordination between compartments. Another possibility is that dILP8 (or another unknown signal) secreted from the growth-perturbed tissue acts directly on the unperturbed compartment and affects a reduction in

growth rate. However, if this were the case, it is unlikely that dILP8 would be mediating this via Lgr3 since Lgr3 is not expressed in imaginal discs [13]. Further, if direct signaling between compartments is relevant, then the indirect effects of dILP8 on the disc mediated through the prothoracic gland and/or CNS must be overridden by ecdysone treatment.

Lastly, the counterintuitive nature by which IIS organ-autonomously perturbs growth coordination in growth-perturbed imaginal discs is extremely puzzling. Our study finds that organ autonomous upregulation or down regulation of insulin signaling by manipulating expression of *InR* has no effect on the ontogenetic allometry between compartments in WT larvae. A recent study demonstrated that manipulating IIS in one compartment by upregulating expression of *Dp110,* the *Drosophila* homolog of PI3K resulted in a skewed compartment size ratio [14]. In agreement with this, my study suggests that compartment-specific perturbation of IIS causes an alteration in the compartment size ratio—however, the relative growth rate of each compartment remains the same. A likely explanation of these data is that growth rates of each compartment are maintained even if cell proliferation rates are not, through compensatory changes in cell size.

My research is part of a growing number of papers in the field demonstrating how localized growth perturbation or changes in the environment affect humoral signaling and how this in turn affects body and organ size. Figure 4-1 highlights some of the most recent research demonstrating the interconnectivity between different organs and the role of humoral signals in maintaining body homeostasis in *Drosophila*. It is important to remember that while these signaling mechanisms have been discovered in *Drosophila,* there are mammalian homologs for a majority of the components involved. For example, IGF-1 regulates growth in a manner that is similar to the action of dILPs in *Drosophila*. Therefore, understanding the connections between

these growth control pathways in flies would be of interest not just to developmental biologists, but also to clinical research focusing on growth abnormalities such as cancer.

Figure 4-1 Systemic signaling pathways regulating body and organ growth during development in *Drosophila melanogaster*

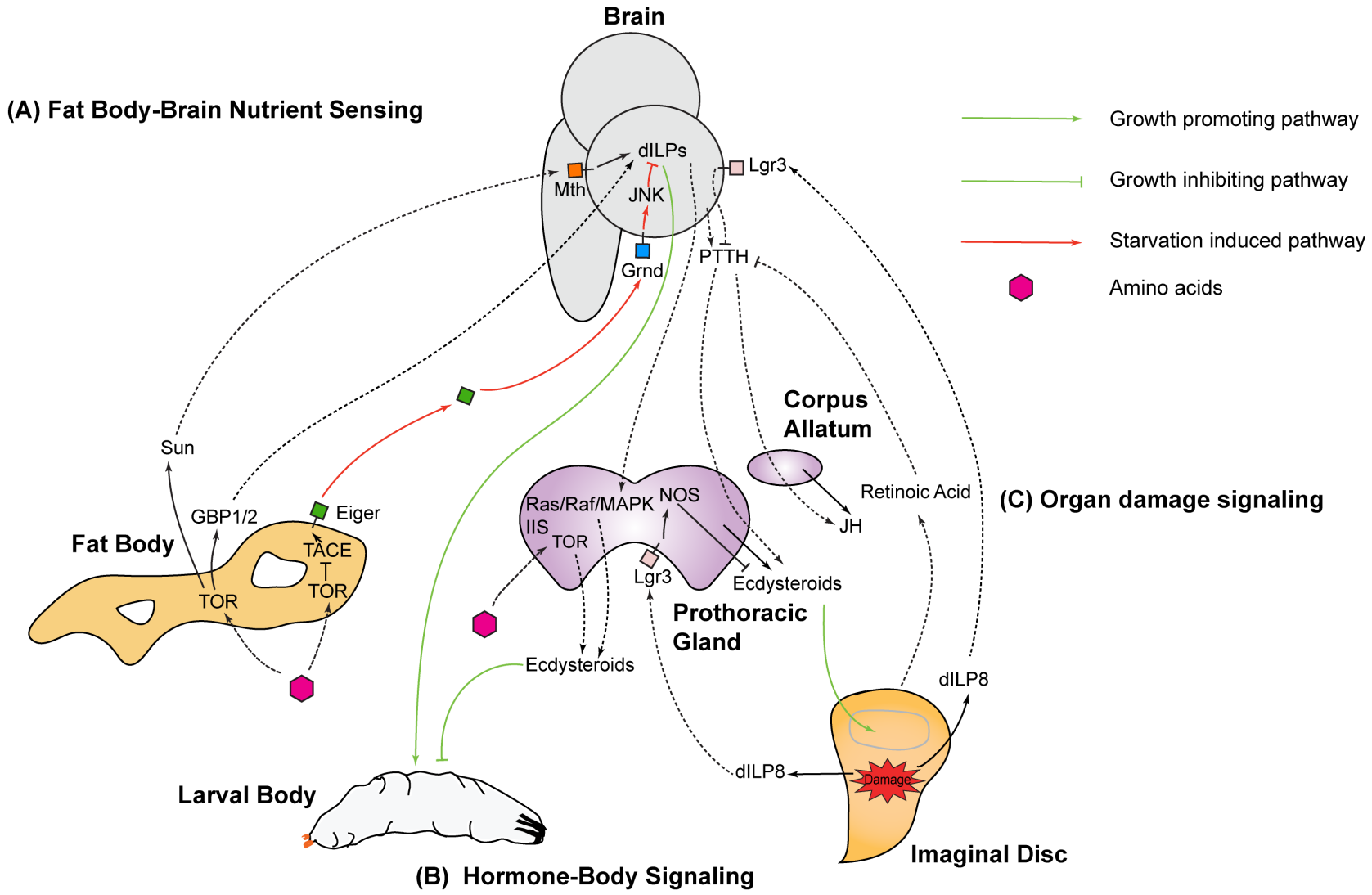

Figure 4-1 (cont'd)

(A) Fat body-Brain Nutrient Sensing Pathways. Developmental nutrition regulates secretion of *Drosophila* Insulin-like peptides (dILPs) from the brain, which promotes larval body growth and ecdysone synthesis in the prothoracic gland. The larval fat body serves as a sensor for sensing amino acids in the diet, and this is communicated to the brain which promotes releases of dILPs. In well-fed flies, the fat body secretes a peptide called as Stunted (Sun) which activates its receptor Methuselah (Mth) in the dILP secreting cells in the brain. Under amino acid starvation, the TNF homolog Eiger is converted into a soluble form (green squares) and interacts with its receptor Grindlewald (Grnd) to upregulated JNK signaling in the brain. Increased JNK signaling inhibits dILP secretion and thereby inhibits further growth. (B) Hormone-Body Size Signaling. The larval ring gland comprising the Prothoracic Gland (PG) and the Corpus Allatum (CA) acts as a central coordinator of hormone secretion in response to various environmental and physiological inputs. Circulating amino acids and brain-derived dILPs regulate Ras/Raf/MAPK signaling and IIS/TOR in the PG which further regulates the synthesis of ecdysone. Ecdysone secretion is also controlled by the Prothoracicotropic Hormone (PTTH) secreted from the larval brain. PTTH also regulates secretion of Juvenile Hormone (JH) from the CA, which acts antagonistcally to ecdysone in regulating body growth. Ecdysone secretion controls developmental timing, promotes imaginal disc growth and inhibits larval body growth. (C) Organ-Damage Signaling. Imaginal disc damage is signaled to the brain and PG by dILP8 interacting with its receptor Lgr3. In the PG, dILP8 signals through Lgr3 to upregulate the expression of Nitric Oxide Synthase (NOS) which inihibits ecdysone synthesis, thereby preventing further growth of imaginal discs. In the larval brain, dILP8 suppresses PTTH expression thereby also preventing ecdysone secretiong by the PG. Imaginal discs also signal

Figure 4-1 (cont'd)

through an unknown retinoic signaling mediated pathway to suppress PTTH secretion from the brain. This complex network showing the roles of different secreted molecules highlights inter-organ communication during development and its involvement in the determination of final body and organ size in response to environmental and growth perturbations.

The role of feedback regulation in organ size regulation

In chapter 3, I described preliminary efforts to understand the *cis*-regulation of *InR* in *Drosophila.* This preliminary effort is part of a much broader goal to understand the role of feedback regulation of *InR* by the transcription factor dFoxo and the physiological relevance of this feedback loop to signal output through the pathway.

As mentioned earlier, dFoxo forms a feedback loop to *InR* under conditions of nutrient deprivation, when dFoxo is active [15]. Feedback regulation by dFoxo in the IIS pathway had previously been proposed as a mechanism by which cells could be 'primed' to produce additional InR and therefore detect and respond to even small changes in nutrient levels. Thus, dFoxo was proposed to control the sensitivity of the IIS pathway[15] to levels of circulating dILPs. Research in *Drosophila* has validated this prediction with the demonstration that the expression levels of dFoxo in different organs makes them more or less sensitive to changes in nutrition thereby regulating the size of different organs in response to changes in nutrition [16]. However, the role of the feedback regulation in mediating this difference in sensitivity has not been clearly elucidated. More importantly, whether feedback regulation has any influence on final organ size and nutritional plasticity is not clear. An *in silico* model of dFoxo feedback suggests that increasing dFoxo expression and feedback would result in an increase in the nutritional plasticity (Y. Liang, personal communication)—though experimental validation of this prediction is still lacking. Overall, the dFoxo feedback regulation highlights an often-ignored aspect of signaling pathways: the contribution of transcriptional regulation to signal output through the pathway.

In this context, genome engineering approaches with CRISPR/Cas9 developed in the last few years provides an attractive solution. Using this approach, precise genetic editing can be achieved and the role of particular transcriptional circuit can be tested without the confounding

effects of non-specific protein knockdown or overexpression. If one were to apply this approach to the dFoxo-*InR* feedback regulation, I would be interested in determining the effect of increasing or eliminating feedback to *InR* on nutritional sensitivity of different organs. The experimental approach would involve generation of transgenic flies in which the endogenous *InR* *cis*-regulatory locus has been modified to include additional dFoxo binding sites (increasing feedback) or to completely delete predicted dFoxo-regulated binding sites (decreasing feedback). If increased feedback in the presence of high levels of dFoxo results in an increase in nutritional plasticity, one would expect that organs with high dFoxo expression such as the wings would be more sensitive to changes in nutrition. Consequently, a decrease in nutrition should result in wings decreasing in size to a much greater degree than the rest of the body.

Alternatively, a simpler transgenic approach as outlined in Chapter 3, Appendix II can be used to answer the question of how much the dFoxo feedback loop contributes to total endogenous *InR* expression in vivo. In this approach, a basal promoter and a Foxo Responsive Element (FRE) containing four or eight dFoxo binding sites drives *InR* cDNA expression. Transgenic flies containing this construct can then be assayed under varying nutritional conditions to determine the contribution of dFoxo feedback to total *InR* expression under starvation.

The role of transcriptional regulation on gene expression has been extensively studied in *Drosophila.* However, studies so far have almost exclusively focused on patterning genes[17], or on transgenic reporter gene expression patterns[18, 19]. The effect of systematic and specific rewiring of transcriptional regulation to components of signaling pathways is a poorly understood area of research, especially in multicellular organisms. Transcriptional regulatory networks involving metabolic pathways and cellular signaling pathways have been well studied in yeast, and have revealed basic design principles of a gene network [20]. Understanding the role

and effect on phenotype of perturbations of these regulatory networks in a multicellular model organism such as *Drosophila* will be an exciting area for future research in the field.

Organ size regulation: a mammalian perspective

Much of the research on size control mechanisms has been conducted in the invertebrate model system of *Drosophila*. However, size regulatory pathways are conserved; similar signaling pathways regulate organ size in vertebrates and mammals. In a nutshell, the final organ size in an adult individual is the result of growth and proliferation of cells during development. Consequently, organ size can simplistically be considered to be a composite measure of cell size and cell number. However, organs do not grow in isolation—they are subject to regulation by environmental factors and physiological factors. Indeed, cells sense their surroundings and alter their growth and proliferation in response. This is mediated by growth factors, survival factors and mitogens that activate intracellular pathways. Along with a proliferative response, programmed cell death pathways are also essential to control final organ size. Organ growth and size control is therefore, a tightly regulated development process. This is perhaps the most fundamental principle of size control—conserved from *Drosophila* to mammals. Organ size control mechanisms can be broadly classified as organ-autonomous or organ non-autonomous. In *Drosophila,* autonomous regulation of organ size occurs during development through compensatory and competition-based mechanisms (See Chapter 1 for more details). A similar mechanism of cell-competition mediated elimination of cells has recently been identified in the early mouse embryo [21, 22]. Broad parallels also exist between *Drosophila* and mammals for non-organ autonomous growth control mechanisms. For example in *Drosophila,* systemic growth control involves the environmentally regulated IIS/TOR pathway, ecdysone signaling and JH signaling along with other secreted factors such as Sun and Eiger. In mammals, Growth

Hormone (GH) signaling, in addition to IIS/TOR signaling plays a critical role in regulating body and organ size. In fact, in the absence of adequate GH signaling, organs fail to achieve to achieve a normal body size [23, 24]. Thus, another common feature of all animal growth seems to be an integration of local organ growth with that of the whole organism to result in the achievement of correct final size.

While a significant part of our understanding of size control mechanisms comes from *Drosophila,* a number of vertebrate model systems have also been used to elucidate the principles and overall generalizability of size control mechanisms. These include the amphibian model system of the salamander, and the mammalian model systems of the chick limb, mouse embryo and human liver. Similar to invertebrates, early transplantation studies in the large and small salamander species *Amblystoma tigranum* and *Amblystoma punctatum* demonstrated that organ growth is controlled by two factors: an organ-autonomous "growth potential" and a non-organ autonomous circulating "regulator" synthesized by the animal [25]. Later, the "positional identity" model derived from chick limb development provided an insight into how these disparate mechanisms could be integrated into one holistic model of growth control. This model posits that growth and differentiation in the limb is critically dependent on the precise assignment of progenitor cells in the early organ bud [26]. An internal clock is thought to measure the time that a cell spends in the "progress zone"—an instructive region that dictates the fate and growth potential of a progenitor cell [26, 27]. Systemic factors are thought to regulate the size of the pool of progenitor cells, whereas late growth of the organ bud is thought to be largely organ-autonomous [28]. This latter aspect was demonstrated with the finding that pieces of limb bud exchanged between embryos of different ages grow at a rate determined by the donor—irrespective of the host [29].

The human liver is perhaps the most well studied model system for understanding mammalian organ size control mechanisms. Research over many decades has elucidated a few common principles of size control using this model system. Firstly, as described previously, final organ size is determined by the integration of extrinsic and intrinsic cues. These include extracellular signals such hormones, cytokines, soluble factors as well local cell-cell interactions. Secondly, while growth regulatory pathways are common to all organs, the ability of each organ to regulate its final size varies among different organs [30]. For example, the human liver (as opposed to the pancreas) has a higher degree of autonomy in regulating its size—it is able to sense size deficiencies at later developmental time points and regenerate to its correct size [31]. The "positional identity" model has been suggested to explain these unique observations [32]. This model suggests that the ability of different organs to compensate or regenerate (after injury) and achieve a correct final size may be related to the timing of the systemic regulatory inputs. If the developing organ progenitor is exposed to regulatory inputs exclusively early in development, growth control program may appear to be largely fixed (and organ-autonomous) with little ability to adjust or compensate in response to later size deficiencies. Conversely, if an organ progenitor is exposed to persistent regulatory inputs in early development or to those that can be re-invoked later on, it may retain a greater regenerative potential and exhibit a more plastic growth program. For example, under this paradigm, it is likely that the human liver is subject to extensive systemic regulatory inputs signals throughout development that contribute to its greater regenerative potential in embryonic development and later adult life. On the other hand, the pancreas (similar to the chick limb paradigm) may receive regulatory inputs exclusively early in development dictating the fate and limited growth potential of the organ in later life. In support of these hypotheses, a recent study found that reducing the number of progenitor cells in the

early liver and pancreas had markedly different consequences [31].. The liver responded rapidly to compensate for the reduction in the progenitor pool (consistent with its regenerative ability), whereas the pancreas did not compensate but instead remained small throughout gestational and adult life [31]. However, we still do not clearly understand the mechanism by which the liver senses a size deficiency, nor the mechanism by which liver cells stop proliferating after sensing normalization of size. Moreover it is not clear why the growth program in some organs appears fixed, but appears regulated in others and whether either of these represents a "default" mechanism.

The molecular mechanisms by which growth control is effected in mammals shares a great degree of overlap with *Drosophila.* Broadly, organ growth is regulated by soluble growth factors, localized cell interactions and cell autonomous mechanisms. Of all the soluble growth factors, GH/IGF-mediated growth is perhaps the most important mechanism of body size regulation in mammals. GH functions as a mitogen in promoting growth of tissues and also acts through IGF-1 in mediating organ growth [23]. Soluble growth factors regulate both the generation as well as proliferation of progenitor cell populations that contribute to the final organ. For example, Fibroblast Growth Factor (FGF) secreted by the apical limb bud cells drives the early growth of the limb bud[33]. An alternate mammalian mechanism by which organs may auto-regulate their growth is by the secretion of organ-specific growth inhibitors called "chalones" [34, 35]. For example, myostatin, a member of the TGF-beta family is secreted from muscle cells and acts systemically to inhibit further muscle growth [36, 37] More recently, a second member of the TGF-beta family, GDF11, produced by embryonic olfactory epithelium cells was shown to act as a chalone by inhibiting progenitor cell proliferation [38]. Interestingly, however, no other chalones or growth inhibitory molecules have been discovered so far, leaving open the question of how

common this mechanism may be. Lastly, environmentally modulated growth takes place through the activation of the IIS/TOR phosphorylation cascade in both *Drosophila* and mammals, albeit through the activation of different receptor molecules. In mammals, the IIS/TOR cascade is activated by IGFR as well as several other classes of growth factor receptors including tyrosine kinase, cytokine and TGF-beta receptors [39], whereas in *Drosophila* it is mediated through a single Insulin Receptor. Localized cell interactions were first shown to be involved in mammalian size regulation with the observation that rat embryonic liver progenitor cells grafted into adult livers increased their number over time. This was accompanied by increased apoptosis of recipient cells to result in no net change of the total liver mass [40]. Subsequently, a c-Myc dependent cell competition mechanism similar to that in *Drosophila* was identified in the mouse embryo raising the possibility that early mammalian embryo size may, at least partly, be regulated by cell competition. These studies demonstrate that differences in the expression of c-Myc amongst cells in a population or tissue results in cell competition causing elimination of cells with lower expression of Myc [21, 22, 41-43]. Cell-cell contact and cell polarity also regulate the Hippo signaling pathway, a major organ size regulatory pathway. In both *Drosophila* and mammalian cells, for instance, cell-cell contact at high cell density produces a growth inhibitor signal that is mediated through the Hippo pathway [44, 45]. Similarly, the regulation of the Hippo pathway by apical-basal polarity proteins and planar cell polarity proteins is a common feature in both *Drosophila* and the mammalian system [46-51]. Lastly, there is some limited evidence from the mammalian system to suggest that cell autonomous mechanisms such as telomere shortening and global epigenetic modifications have an impact on cell proliferation rates [Lui and Barone 2011). However, more concrete evidence linking such genomic changes to impacts on organ size has yet to be demonstrated.

Collectively, mammalian growth control mechanisms regulating organ size share a number of similarities with those already well elucidated in *Drosophila.* Firstly, size regulatory pathways are highly conserved at the molecular level, and function to regulate organ size in response to similar inputs in both *Drosophila* and mammalian organs. For instance, in both invertebrate and vertebrate systems, the IIS/TOR and Hippo pathways regulate organ growth in response to extracellular inputs and localized cell interactions, respectively; each of these pathways consist of a highly conserved core signaling cascade. Secondly, if one considers *how* these pathways are regulated in vertebrate systems, we see that once again, there are some highly conserved features to those in invertebrate systems. For instance, in both *Drosophila* and mammals, cell polarity and cell-cell contact regulate Hippo signaling and cell competition. Lastly, despite the vast size differences between and within species amongst vertebrates, there is little variation in the overall pathway architecture of growth regulating pathways between vertebrates and invertebrates. Size variation within and between species can instead be better explained for instance, by allelic differences within species (in the case of different dog breeds) [52], or by the utilization of a variety of physiological controls that regulate and limit organ growth differently in different species [53]. Nevertheless, despite the great progress in understanding growth control mechanisms across different model systems, several fundamental aspects remain as yet unclear. For instance, how is target size sensed to initiate or arrest growth of organs? How does the DNA content/ploidy of a cell impact its size and/or growth regulation? How is autonomous growth of mammalian organs achieved? From a more clinical perspective, how are some of these size control checkpoints overcome and tissue growth dysregulated to result in tumorous overgrowth? Future research directed at answering these and other open questions would likely yield insights not just into organ function but also into the pathology of growth control diseases like cancer.

Organ size regulation: perspectives for future research

The research described in the previous chapters can best be appreciated if one considers organ development from the point of view of a synthetic biologist. Both of the aspects of growth control described in this thesis play unique roles in specific developmental and physiological contexts, which enable the organism to adapt and effectively respond to the fluctuating environment while still ensuring adequate functionality of the organ. A growing area of research today is the field of regenerative medicine—which aims to improve health and disease outcomes by repairing and regenerating cells, tissues and organs. The ultimate goal of regenerative medicine and tissues engineers is the synthesis of a fully functional organ in the laboratory. However, to build a functional organ, a solid understanding of the physiological processes involved in size determination—both at the molecular level as well as the whole-organismal level is crucial.

One of the main challenges of regenerative medicine has been poor *in-vitro* ability of cells to aggregate and self-organize to form tissues mimicking the complex organization *in vivo*. The primary problem is size—as the tissue grows in size, the accompanying vasculature must keep up with it in order to sustain further growth of the tissue. Research over the last few years has demonstrated that organ growth involves multiple different types of cells growth together in harmony and interacting with each other through different signaling pathways to regulate the growth of the organ. Replicating these pathways *in vitro* will be key to synthesizing a truly functional organ. Future research directed at understanding the minutiae of growth control pathways will not only give us a deeper understanding of the different molecular processes involved in size control but also solve some of the most pressing challenges of regenerative medicine.

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