Functional and Molecular Characterization of Targets of Ultrabithorax in Drosophila

A thesis
Submitted in partial fulfilment of the requirements

of the degree of
Doctor of Philosophy

By

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CERTIFICATE

Certified that the work incorporated in the thesis entitled **"Functional and Molecular Characterization of Targets of Ultrabithorax in *Drosophila* "** Submitted by **SAVITA SINGH** was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.

(Prof L S Shashidhara)

Date: 14-10-2014

Declaration

I declare that this written submission represents my ideas in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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Appendix:

Publication

Synopsis

Synopsis

Title: "Functional and Molecular Characterization of Targets of Ultrabithorax in *Drosophila*"

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Introduction

Drosophila is a most favored model organism for better understanding of the molecular mechanism of pattern formation during development, identifying various signaling pathways and to elucidate how various tissues and organs are developed. Wing and haltere are the dorsal appendages of second and third thoracic segments, respectively. They are homologous structures, but differ greatly in their morphology. The Homeotic gene *Ultrabithorax (Ubx)*, which is expressed in third thoracic segment, is known to regulate many wing patterning genes to specify haltere but the complete mechanism is still not clearly understood.

Ubx expression in developing haltere causes its cells to have reduction in cell size and number. It also causes haltere to take up a different shape by conferring different cellular affinities compared to wing. Haltere lacks wing type vein and sensory bristles. Haltere cells also differ from wing cells in morphology and arrangement of trichomes. The current understanding of mechanisms by which wing and haltere differ at cellular, tissue and organ level is ambiguous (Sánchez-Herrero, 2013). Our aim is to identify functional and molecular mechanisms by which Ubx regulates genes/pathways to provide haltere its distinct morphology.

While removal of Ubx from the entire haltere, or at least from one entire compartment, leads to haltere to wing transformation with increased growth of Ubx^{-} tissues, mitotic clones of *Ubx* (using the null allele $Ubx^{6.28}$) show similar sized twin spot in small clones. Only when very large clones of $Ubx^{6.28}/Ubx^{6.28}$ are generated, one can see higher growth potential as compared to their twin spots. This suggests that unless a certain threshold levels of

growth factors are de-repressed, haltere doesn't show any overgrowth phenotype.

Decapentaplegic (Dpp), Wingless(Wg), epidermal growth factor receptor (EGFR) are some of the major growth and pattern regulating pathways which are repressed by Ubx in the haltere (Shashidhara et al., 1999; Mohit et al., 2003, 2006; Crickmore and Mann, 2006; Pallavi et al., 2006; de Navas et al., 2006; Makhijani et al., 2007), However, over-expression of pathway components like Dpp, Wg, Vestigial (Vg) and Vein provides only marginal growth advantage to haltere compared to the wild type. In this context, we studied additional growth regulating pathways amongst the targets of Ubx. Genome wide studies to identify targets of Ubx have identified many components of Hippo and Insulin-insulin like signaling (IIS) pathways as potential targets (Mohit et al., 2006; Hersh et al.,2007; Pavlopoulos and Akam, 2011; Slattery et al., 2011; Choo et al., 2011; Agrawal et al., 2011). The Hippo pathway is a crucial determinant of organ size in both *Drosophila* and mammals (reviewed by Halder and Johnson, 2011). It regulates cell proliferation, cell death, and cell fate decisions and coordinates these events to specify organ size. Recent studies have revealed that Hippo pathway networks with other signaling pathways (Irvine, 2012; Kwon et al., 2013).

Objectives

The objectives of the current study were

1. Regulation of IIS Signaling and Hippo pathways by Ubx and its significance in haltere organogenesis.
2. Cross-talk between Hippo and other signaling pathways during haltere organogenesis.

This work involved

(A) To identify the components of pathways differentially regulated between wing and haltere and regulation of them in haltere by Ubx.

(B) Functional implication of components of pathways and integration of various pathways in regulating differential growth response at cellular,

compartment, organ levels and cell fate determination during haltere development.

Results

1. Insulin /insulin-like Growth Factor Signaling Pathway in Developing Wing and Haltere

Akt (also called Protein Kinase B, PKB) a central component of IIS signalling pathway is down-regulated in the haltere. Miss-expression of IIS pathway components in haltere caused moderate increase in growth of haltere disc at the third instar larval stages. At adult level, components of IIS pathway induced marginal effect on haltere size. This suggests that IIS pathway, while is down regulated in the haltere, modulating this pathway alone is not sufficient to induce major changes in morphology of haltere.

2. Ultrabithorax, Hippo pathway and haltere specification

Output of Hippo signaling is mediated by Yorkie (Yki), a transcriptional co-activator protein and is regulated differentially between wing and haltere. We observed higher levels of nuclear Yki in haltere cells than in wing cells. Mitotic clones for *Ubx* null allele suggested that Yki localization is regulated by Ubx in cell autonomous manner. Interestingly, many of the targets of Yki are down regulated in the haltere except *bantam* micro-RNA. This and additional experiments suggest that nuclear localized Yki is not an activated form. Interestingly, we have observed a possibility that Ubx may cooperate with Yki in haltere to regulate Yki itself and its downstream targets the haltere. Haltere-to-wing transformation at the level of trichomes, organ size and bristles were observed in flies over-expressing or down-regulating some of the positive and negative components of the Hippo pathway, respectively.

3. Integration of Hippo and Other Signaling Pathways

As compared to wing, haltere is reduced in size and is less responsive to majority of growth regulating pathways such as Dpp, Wg, EGFR and IIS pathway except Hippo pathway (described above). Co-expression of pathway

components such as Vein and Akt with Hippo pathway components de-represses this resistant growth response of haltere, both at the levels of imaginal disc and adult cuticle. Akt when over-expressed in the background of down regulation of *expanded* is able to induce increase in cell size and differentiation of haltere trichomes to wing type. Therefore, Ubx-mediated regulation of Hippo pathway in the haltere appears to be critical in specifying haltere size, trichome morphology etc. The effect is compounded due to the regulation by Ubx of EGFR and IIS pathways, which interact with Hippo pathway during organ specification.

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Chapter 1

Chapter 1: Introduction

Summary

Homeotic/Hox genes are known to generate diversity in morphology along the body axis in animals. Diversity involves differences in morphology of structures in terms of organ/cell size and shape. Understanding the link between Hox gene expression patterns and their function in conferring diversity in morphology is an intriguing developmental biology question. Here we address this problem using the model organism *Drosophila melanogaster*. Ultrabithorax expression in thoracic segment three of insects causes modification of wing fate into haltere fate. The objective of this study is to understand the mechanism by which Ultrabithorax functions to specify haltere fate. In this chapter, *Drosophila* development as relevant to Homeotic gene function and wing development is discussed.

Introduction

1.1 Homeotic/ Hox Genes

Hox genes determine the development of different structures along the anterior-posterior (A/P) axis of Bilaterans. Hox genes are evolutionary conserved across distant animal phyla and have role in providing diversity to the morphology of animal body plans and body parts. The term Homeotic transformation was coined by Bateson in which the morphology of a given structure is transformed into the likeness of another structure of the same organism (Bateson, 1894). Homeotic mutations in *Drosophila* were first described by Bridges and Morgan (1923). The Detailed studies on these mutations in subsequent decades lead to the discovery of Hox genes.

In *Drosophila,* Hox genes are present on 3rd chromosome in two separate clusters: the Antennapedia complex (ANT-C), comprising the Hox

genes *labial (lab), proboscipedia (pb), Deformed (Dfd), Sex combs reduced (Scr),* and *Antennapedia (Antp)* and the Bithorax complex (BX-C), including the Hox genes *Ultrabithorax (Ubx), abdominal-A (abd-A),*and *Abdominal-B (Abd-B)* (Lewis, 1978; Kaufman et al., 1980). Vertebrates, such as mouse and human, consist of 39 Hox genes, which are organised in four clusters (Graham et al., 1989). Hox gene expressions correspond to the order of the genes within the Hox complex on the chromosome, a characteristic generally known as spatial collinearity (Reviewed in Durston et al., 2011) (Fig. 1.1).

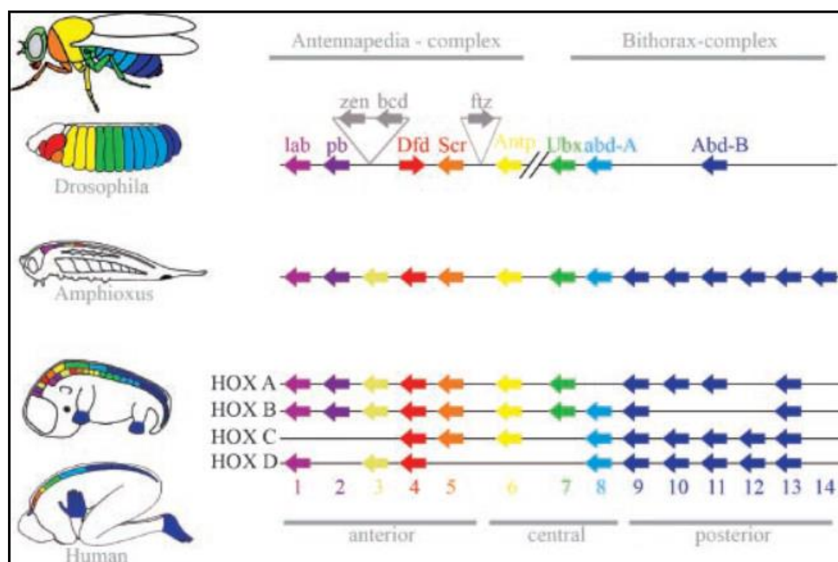


Figure 1.1 Hox gene organization and expression in different organisms

The *Drosophila* Hox genes (top), Amphioxus (middle) and Mouse/Humans are shown. In case of *Drosophila,* Hox genes are grouped into two genomic clusters: the Antennapedia (ANT-C) and Bithorax (BX-C) clusters and they both are on the 3rd chromosome. The vertebrates Hox complex are organized into four complexes present on different chromosomes and consist of 39 genes. Expression of Hox genes along the A/P axis of the adult or embryo match the order of the genes along the chromosome, displaying a property termed collinearity Source: Hueber and Lohmann, 2008

1.2 Insects wing diversity: regulation by Hox gene

Ultrabithorax (*Ubx*)

Insects are amongst the highly successful animals on the earth in terms of number of species and their diversity in habitats. One of the interesting features of insect diversity is in the number and morphology of their wings. Most of the modern insects bear two pair of wings: forewing on the second thoracic segment and hindwings on the third thoracic segment. Hox genes have role in regulating morphology of serially homologous structures within a species and the homologous structures of different species (Carroll et al., 1995).

Ubx is expressed in hind wings of all the insects so far studied (Warren et al., 1994). Role of Ubx in providing diversity to hindwings can be understood by comparing the morphology of hindwings of arthropods from each other and their respective forewing. Some of the selected model organisms like *Drosophila melanogaster* (fruitfly), *Apis Mellifera* (Honey bee), *Bombyx mori* (Silkworm), *Precis coenia* (Butterfly) and *Tribolium castaneum* (Beetles) have been used by our and other labs to understand the role of Ubx in imparting different morphologies to hind wings (Weatherbee et al., 1999; Warren et al., 1994; Tomoyasu et al., 2005; Naveen Prasad; unpublished,, Harsha TT unpublished,).

The role of Ubx in conferring differences in morphology to hindwing is a well-studied phenomenon. Removal of Ubx from thoracic segment T3 results in four winged flies in *Drosophila* and transformation of wing to elytra in Beetles (Lewis, 1978; Tomoyasu et al., 2005). In our study, we have used *Drosophila* as a model system to understand the mechanism of Ubx mediated organogenesis. The forewings in *Drosophila* or elytra in Tribolium develop without any Hox input. Interestingly, expression of Ubx of a sister phylum member *Onychophoran* in T2 segment of *Drosophila* is sufficient to transform wing to haltere and can activate/repress many of the target genes (Grenier et al., 2000). Assuming that Ubx itself has not evolved across the species, it is proposed that evolution of cis-regulatory regions in the targets of Ubx may be

the reasons for the diversity in hindwing morphology in insects (Weatherbee et al., 1999) (Fig. 1.2).

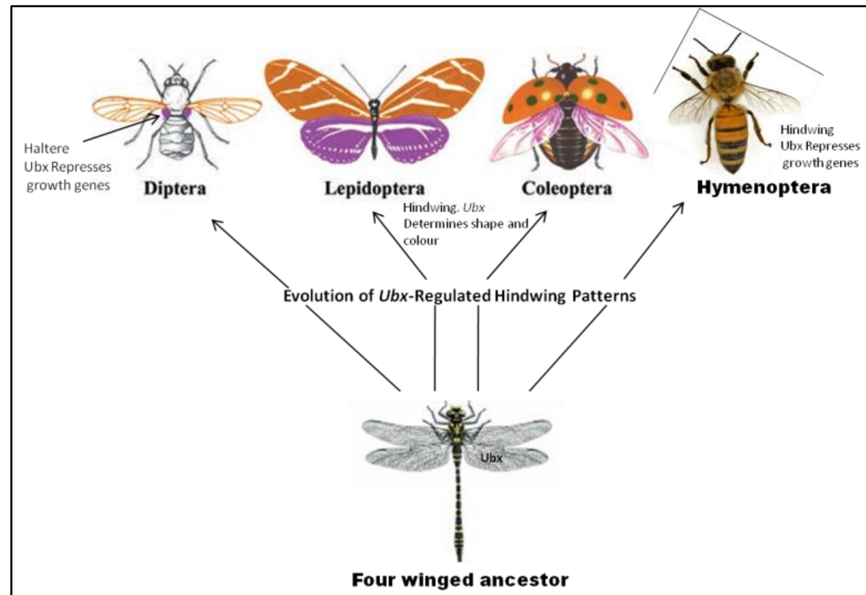


Figure 1.2 Ubx confers diversity to insect's hind wing morphology
Morphological diversity in insect's hind wings evolved from a common ancestor having similar fore and hind wing. Ubx is expressed in the third thoracic segment of all insects studied so far. It is likely that diversity is provided by evolutionary changes in Ubx target genes involved in wing development programme Modified from: Carroll et al., 2004.

1.3 Experimental System: Fruit Fly or *Drosophila melanogaster*

Major feature that makes *Drosophila* as popular model organisms is its short life cycle, which is around 10-12 days at 25^{0}C and availability of diverse experimental tools. Flies are holometabolous insects, which means they undergo a complete metamorphosis during larval to adult transition. After fertilization, eggs develop into larvae, pupae and adult. The stages between larval molts are called instars. *Flies* have three instars. The wandering stage is after third instar. At late third instar larvae undergo a metamorphic molt to from Pupae. During pupation, adult structures form and replace the larval structures (Fig. 1.3 A). The adult structures are formed from undifferentiated nests of cells called as imaginal disc, which are kept aside during the embryonic stages itself as progenitors of adult tissues and organs. In

Drosophila, there are ten pairs of imaginal discs and an unpaired genital disc, which further proliferate and differentiate into respective adult structures during metamorphosis (Fig. 1.3 B).

Availability of diverse set of genetic tools makes possible the various kinds of genetic manipulations to be performed in this model organism. Some of the highly used genetic tools include UAS-GAL4 system, yeast FLP/FRT system, insertional mutagenesis using P-element, enhancer trapping etc. which help to control and monitor temporal and spatial expression of genes.

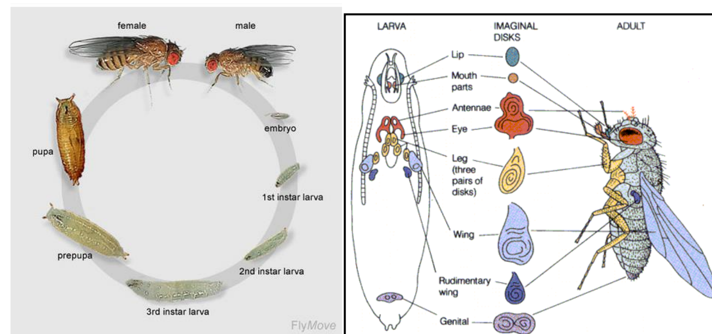


Figure 1.3 Life cycle of fly
(A) The life cycle of *Drosophila* consists of embryonic stage, which lasts for ~ 24h at 25°C, three larval stages also called instars each having duration of about 1 day long and the pupal stage about 5 days long (https://www.flickr.com/photos/11304375@N07/2993342324). (B) Imaginal discs of *Drosophila* (http://oregonstate.edu/dept/biochem/hhmi/hhmiclasses/bb450/winter2002/ch28/fi28p45.htm).

1.4 Early Development in *Drosophila*

During early embryogenesis, the anterior-posterior (A/P) and dorsal-ventral (D/V) axes are determined by maternal genes. Gradient of maternal proteins such as Bicoid and Nanos pattern the A/P axes, while maternal genes like *dorsal* establishes the D/V axes. Bicoid and Nanos activate zygotic gap genes such as *kruppel, knirps*, giant along the A/P axis, while *twist, snail, rhomboid* etc. are activated along the D/V axis by dorsal. The gap-genes activate the pair rule genes, which define the 14 parasegments, in an intriguing mechanism. Pair rule genes such as *even-skipped* define odd number parasegments, whereas others such as *fushi tarazu* define even numbered parasegments. Parasegments are further divided in to two compartments by segment polarity gene, which define the posterior compartment of a segment. Identity to each

segment is provided by a class of master regulatory genes, the Homeotic selector genes described in the previous section. All three classes of zygotic segmentation genes Gap proteins, pair rule proteins and segment polarity proteins are involved in Hox regulation.

The Bithorax complex controls the development of parasegments 5-14, while the Antennapedia complex controls the identity of the more anterior parasegments. These genes are expressed in combinatorial manner e.g. *Ubx* expressed in all parasegments from 5-12, *abd-A* parasegments 7-13, and *AbdB* more posteriorly parasegment 10 onwards (Fig. 1.4).

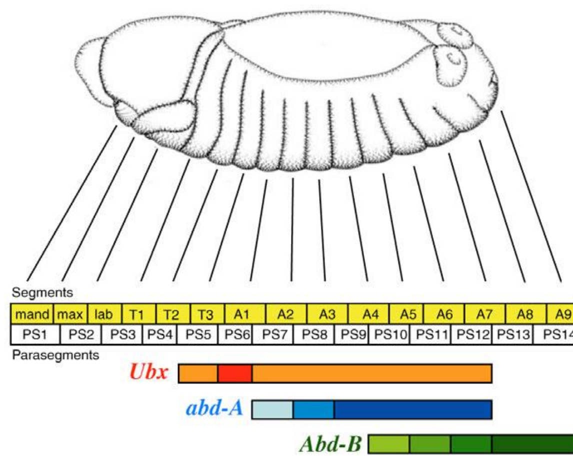


Figure 1.4 The Bithorax complex of *Drosophila melanogaster*
Drosophila embryo consists of 14 parasegments defined by the maternal, gap and pair-rule genes that form the three head, the three thoracic and the eight abdominal segments. The expression pattern of each BX-C homeotic gene is shown as darker shades of color indicate higher expression levels. Source: Maeda et al., 2006

1.5 Molecular mechanism controlling Hox Gene expression in *Drosophila*

How similar sets of developmental genes result in diverse developmental programmes can be partially understood by studying the regulation of Hox gene expression in various model organisms. The very fact that a given Hox protein specifies a particular developmental pathway, necessitates that the expression of Hox proteins have to be extremely well regulated and with a clear boundaries defining their expression domains.

In vertebrates and invertebrates, Hox gene expression are controlled by diverse mechanism including nuclear dynamics, RNA processing, microRNA and translational control (Mallo and Alonso, 2013). In case of Vertebrates the

architecture of chromatin is associated with the early global repression and subsequent collinear activation of Hox gene expression (Durston et al., 2011).

In *Drosophila,* during the early stages of embryogenesis, transcription factor encoded by maternal, gap and pair-rule genes interact with elements in each of the cis-regulatory regions of the BX-C genes to determine their ultimate expression patterns. Homeotic gene expressions are further maintained by a cellular memory system based on the action of Polycomb (PcG)/trithorax (trxG) group of proteins (Beck et al., 2010). PcG activity is also closely linked to modulation of specific chromatin states, which are usually not restricted to specific genes, but rather affect large chromosomal domains.

The Hox genes themselves cross-regulates each other i.e. the more posterior Hox genes are able to repress the expression and function of more anterior genes, a process termed ‘posterior prevalence’.

Long non-coding RNAs are known to regulate the Hox gene expression by interacting with transcription factors and chromatin modifiers. lncRNA bithoraxoid (*bxd*) controls the Ubx transcription by recruiting trxG proteins (Petruk et al., 2006; Sanchez-Elsner et al., 2006). lncRNA *iab-8* represses abd-A transcription and is suggested to have role in the process of posterior prevalence (Gummalla et al., 2012).

Role of several micro-RNA have been found to control the Hox gene expression. In *Drosophila miR-iab-4, miR-iab-8, miR-10* and *miR-993* are present within the Hox clusters. *miR-iab-4* and *miR-iab-8* are known to target several genes including *Ubx, Antp, abd-A* and *Abd-B* (Mallo and Alonso, 2013). *miR-iab-4* has a specific role in regulating the Ubx expression during haltere development (Ronshaugen et al., 2005).

1.6 Structure and function of Hox proteins

Hox genes code for proteins that contain a highly conserved DNA-binding homeodomain. In vitro studies have identified a short DNA binding motif. In contrast to their highly specific in vivo activity, they show a weak in vitro

binding specificity and recognise a ubiquitous sequence. This condition is defined as Hox paradox.

Various models have been proposed to understand the Hox paradox. For example, Hox proteins do not act alone but they require other cofactors or collaborators for activity (Fig. 1.5). The best characterized cofactors belong to the TALE class of homeoproteins including Extradenticle (Exd) and Homothorax (Hth) in invertebrates and Pbx and Meis in vertebrates. However, the cooperative binding of Hox-Exd-Hth interaction explain the high degree of DNA binding specificity to some extent only as Hox proteins are able to regulate target genes in the absence of these cofactors. Role of two segmentation proteins, Engrailed (En) and Sloppy paired 1 (Slp1), have been shown to be required for the repression of the Hox target gene *(Distelless) Dll* by Ubx (Gebelein et al., 2004).

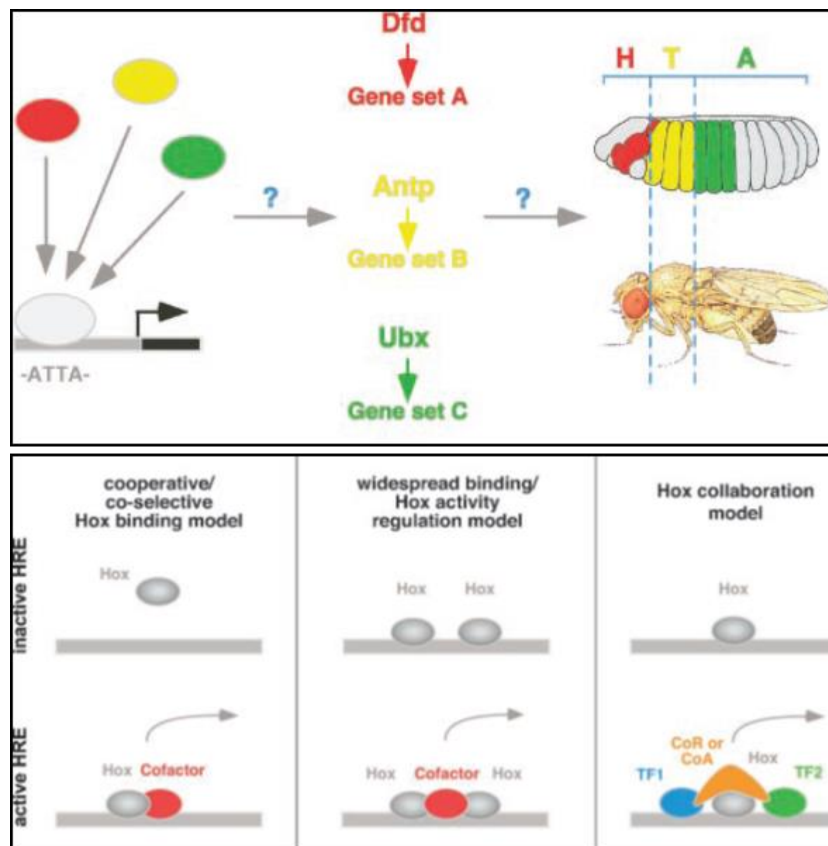


Figure 1.5 Schematic presentation of Hox paradox

(A) Hox proteins are transcription factors that bind to very similar sequences and regulate different set of target genes to provide different morphological output. (B)

Three models explaining hox target gene regulation. Source: Hueber and Lohmann, 2008

1.7 Regulation of organogenesis by Hox genes (Function of Hox genes)

Hox genes primarily function as modifiers of pre-existing developmental platforms. Their function may result in two different types of effect as reflected in the phenotypes observed when they are mutated: first, mutation in a Hox gene causes transformation of one structure to another of the same body plan e.g. wing-to-haltere or antenna-to-leg transformations; second mutations in certain Hox genes result in new types of organs e.g. *Dfd* or *lab* mutations in *Drosophila* often show abnormal structures. In both cases, Hox genes must modify various cellular events such as cell death, cell affinity, cell proliferation, cell size, cell shape and cell migration (Reviewed by Sánchez-Herrero, 2013).

Here, our study involves the role of Ubx in providing haltere identity to dorsal appendage of the third thoracic segment.

1.8 Ultrabithorax

1.8.1 Expression: During embryogenesis in *Drosophila*, Ubx is expressed in parasegments (PS) 5-13 with highest level of expression in PS6, thereby determining the identities of PS5 (that corresponds to T2p+ T3a) and PS6 (that corresponds to T3p+A1a). Different cis-elements of Ubx regulate its expression at parasegment boundaries.

During larval stages, Ubx is expressed in both the dorsal (wings and halteres) and ventral (legs) imaginal discs of the thoracic T2 and T3 segments. In the wing imaginal disc, Ubx is expressed only in the peripodial membrane, but not in the disc proper. Although Ubx is expressed throughout the development of haltere, presence of Ubx at late larval and early pupal development are sufficient to provide identity to haltere (Roch and Akam, 2000).

1.8.1 Structure: The 77-Kb long transcription unit of Ubx generates family of six protein isoforms Ubx isoforms through alternate splicing. All isoforms include most of the N-terminal domain and the C-terminal homeodomain containing region. All isoforms have similar DNA binding activities and splicing affects the regulatory capacity of the resultant protein (de Navas et al., 2011). During embryogenesis, Ubx isoforms are not functionally interchangeable and they show differential binding capacity in presence of co-factor Exd (Reed et al., 2010).Thus, alternate splicing produces Ubx protein of different functions. In *Drosophila*, these isoforms are phosphorylated on serine and threonine residues to form 5 phosphorylated states per isoforms and are phosphorylated throughout the embryogenesis, although the functional relevance of this is poorly understood (Gavis and Hogness, 1991).

1.8.2 Activation and repression Activity: Ubx proteins have both transcriptional repression and activation activity. The YPWM motif is known to be associated with repression and SSYF motif at N terminal is known to associate with activation function of Ubx protein (Tour et al., 2005). A link between transcriptional activation and repression function of Ubx comes from the interaction of Med19 Subunit of RNA polymerase III machinery. Med19 can directly bind to Hox homeodomain and can activate Ubx target gene (Boube et al., 2014).

1.8.3 Target gene recognition and regulation: Various in-vitro approaches to identify Ubx binding element show a similar –T-A-A-T- binding sequence. Studies using random sequence oligonucleotides have shown that the sequence 5 '-T-T-A-A-TG>T-G>A-G-G 3' as the optimal binding site for the Ubx homeodomain (Ekker et al., 1991). Other in vitro approaches used to identify the Ubx binding motif include using a bacterial one-hybrid system (Noyes et al. 2008) also showed the same binding element for Ubx binding.

In-vivo approach to identify Ubx binding element includes Chromatin immunoprecipitation coupled with microarray analysis. Chip experiments by three independent labs were carried out using Immunoprecipitation of 0-16 hrs embryo, 3rd instar larval haltere imaginal discs (Choo et al 2011; Slattery et al

2011; Agrawal et al 2011). These studies used different methods to pull down Ubx. One study used anti-GFP antibody on Ubx::YFP protein trap line, while a study form our laboratory used Ubx-specific antibodies (raised against N-terminal domain) on *Cbx* wing discs. Yet another study used antibodies raised against full-length protein on T3 leg and haltere discs. None of these studies identified a specific motif as target recognition motif for Ubx. However, ChIP data revealed enrichment for binding sites for some of the other transcription factors suggesting that Ubx may recognize its targets through these transcription factors. Ubx is shown to interact with many co-factors such as Hth and Exd which provide the specificity and affinity to bind the target elements. However, in the haltere imaginal disc, Exd and Hth are required for Ubx function only in the hinge and notum region that gives rise to proximal region of the appendage and the body wall. Although, Exd is expressed in the pouch region of haltere, lack of Hth has no effect on Ubx function (Casares and Mann, 2000). Genome wide approach to understand the mechanism of target gene regulation further strengthens the Hth independent regulation of Ubx target genes (Choo et al., 2011).

A yeast two-hybrid screen identified an array of Ubx interacting proteins, including transcription factors and components of signalling pathways (Mastick et al., 1995). Analysis of pulled down sequence using bioinformatics tools reports several potential cofactors for Ubx such as Pho, Brk (Choo et al., 2011), GAGA, MAD (Agarwal et al., 2011), GATA, MAD (Slattery et al., 2011) etc. Specific functional studies have reported that Ubx directly represses *spalt (sal)*, also known as *spalt major (salm)* gene, in the haltere by Mad and Med collaborating factors (Galant et al., 2002; Walsh and Carroll, 2007). Nonetheless, the precise mechanism by which Ubx recognizes, binds and regulates its targets is far from understood.

1.9 Ubx-mediated Haltere Specification

Role of Ubx in providing distinct morphology to haltere is evident by its gain of function and loss of function phenotypes. Loss of function mutations (known as bithorax (*bx*)) results in haltere-to-wing transformation giving rise to the four winged fly phenotype. Conversely, dominant gain-of-function

mutations (known as contrabithorax (*Cbx*)) that cause ectopic expression of Ubx in the developing wing disc results in wing-to-haltere transformation (Lewis, 1978).This kind of transformations provides a useful model system to understand the molecular basis of differential tissue and organ development. Previous studies suggest that Ubx regulates numerous target genes with a wide range of functions to modulate the wing program. It interfere the wing transcriptional network at multiple levels and over many developmental stages (Fig. 1.6).

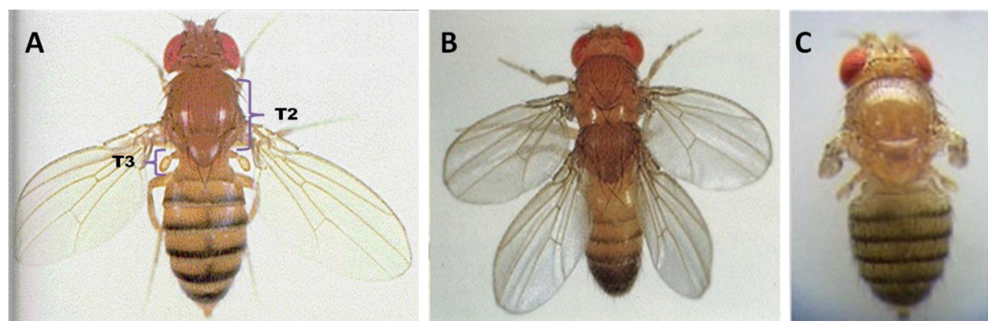


Figure 1.6 Homeotic transformations of wing and haltere in *Drosophila*
(A) Wing and haltere are the dorsal appendages of the T2 and T3 segments, respectively. (B) Loss of function of the homeotic gene *Ubx* in haltere causes transformation to wing suggesting that *Ubx* suppresses wing development in T3 to specify haltere fate. (C) Ectopic expression of Ubx causes wing-to-haltere transformation suggesting that Ubx is sufficient to specify haltere development.

1.10 *Drosophila* Wing Development

1.10.1 The adult wing

The adult wing of *Drosophila* is formed of two tightly adhered epithelial sheets. The wings have longitudinal veins (L1-L5) which run along the proximal-distal axis and the cross-veins (CV), anterior (ACV) and posterior (PCV), which run perpendicular to the longitudinal veins. Veins provide structural support to the wing membrane during flight, as well as acts as channels for haemolymph, axons of sensory neurons and trachea (Fig. 1.7).

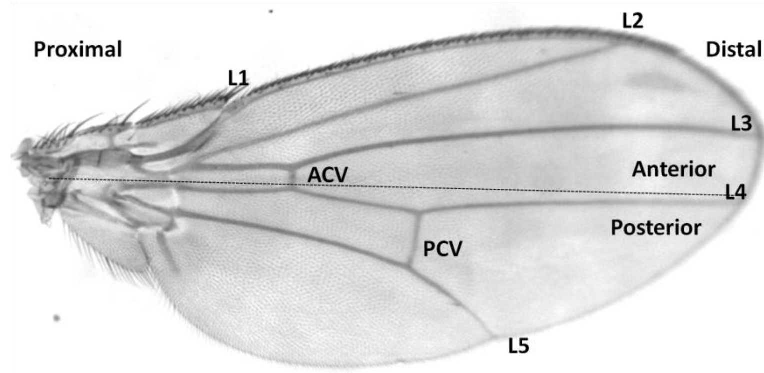


Figure 1.7 An adult wing of *Drosophila*
Showing 5 longitudinal veins L1-L5 and cross-veins (anterior cross-vein (ACV) and posterior cross-vein (PCV).

1.10.2 Early Development of *Drosophila* wing

Progenitors of Wing or the wing primordia are determined during early embryogenesis. After cellular blastoderm, ectodermal cells are selected to form imaginal precursors and are specified in response to *wingless (wg)*, a segment polarity gene and *decapentaplegic (dpp)* (Cohen et al., 1993). Both Wing and leg primordia originate from the Dll expressing precursor cells. At about 10 hours after embryogenesis, a group of about 30 Vestigial (Vg) -expressing cells give rise to wing and haltere primordia in T2 and T3 (Cohen 1993).These cells move more dorsally and get spatially separated from the ventral leg primordia.

During the four days of larval growth, numbers of wing cells increase from 40 cells to 50,000 and this involves about 10-11 divisions (García-Bellido and Merriam, 1971). The larval wing imaginal disc consists of two layers of cells. Columnar epithelial layer of disc proper cells, which give rise to the wing blade, notum and the hinge of the adult and a squamous epithelial layer of peripodial cells, which do not directly contribute to any wing structure but have supporting role in disc eversion and in notum formation (Fristrom and Fristrom, 1993). All the structures specified by the wing disc are patterned de novo during larval stages, which are built on the A/P and D/V axis determinants carried from the embryonic stages (Fig. 1.8).

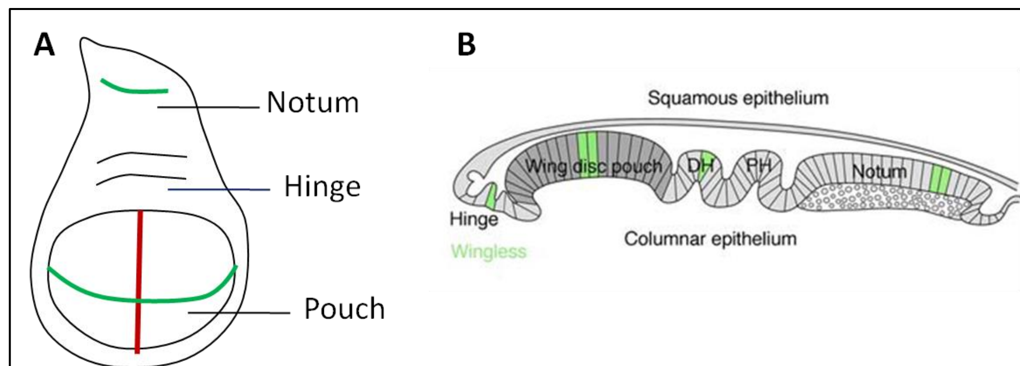


Figure 1.8 Schematic drawings illustrating the organization in a wing disc
(A) Fate map showing pouch, hinge and notum parts are labelled. Red line: the A/P compartment boundary of the pouch. Green line: the D/V compartment boundary of the pouch. (B) Cross-section of a wing disc. The disc is made up of two epithelial sheets, one columnar and the other squamous. Modified from Widmann and Dahmann, 2009.

(A) Anterior/Posterior patterning

The posterior compartment of the disc is marked by the expression of homeodomain gene *en* which activates the *hedgehog (hh)* gene in the cells of posterior compartment. Hh protein acts as a short range signal and binds to its receptor Patched (Ptc). In the absence of Hh ligand, Ptc represses the activity of transmembrane protein Smoothened (Smo) by a mechanism which is not completely understood, which initiates a series of post-translational modifications of components of the Hh signaling transduction pathway (reviewed by Wilson and Chuang 2010).

This cascade of events involves the modulation of activator and repressor forms of the Cubitus- interruptus (Ci) transcription factor (Aza-Blanc et al., 1997). In the Smo inhibited state full length Ci is phosphorylated to generate a truncated repressor form to block the expression of Hh responsive genes. Hh signaling inhibits the formation of repressive form by preventing the Ptc mediated inhibition of Smo, which through downstream events retains the active form of Ci (reviewed by Wilson and Chaung 2010). This active form of Ci translocate to nuclei to induce the expression of target

genes such as *ptc, dpp, (vein) vn*, *(knot) kn* (Basler and Struhl 1994; Tabata and Korenberg 1994; Schnepp et al., 1996). vn is a ligand for EGFR, required to specify veins (Wessells et al., 1999). Dpp is a secreted protein of the TGF-beta family which acts as a long range signal to regulate growth and patterning along the A/P axis (Basler et. al., 1994) (Fig. 1.9A).

(B) Dorsal/Ventral patterning

D/V pattering is regulated by Apterous (Ap), Notch (N) and Wg signaling pathways. Cell lineage analysis has shown that D/V boundary is determined much later in development, compared to the A/P boundary (Garcia-Bellido et al., 1976). The first step in this process is the separation of dorsal and ventral cells by the expression of Ap in the dorsal compartment, which is visible from the early second larval instar stages (Cohen et al., 1992). Ap induces expression of Fringe (Fng) and Serrate (Ser) in the dorsal compartment. Ser activates N only in the ventral cells at the D/V boundary, while Fng inhibits the activation of N by Ser in dorsal cells (Irvine and Wieschaus 1994). At the same time Delta (Dl) activates N to reinforce the effect of Ser.

Once activated, N also induces Dl expression in the DV boundary (Panin et al. 1997). Notch further activates *wg, cut (ct)* and *vg* in the DV boundary (Couso et al., 1995; Kim et al., 1996; Neumann and Cohen, 1997). Wg is a morphogen which activates downstream target gene in concentration dependent manner. Target genes *achaete (ac), Dll* and *vg* require high, moderate and low levels of Wg respectively, for activation (Neumann and Cohen, 1997). Vg expression is regulated by two enhancers; Boundary enhancer (regulated directly by N) and quadrant enhancer (regulated by Wg) (Fig. 1.9 B).

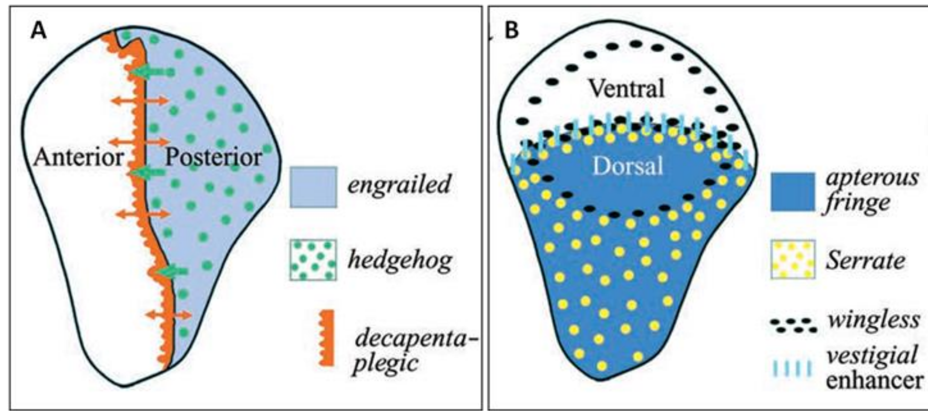


Figure 1.9 Patterning in the A/P and D/V axes of the wing
(A) A/P signaling involves sequential activity of three main proteins En, Hh and Dpp. (B) The D/V coordinate system includes the sequential organizing activities of Ap and the N and Wg pathways. Modified from: Carroll et al., 2004

1.10.3 Later stages of wing development

During the pupal stage, the wing imaginal disc develops internally within the larvae and further evaginates to lie outside of the body wall. During apposition process of wing development, the monolayer wing pouch forms the bilayer structure by coming together of the upper (dorsal) and lower (ventral) layers with their basal surfaces facing each other.

At about 12 Hours after puparium formation (APF), influx of haemolymph causes the two layers to separate. Re-apposition is initiated at around 16-18h APF. After this the vein and intervein domains are defined. At about ~32h APF called as 'definitive stage', wing appears similar to adult wing but is reduced in size. The subsequent stages involve the achievement of adult wing size and flattening of cells. The final stage of wing development is wing maturation, the process of expanding the folded wing after eclosion of the adult (Fristrom et al., 1993; Blair, 2007) (Fig. 1.10).

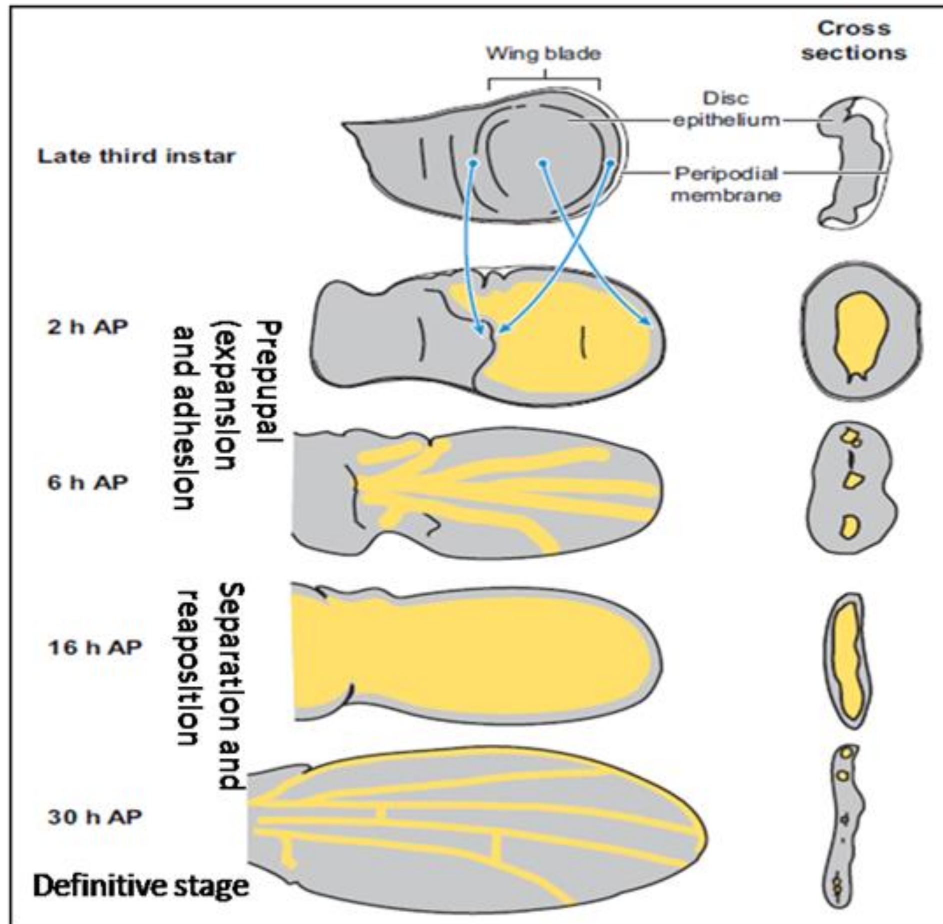


Figure 1.10 Wing development at pupal stages

Transverse sections of wings at different stages of pupal development. Modified from: Fristrom et al., 1993 and Blair, 2007.

1.11 Ubx modulates wing development pathway to specify haltere

As discussed earlier, Ubx suppresses wing development and specifies haltere in the third thoracic segment. With the detailed understanding of wing development, there have been multiple efforts to identify targets of Ubx amongst the wing development genes to understand the mechanism of Ubx function. Various approaches have been used to identify targets of Ubx that are expected to differentially express between wing and haltere, e.g. loss-of-function genetics, deficiency screens, enhancer-trap screening, proteomics, microarray analysis and other genome wide approach that is chromatin immunoprecipitation (ChIP). Targets include genes involved in diverse cellular functions like components of the cuticle and extracellular matrix, genes involved in cell specification, cell proliferation, cell survival, cell adhesion, or cell differentiation, structural components of the actin and microtubule filaments, and accessory proteins controlling filament dynamics (Reviewed by Sánchez-Herrero, 2013).

Table 1.1 Methods used for whole genome identification of Ubx target genes in haltere

Genome wide Approach	Total number of targets	Sample	References
Microarray	542 genes	Haltere and wing imaginal discs	Mohit et al., 2006
Microarray	308	Haltere and wing imaginal discs	Pavlopoulos and Akam, 2011
Microarrays	344	Haltere and wing imaginal discs	Hersh et al., 2007
ChIP	1147	Haltere imaginal disc	Choo et al., 2011
ChIP	3400 genes	Haltere and third leg imaginal discs	Slattery et al., 2011
ChIP	493 Genes	Haltere imaginal disc (Cbx gain of function mutant)	Agrawal et al., 2011

1.12 Modulation of wing patterning pathways at multiple levels by Ubx

Previous work has shown that several wing patterning genes comes under the regulation of Ubx to specify haltere. Also Ubx modulates these signaling pathways at multiple levels (Fig. 1.11). Many of the genes which are targets of Ubx have role in controlling wing size, others its patterning and few affecting both wing size and patterning. Wg and Dpp signaling are critical regulators of growth and patterning along the A/P and D/V axis of *Drosophila* wing imaginal disc. Ubx down regulates D/V and A/P signaling at multiple levels to specify haltere fate (Weatherbee et al., 1998; Shashidhara et al., 1999).

In Haltere imaginal disc Wg is expressed only in the anterior compartment and Ubx down regulates its expression in the posterior compartment. Although Wg is expressed in the anterior compartment, downstream Wg signaling is kept in a repressed state by down regulation of downstream targets. Expression studies have shown that Ac, Dll and Vg-QE are down regulated in haltere imaginal disc, (Weatherbee et al., 1998; Shashidhara et al., 1999) also Ubx functions downstream of Shaggy/GSK3b to enhance the degradation of Armadillo (Arm) (Prasad et al., 2003).

Expression patterns of Dpp, Thick-vein (Tkv), mother of thick-vein (Mtv) and Dally show differential regulation in wing and haltere imaginal disc. Dpp transcript levels are down regulated in haltere although *dpp* does not appear to be a direct target of Ubx. Dpp signaling is down regulated at multiple levels in haltere imaginal disc by its reduced diffusion, at the levels Mtv and Dally, which are down regulated and Tkv, which is up regulated in the haltere (Crickmore and Mann, 2006; de Navas et al., 2006; Makhijani et al., 2007).

Egfr/Ras signaling pathway is one of the other major regulators of growth and patterning in wing imaginal disc which is down regulated in haltere imaginal disc at multiple levels. Ubx negatively regulates the expression of the ligand *vn* as well as the receptor *Egf-r* to down-regulate the signaling pathway (Pallavi et al., 2006).

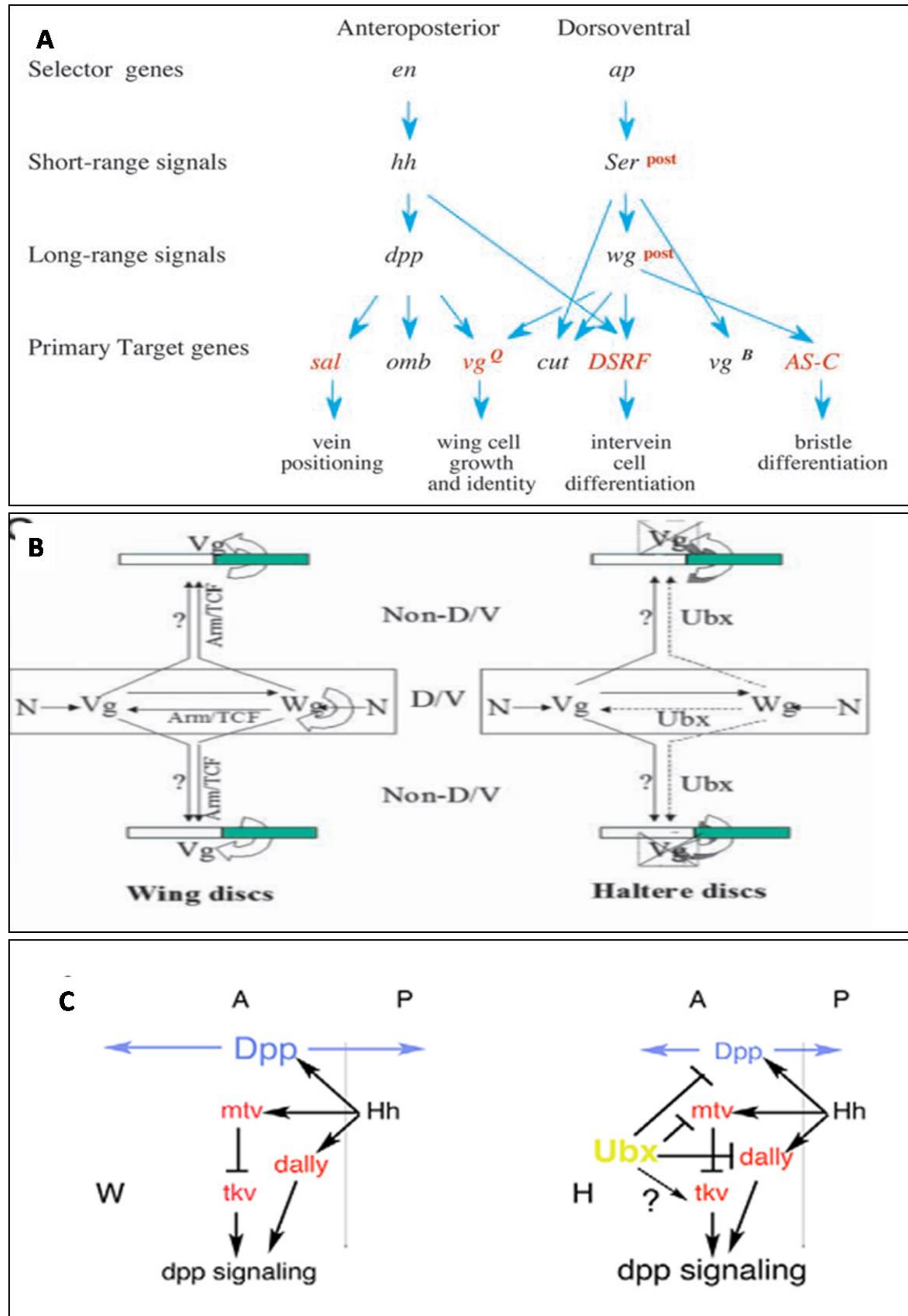


Figure 1.11 Ubx protein selectively modifies the wing regulatory hierarchy at multiple levels

(A) Genes that are Ubx-regulated in the haltere are shown in red. Source: Weatherbee et al., 1998. Ubx differentially regulates (B) Wg (Source: Prasad et al., 2003) and (C) Dpp signaling pathways in haltere. Source: de Navas et al., 2006.

1.13 Regulation of Ubx target genes in developmental stage specific manner

Microarray approach has identified many genes to be regulated by Ubx, although this approach does not distinguish between direct and indirect targets (Table 1.1). Development-stage specific identification of Ubx targets has been done by Gal4/Gal80ts system (Pavlopoulos and Akam, 2011). Ubx-dependent differential gene expression was analysed at three stages; late third instar larvae, pre-pupa and early pupa and found distinct sets of target at different stages (Pavlopoulos and Akam, 2011). They observed that Homeotic transformations are stronger in earlier temperature shifts. Comparisons of Ubx bound genes and Ubx-regulated genes at different stages suggested that genes having function at pupal stages are already bound by Ubx at earlier stages (Choo et al., 2011)

1.14 Plan of work

As described in the previous sections, multiple genes and pathways involved in wing development are regulated by Ubx in the haltere. Ubx regulate cellular processes such cell division, cell proliferation, cell differentiation, cell size, and cell affinity in developmental stage specific manner to provide haltere a distinct morphology than wing (Fig. 1.12). However the functionality of genes and pathways required by Ubx to show cellular differences are not completely known.

Reports from various labs have shown that expression of components of major growth and pattern regulating pathways like Dpp, Wg, and EGFR in haltere causes no or moderate growth of haltere compared to wing (Shashidhara et al., 1999; Mohit et al., 2003, 2006; Crickmore and Mann, 2006; de Navas et al., 2006; Makhijani et al., 2007). Even the co-expression of Vg and Dpp is even not enough to cause a dramatic growth of haltere capitellum (Fig. 1.13). Therefore, current knowledge of genes regulated by Ubx is clearly incomplete because expression of any of the known targets fails to mimic the effect of loss-of-*Ubx* in driving tissue growth.

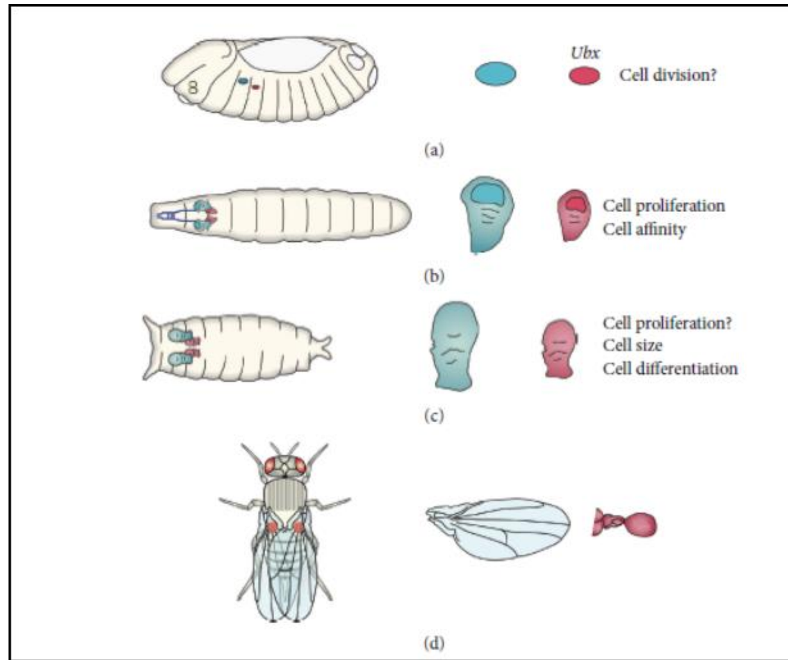


Figure 1.12 Ubx modifies various cellular processes at development stage specific manner to form haltere (a) embryo (b) larval (c) Pupal and (d) at adult stage. (Adapted from Sánchez-Herrero, 2013)

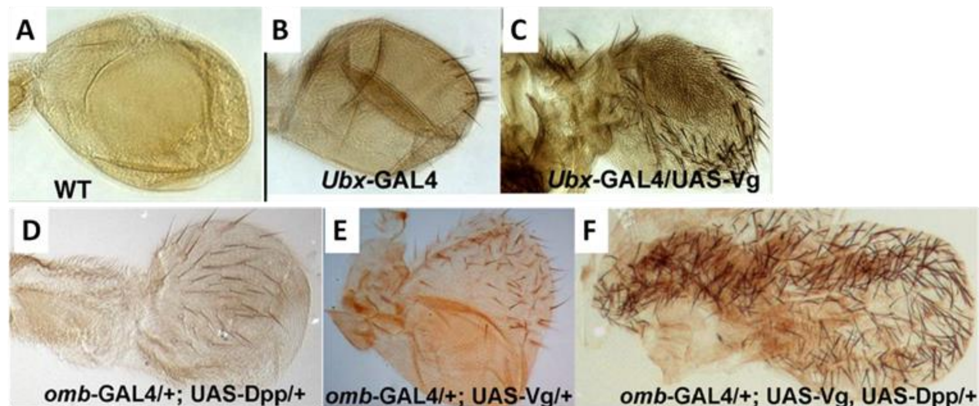


Figure 1.13 Effect of the expression of the major signaling pathways that regulate growth and patterning Source: Mohit et al., 2003, 2006 and Pallavi et al., 2006.

A considerable amount of research has been performed to identify the targets of Ubx in haltere (Table1.1). Recent, transcriptome and genome wide studies from various labs have revealed many components of two major organ size controlling pathways as potential targets of Ubx; The Insulin/insulin like growth factor signaling (IIS) and the Hippo signaling pathways. However, the function of their regulation by Ubx during haltere development has not been

investigated. In this study, we examined the status of IIS and Hippo pathways during haltere development.

In this context, Specific Objectives of study were:

1. Regulation of IIS Signaling pathway by Ubx and its significance in haltere organogenesis.

2. Regulation of Hippo pathway by Ubx and its significance in haltere organogenesis.

3. Cross-talk between Hippo and other signaling pathways during haltere organogenesis.

Chapter 2

Chapter 2

Experimental procedures

Drosophila Genetics

2.1 General Fly Maintenance

The required fly stocks for crosses were grown on standard cornmeal-sugar-agar media and were maintained at 25^0C. The wild type strain used during study is Canton-S. All the crosses were set up at 25^0C, unless specified otherwise. To avoid overcrowding to enable comparative organ/compartment/size analysis, number of male and female flies were kept constant within a set of experiments. They were allowed to mate for two days and then were transferred to fresh vials for lying eggs for 4 more days; thereafter adult parental flies were removed.

2.2 Mutant Alleles used:

$Ubx^{6.28}$ is a null allele of *Ubx* (and the *Df109* deletion eliminates the *Ubx gene (Lewis, 1978,* Beachy et al., 1985, cited in de Navas et al., 2006*).*The *pbx* mutations causes' loss of Ubx expression in the posterior compartments of the haltere disc.

2.3 GAL4-UAS system for manipulating gene expression

We employed GAL-UAS system (Brand and Perrimon, 1993) for over-expression or knock-down of gene expression.

2.3.1 GAL4 drivers

Following GAL4 drivers were used in this study. *Ubx-GAL4 (*Pallavi and Shashidhara, 2003*), omb*-GAL4 (Lecuit et al., 1996), *MS1096*-GAL4 (Capdevila and Guerrero, 1994)*, en*-GAL4 (Brand and Perrimon, 1993)*, hh*-GAL4 (Bloomington stock list; originally developed by Andrea Brand).

2.3.2 UAS lines used for over-expression studies

UAS-Rheb, UAS-Akt, UAS-FOXO, UAS-4EBP OR UAS-THOR (Bloomington exelixis, Inc),hs-FLP122; UAS-dAKT (Verdu et al., 1999), UAS-Inr^{wt} (Bohni et al., 1999), UAS-Dp110 (Leevers et al., 1996), UAS-Dp110^{D945A} (Leevers et al., 1996), UAS-dPTEN (Gao et al., 2000), UAS-DS6K , UAS-Tsc1 and UAS-Tsc 2 (Tapon et al., 2001),UAS-Yki (Huang et al., 2005), UAS-yki-GFP (Oh and Irvine, 2008), UAS-yki^{S168A}(Oh and Irvine, 2009), UAS-Diap1, UAS-CycE , UAS-Bantam (on third from Irvine lab), UAS-Dpp(Bloomington), UAS-Vg (Kim et al.,1996), UAS-vein (Schnepp et al., 1996), UAS-*bantam*-GFP (Brennecke et al., 2003), UAS-*bantam*-sponge (Herranz et al., 2012) and UAS-Nuclear lacZ, UAS-Ubx .

2.3.3 UAS-hairpin transgenes used for gene knock-down studies

UAS-*Tsc1*^{RNAi} (TRiP.JF01484)/(TRiP.JF01262), UAS-*Tor*^{RNAi,} (TRiP.HMS00904), UAS-*foxo*^{RNAi} (TRiP.JF02019), UAS-*ex*^{RNAi,} (TRiP.JF03120), UAS-*hpo*^{RNAi} (TRiP.JF02740), UAS-*wts*^{RNAi} (TRiP.JF02741), UAS-*ds*^{RNAi} (TRiP.JF02842), UAS-*mer*^{RNAi} (TRiP.JF02841), UAS-*ft*^{RNAi} (TRiP.JF03245), UAS *yki*^{RNAi} (TRiP.HMS00041) / (TRiP.JF03119) (KK109756),UAS-*Ubx*^{RNAi} (v37823), UAS-PI3K*RNAi* (TRiP JF02270)..

2.4 Temporal control of Gene expression

The *tub*-Gal80^{ts}/Gal4 system (McGuire et al., 2003) was used to temporally control the induction of transgenes with the Gal4-UAS method. GAL80 is a protein from yeast that binds to and represses the activity of GAL4. A temperature sensitive version of this protein was used to switch-off or induce GAL4 activity at various stages of development. In experiments with tub-GAL80^{ts}, flies were allowed to grow at the restrictive temperature (19°C) at different stages of development and then shifted to the permissive temperature (28°C).

2.5 Reporter constructs Used

To monitor expression patterns/levels of a given gene, following reporter transgenes were used. *cycE*-lacZ, *bantam*-lacZ (P{lacW}banL1170a) described in flybase, *vg*-quadrant enhancer-lacZ (Kim et al., 1996), *expanded*-lacZ (Hamaratoglu et al., 2006), *diap*-lacZ (Huang et al.,2005) and *bantam*-sensor-GFP (Brennecke et al., 2003)

2.6 Mosaic Analysis

Ubx^{-} clones: Mitotic clones of a null allele of *Ubx* were generated using FLP-FRT method (Xu and Rubin, 1993) using FRT82B *Ubx$^{6.28}$* (Weatherbee et al., 1998, cited in de Navas et al., 2006). Clones were generated using hsFLP and Ubi-GFP was used as the clonal marker. Clones were induced by giving heat shock for 1 hr at 37^{0}C during the larval period. Wandering third instar Larvae were dissected after clonal induction. The genotype of the larvae was: y hs-flp122; FRT82B *Ubx$^{6.28}$*/FRT82B Ubi-GFP.

Ay-Gal4 flip-out clones: Flip out clones over-expressing a gene of interest (Struhl and Basler, 1993) were generated by crossing UAS-transgene to hs-flp; AyGal4 UAS-GFP and heat shock was given for 15min at 37^{0}C , 48-72 hrs after egg lying(AEL). Whenever this experiment was done for the purpose of measuring the influence of gene expression on growth of cells within a clone, hs-flp; AyGal4 UAS-GFP female flies were crossed to males of both UAS-transgene of interest and UAS-nuclear-LacZ in the same vial. The latter was used as control. They were allowed to mate for 48 hrs and then the females were transferred to a separate vial to lay eggs. Heat shock was given at 36-48 hrs or 65-77 hrs after AEL for 15min at 37^{0}C. Discs were stained with both anti-β-gal (red) and anti-GFP (green) to differentiate between the larval discs bearing test and control clones within same set of experiments.

2.7 Immunohistochemistry

Immunohistochemistry in larval and pupal discs were essentially as described in Patel et al. (1989). The primary antibodies used are, Monoclonal anti-Achaete (1:10; Developmental Studies Hybridoma Bank (DSHB),anti-Armadillo (1:100; DSHB), anti-β-galactosidase (1:100, Sigma,), anti-Engrailed 4D9 (1:200; DSHB), anti-Ultrabithorax (1:30; White and Wilcox, 1985), anti-Wingless (1:1000; DSHB); anti-GFP (1:10,000; invitrogen) , p-Akt 473(1:100; cell signaling), antiphospho-4EBP(1:10; cell signaling), anti-S6k(1:10; cell signalling), anti-RHEB (1:1000; Abcam), anti-Yki(1:100; a gift from Ginés Morata)and polyclonal anti-Armadillo (1:100; Abcam) and anti-N-terminal Ubx(1:1000; Agarwal et al., 2011).

All secondary antibodies used during this study were all obtained from Invitrogen.

2.7.1 Antibody staining of larval and pupal imaginal discs

Staining larval discs:

Wandering third instar larvae were collected and were washed with Phosphate buffer saline (PBS, pH7.4 from sigma) in a glass cavity block. The gonadal primordia of larvae were used to identify the sex of the larvae. The male gonad primordia are a lateral pair of translucent discs, easily seen through the larval cuticle approximately 2/3 down the length of the body. The female gonad primordial is smaller and not easily identified. Larvae were given a transverse cut at 1/3 length from the anterior tip. The anterior part, which contains the discs of interest, was turned inside out. At least 10/12 such heads were fixed for 20 minutes in PBS with 4% Para formaldehyde. Fixed larval heads were given 2 washes of PBS for 10min each and were blocked for 1hr at room temperature or overnight at 4^{0}C on a rotating platform. They were then incubated with primary antibodies of desired dilution overnight at 4°C or 2hrs at room temperature. This followed washing 3 times for 20 min each with PBTX and incubation with secondary antibody of desired dilution in blocking solution for 1 hr at room temperature or overnight at 4°C in dark. Then larvae were again given 3 washes of 20 min each with PBTX. Final wash with PBS

was given to remove all PBTX. Imaginal disc of interest were obtained by dissecting them from anterior heads in cavity block and were carefully transferred to microscopic slides with spacers or without spacer depending on the nature of the experiment. Imaginal disc were then mounted with anti-fade with or without DAPI (Invitrogen) and then was covered with cover slip.

Reagents

Phosphate buffer saline PBS pH7.4 Sigma

Blocking solution: PBS + 0.1% TritonX-100 (sigma) + 0.5% BSA (Sigma).

PBTX : PBS + 0.1% TritonX-100 (sigma)

Pupal Dissections and staining

The larval/pupal transition is marked by the formation of the pre-pupae that are usually white (considered 0 hours after puparium formation-APF). They are oblong round shape and have protrusion of anterior spiracles. 0 hr pupae from culture vial or bottles were gently removed with wet paintbrush. The pupae were placed on slide with glue side facing up tape, on their side for dorsal-ventral dissection or their back for sagittal dissection. The cut was given with a double side razor blade (e.g. Wilkinson brand). After that two half cut pupae with disc of interest were transferred to cavity block and were washed with PBS. Then fat body was removed gently with blow of PBS or with forceps, thereafter they were transferred to microfuge tubes and were fixed with PBS containing 4% paraformaldehyde and 0.1% triton X 100 for 1 hrs at room temperature for 50 APF pupae and for 3 hrs for later staged pupae. Rest of the protocol was similar to larval staining.

2.8 Adult cuticle preparation

After collecting adult flies of particular genotypes they were stored in microfuge tubes in 70% ethanol. Flies were cleared by boiling in 10 % KOH for 15 minutes. They were then sequentially dehydrated in 10%, 50%, 70%, and 100% ethanol and were finally left in clove oil for overnight. The

required body parts were then dissected and mounted in 70% glycerol or clove oil.

2.9 Microscopy

Fluorescence and Confocal microscopy: Fluorescence images were obtained on Zeiss 780 LSM Confocal/Multiphoton microscope, Zeiss LSM 710 Confocal microscope or Zeiss apotome upright microscope. Images were processed using Zen light software, Axiovision 4.8.2 software or NIH Image J. Bright field images of haltere capitellum were obtained with Zeiss apotome upright microscope.

Scanning Electron microscopy (SEM) was carried out on Carl zeiss EVO LS10 Scanning Electron Microscope Zeiss using Axiovision 4.8.2 software to operate the microscope and for image analysis. Fresh samples of flies were cleaned with 70% ethanol and were directly used for imaging.

2.10 Measurements of Surface area of Capitellum/ Compartment/ Clone/ Cell:

Measurements of anterior and posterior compartment ratio

The posterior compartment of imaginal disc was marked by staining it with Engrailed and the whole disc with armadillo or DAPI. The discs were mounted on microscopy slide by keeping spacers so that they do not lose their morphology. Various sections of wing and haltere imaginal discs obtained were stacked together by constructing 3D images. Surface areas of anterior and posterior compartments were measured using NIH Image J. The surface area of compartment was calculated in pixels and then ratio of anterior to posterior compartment was measured.

Surface area of cell

To measure any change in size of cells, wing and haltere imaginal disc were stained with Armadillo and then the surface area occupied by10 cells was calculated in pixels.

Measuring size of AyGAL4 clones in wing and haltere

Various Z-stack of wing and haltere imaginal disc was processed to 3D reconstructions in software of respective confocal microscope. The surface area of clones was outlined using NIH Image J program and the area was estimated in pixels.

Size of adult haltere capitellum

Bright field Images of adult haltere cuticle were taken using Zeiss Apotome microscope and the surface area of capitellum was measured using NIH Image J. Since haltere is a bulbous structure without any major landmarks it was difficult to mount haltere capitellum with similar orientations and shape. Outlines of just the capitellum (the bulbous portion, excluding the stalk) of halteres (10X magnification) were traced by converting them to binary or by setting threshold and the area of capitellum in pixels were estimated using Image J software. Adult haltere bristles counts were performed directly on selected flies of different genotypes.

For all histograms, error bars represent SD and P values were calculated using two-tailed Student's t tests.

Chapter 3

Chapter 3

Insulin/insulin-like Growth Factor Signaling Pathway in Developing Wing and Haltere

Summary

Ultrabithorax (Ubx) modulates various wing patterning genes in developmental-stage specific manner to specify haltere. Ubx expressing haltere cells are fewer in number, smaller in size, have different shape and cellular affinities. Even of decades of research to understand the role of Ubx in haltere and the downstream targets it regulates, our understanding of the molecular mechanism that establish these cellular differences is ambiguous. Insulin/insulin-like growth factor signaling (IIS) pathway is thought to control cell size in response to external cues and thereby organ size in both invertebrates and vertebrates. Cell size in *Drosophila* wing is one of the well-studied systems to understand the role of IIS pathway and to identify its various components. In this chapter, we have investigated the role IIS pathway during haltere development. Here we show that IIS pathway is differentially regulated between wing and haltere. Akt (also called Protein Kinase B, PKB), a central component of IIS signaling pathway, is down regulated in haltere. Miss-expression of components of IIS pathway induces a moderate growth response, both increase in cell size and organ size. Thus, while IIS pathway is an important target of Ubx, modulating this pathway alone is not sufficient to induce major changes in haltere size and shape.

3.1 Introduction

3.1.1 Morphological differences between wing and haltere

Wing and haltere differ dramatically at the whole organ, compartment and cellular levels. At the organ level, the obvious difference between wing and haltere is size. Wing is 2-layered sheet of cells, while haltere is 2-layered globular structure (Fig. 3.1A). Space between two layers of haltere cells consists of a hollow cavity filled with haemocyte (Roch and Akam, 2000). An adult haltere consists of three main parts from proximal to distal, the pedicellum, scabellum and capitellum. The scabellum consists of array of sensory structures called as sensilla campaniformia. The capitellum consists of small bristle like structure called as sensilla trichodea (Fig. 3.1B, E). Haltere lacks the vein, intervein and two rows of marginal bristles present on the dorsal/ventral (D/V) boundary of wing (Fig. 3.1A, B). Each wing cells have 8 fold larger cuticle area than haltere cells (Roch and Akam, 2000). Each wing cell have a single hair while each haltere cells produces 2,3 or 4 hairs which are shorter in length and are thinner in their morphology (Fig. 3.1C, D).

Adult wing and haltere cells have different cell size and shape. The difference between cell size and shape becomes evident at late pupal stages, while the cells of third instar wing and haltere discs are similar in size and shape (Fig. 3.2 and Fig. 3.3). Difference in organization of actin cytoskeleton elements, such as F-actin, also becomes evident at pupal stages (Roch and Akam, 2000).

The third instar wing and haltere imaginal disc differes in terms of number of cells and patterning events. The wing primordium contains ~24 cells and the haltere primordium contains ~12 cells at 9-10h After Egg Laying (AEL). By third instar, a wing imaginal disc has ~50,000 cells and haltere imaginal disc has ~10,000 cells (Held, 2002, cited in Makhijani et al., 2007). At compartment level, the ratio of size between anterior and posterior compartments in the haltere disc is nearly 3:1, while that in the wing disc is 1:1 (Fig. 3.2B, C). The reduced size in the posterior compartment of haltere (and thereby a different shape of the organ) is thought to be due to Ubx-mediated suppression of (Decapentaplegic) Dpp signaling in the posterior

compartment (Crickmore and Mann, 2006; de Navas et al., 2006; Makhijani et al., 2007).

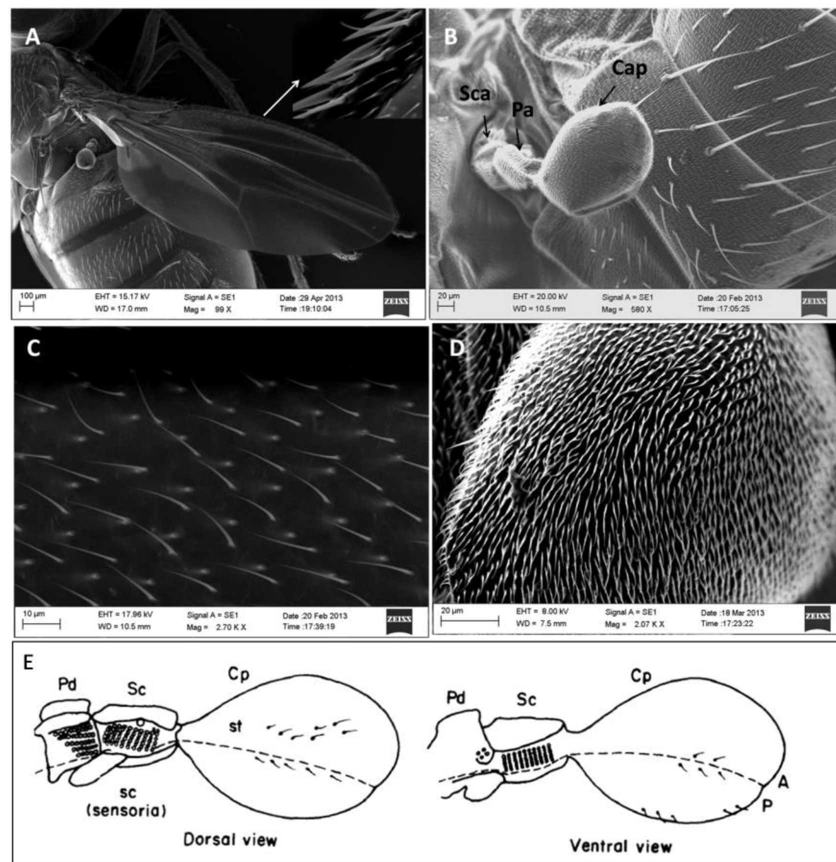


Figure 3.1 Difference between adult wing and haltere

(A) A Scanning electron microscopy (SEM) image of an adult female *Drosophila*. Image represents a wing and a haltere present on thoracic segments T2 and T3, respectively. Compared to wing blade, haltere is highly reduced in size and bulbous in morphology and lack mechanosensory bristles of the wing margin (arrow points to higher magnification image of wing margin).

(B) A higher magnification of an adult haltere. Capitellum (Cap), Scabellum (Sca), Pedicellum(Pa) are different parts of haltere.

(C) A higher magnification image of wing blade showing arrangement and morphology of trichomes. Wing blade is a bilayer structure. The two layers are tightly opposed to each other such that in this SEM image of dorsal surface, trichomes of ventral surface too are visible (arrow).

(D) A higher magnification image of haltere showing highly dense arrangement of trichomes compared to wing (C). Haltere too is made of two layers of epithelium, but are not tightly adhered to each other.

(E) Figure adapted from González-Gaitán et al., 1990 Morphology of the adult haltere of wild type flies showing their dorsal and ventral view. Dashed line separates the A (anterior) and P (posterior) compartments. Pd- pedicellum; Sc- scabellum; Cp- capitellum; sc- sensilla campamiformia; st- sensilla trichodea.

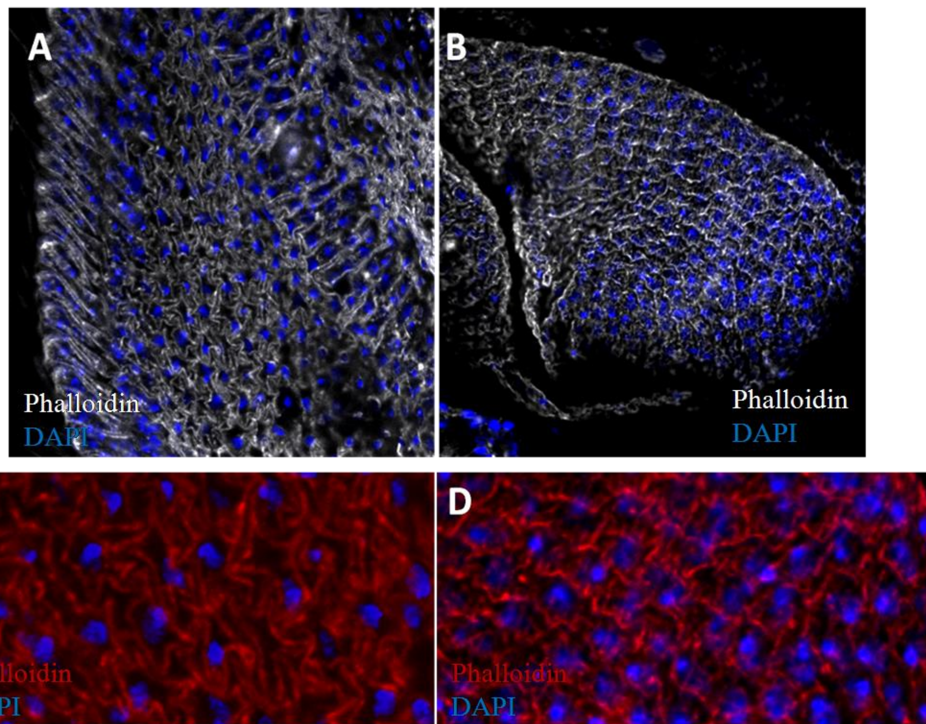


Figure 3.2 Difference in size and shape of cells becomes evident at pupal stage
Confocal image of wing and haltere at 48-52 hrs after pupal formation (APF).(A) wing and (B) haltere disc stained with phalloidin and DAPI (blue), (C) and (D) are the same imaginal discs (A and B, respectively) at higher magnification. The wing cells are star shaped while haltere cells are cuboidal.

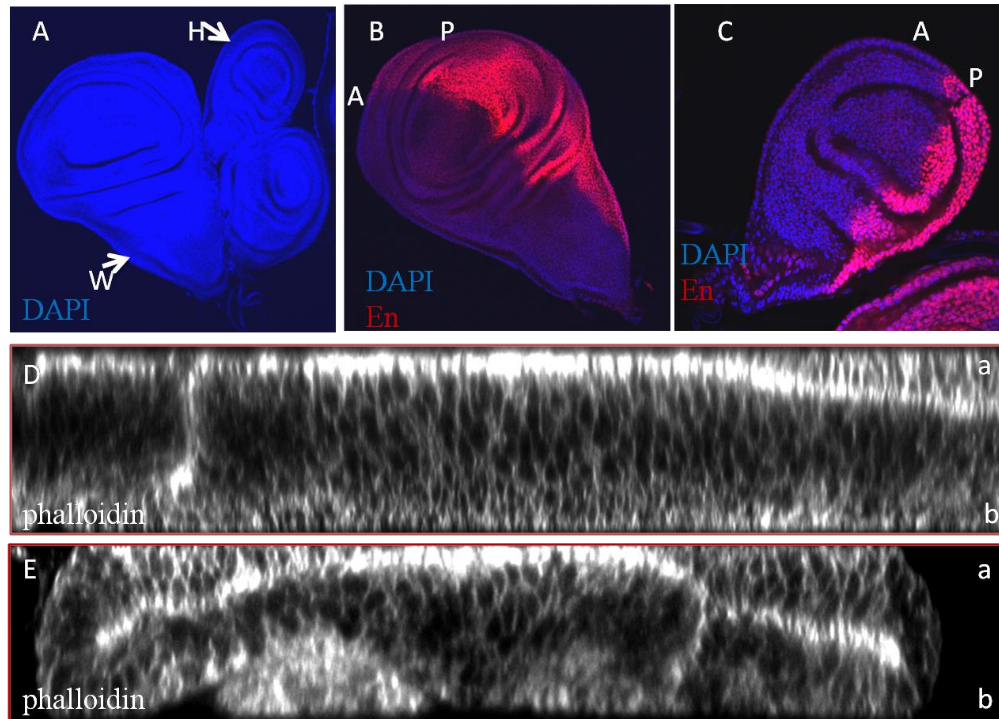


Figure 3.3 Comparison of wing and haltere discs at third instar larval stage (A)Third instar wing (W) and haltere (H) imaginal discs, Haltere disc is similar to wing in structure but is much smaller. (B) Third instar wing and (C) haltere imaginal discs stained with DAPI (blue) and anti-Engrailed (En) antibodies (red) to mark the posterior compartment. The posterior compartment of haltere disc is much reduced in size compared to that in the wing disc. (D) An X-Z section of third instar wing and (E) haltere imaginal discs stained with phalloidin, showing similarity in size and shape of cells. A (anterior), P (posterior), a (apical) and b (basal).

3.1.2 Ultrabithorax and growth control in haltere

To understand the cell-autonomy of growth control in haltere, we induced mitotic clones for an *Ubx* null allele, $Ubx^{6.28}$, using FLP/FRT system. We induced $Ubx^{6.28}$clones of different sizes in haltere by giving heat shock at different stages of development and compared their size with corresponding twin spots. Smaller clones were similar in size as compared to their twin spots (Fig. 3.4A). However, larger clones were larger than their twin spots (Fig. 3.4B). Although we did not observe any induction of growth in Ubx^{+}/Ubx^{+} cells by the neighboring $Ubx^{6.28}$ cells, these observations suggest that unless a critical number of cells are Ubx^{-}, they do not show wing-type growth pattern. This is possible if growth signals secreted by these cells attain a threshold level to suppress Ubx mediated growth in the haltere.

Dpp, Wingless (Wg), epidermal growth factor receptor (EGFR) are some of the major growth and pattern regulating pathways, which are repressed in haltere discs by Ubx (Shashidhara et al., 1999; Mohit et al., 2003, 2006; de Navas et al., 2006; Pallavi et al., 2006; Makhijani et al., 2007). Interestingly, over-expression of pathway components such as Dpp, Wg, Vestigial (Vg) and vein (vn) induce only marginal growth in developing haltere. It is possible that Ubx specifies haltere size by regulating the function of certain organ-size determining pathways.

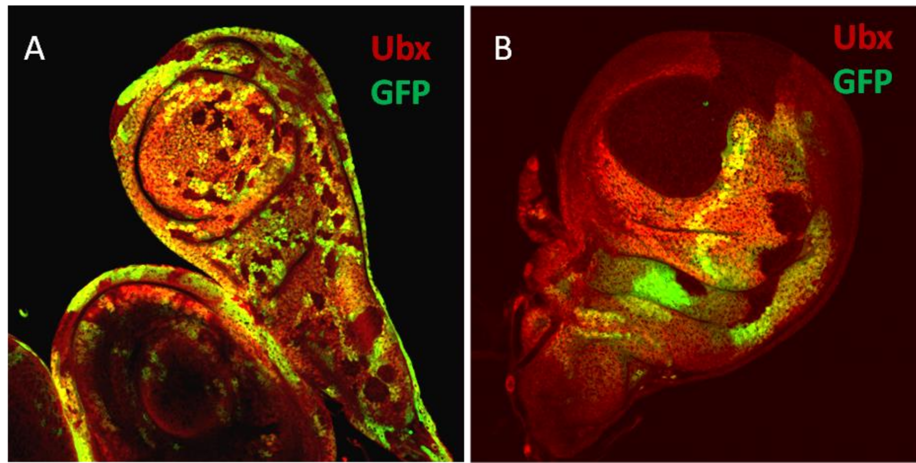


Figure 3.4 Removal of Ubx does not give any growth advantage to individual cells

Confocal images of developing third instar haltere imaginal disc with mitotic clones of *$Ubx^{6.28}$*allele. In order to get clones of different size, Clones were induced at different stages of development using FLP/FRT system. (A) Haltere disc with small *$Ubx^{6.28}/Ubx^{6.28}$*null clones, which are similar in size compared to its *Ubx^{+}/Ubx^{+}* twin spots, which express both GFP and Ubx. (B) A single large *$Ubx^{6.28}/Ubx^{6.28}$* null clone is much larger in size compared to its twin spot. *Ubx^{-}* clones are marked by loss of expression of GFP and Ubx, while their twin spots show higher intensity of GFP and Ubx compared to the background.

3.1.3 The Insulin /insulin-like growth factor signaling (IIS) pathway

The major difference between wing and haltere is their whole organ size.The IIS pathway regulates the rates of cell growth, nutrient use, cell size and body size in both flies and in mammals (Garofalo, 2002; Edgar, 2006). The role of

Insulin signalling pathway components in regulating cell size and cell number during wing development is relatively well studied.

IIS pathway functions are highly conserved across species but the number of insulin-like peptides (ILPs) varies greatly between species. *Drosophila* produces eight ILPs in the central nervous system, gut, imaginal discs and fat body (Kannan and Fridell, 2013). The upstream components of this signaling pathway like, CHICO, the lipid phosphatase PTEN and Class I(A) phosphoinositide 3-kinases (PI 3-kinases), controls both the cell size and cell number (Leevers et al., 1996; Bohni et al., 1999; Weinkove et al., 1999; Goberdhan et al., 1999). Further downstream, ILPs activates a single insulin receptor (InR) in the target tissue. Once activated, InR recruits Chico an adaptor protein, *Drosophila* homolog of Insulin receptor substrates (IRS), and PI3kinase to the plasma membrane (Leevers et al., 1996; Bohini et al., 1999). Further downstream, InR activates Akt through phosphokinase signal transductuction.

Akt is the central downstream effecter molecule of IIS signaling. In response to IIS signaling, Akt through series of downstream targets regulates the organ growth. Akt is activated by PDK1 and Target of rapamycin (TOR) (Alessi et al., 1997; Sarbassov et al., 2005, cited in Ye et al 2012). There is a fine tuned feedback network between the components of this signaling pathway (Edgar, 2006). The negative growth regulator of this signaling pathway consists of forkhead-related transcription factor (FOXO) and Tuberous Sclerosis Complex 1/2(TSC1/TSC2). TSC1/TSC 2 negatively and positively regulates the TOR complex 1 (TORC1) and TOR complex 2 (TORC2) activity respectively (Yang et al., 2006). The small GTPase Ras homologue enriched in brain (Rheb) functions downstream to TSC1/TSC2 and activates ribosomal S6 kinase (S6K) controlling the protein machinery (Dufner and Thomas, 1999; Stocker et al., 2003). Activated TOR Phosphorylates the eukaryotic initiation factor 4E-binding protein (4EBP) (Lawrence and Abraham, 1997). 4EBP is also regulated by the FOXO transcription factor (Tettweiler et al., 2005).

Many components of this signaling pathway like Akt, S6K, FOXO, InR and PTEN are potential targets of Ubx (Mohit et al., 2006; Choo et al., 2011; Agarwal et al., 2011). Our aim in this direction was to investigate how Ubx-mediated regulation of Akt pathway influences this pathway in haltere development

3. 2 Results

3.2.1 Differential regulation of Akt between wing and haltere

Akt is the central component of IIS signaling pathway that positively regulates tissue growth in *Drosophila* (Verdu et al., 1999). Genome-wide studies have suggested that *akt* is a target of Ubx (Mohit et al., 2006; Choo et al., 2011; Agarwal et al., 2011). To further understand the regulation of Akt by Ubx, we examined its expression pattern in third instar wing and haltere imaginal discs. Antibody staining for P-Akt in wing and haltere imaginal disc demonstrated only a subtle difference in level. (Fig. 3.5A, A'). Firstly, the haltere cells showed lower levels of P-Akt and it was restricted to only in the haltere pouch, whereas wing discs show P-Akt in both the pouch and the notum.

This difference was further confirmed within same imaginal disc when we compared anterior and posterior compartments of *pbx/DfUbx*^{109} haltere, in which the posterior compartment is transformed to that of wing-type. The non-transformed Ubx expressing anterior compartment of haltere showed lower levels of P-Akt in the pouch region, transformed posterior compartment not only showed increased levels, we observed P-Akt level in the notum (Fig. 3.5B-B''). Quantitative measurement of intensity of staining further confirmed difference in the levels of level of P-Akt between Ubx expressing and non-expressing compartments (Fig. 3.5C, C') and also as compared to the expression in wild type discs (Fig. 3.5A').

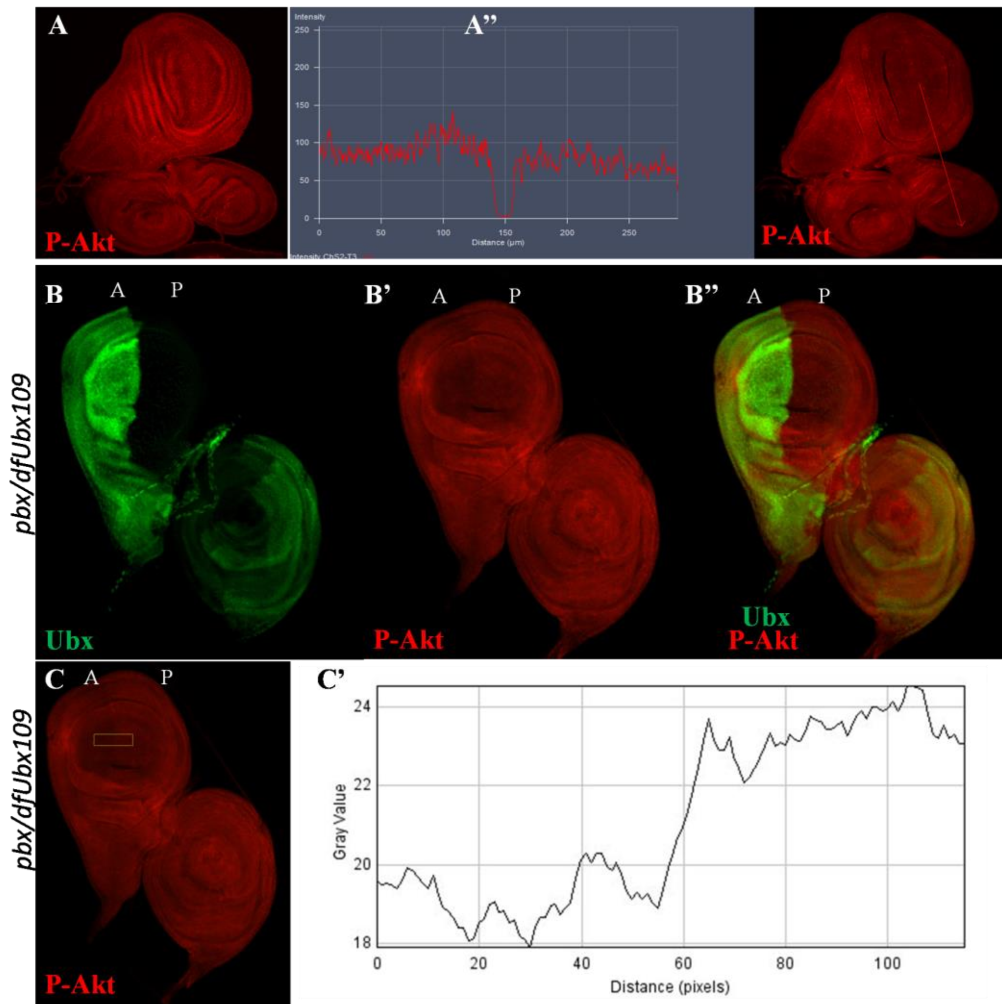


Figure 3.5 dAkt is differentially regulated between wing and haltere

(A) Wild type wing, haltere and leg imaginal discs stained with P-Akt. (A') Intensity graph of wing and haltere imaginal discs stained with P-Akt473. Arrow marks the region selected for quantitative estimation of P-Akt levels. Please note the level of P-Akt in the haltere disc is marginally lower than in the wing disc. (B) Third instar haltere and leg imaginal discs of *pbx/dfUbx109* genotype stained with Ubx (green) and P-Akt (red). Please note these discs do not express Ubx in the posterior compartment. The transformed posterior compartment shows increased P-Akt in the pouch region. (C) Small region of haltere imaginal disc selected for intensity measurement (C') Intensity graph plotted using image J for selected region of haltere imaginal disc depicting quantitative difference in level of P-Akt..

3.2.2 Wing and haltere discs respond differently to over-expression of Akt

Based on above result we next examined the effect of increasing the expression of Akt. We over-expressed Akt using two pouch-specific GAL4 drivers: *omb*-GAL4 and *Ubx*-GAL4 drivers (Fig. 3.6A-A'', B-B'' and C-C'') and examined its effect on the cell size in third instar wing and haltere imaginal discs. Surface area of cells was estimated by calculating area occupied by 10 cells. There is no difference in the area occupied by 10 cells between wild type wing and haltere imaginal discs indicating similar sized cells (Fig. 3.6D, E). o*mb*-GAL4;UAS-GFP wing and haltere cells too were similar in size (Fig. 3.6D). A significant increase in area occupied by 10 cells was observed in *omb*-GAL4; UAS-Akt wing imaginal disc as compared to *omb*-GAL4;UAS-GFP (Fig. 3.6D). Thus, consistent with previous findings, over-expression of Akt resulted in increase in cell size. However, *omb*-GAL4;UAS-Akt haltere imaginal discs caused no such significant change in cell size (Fig. 3.6D).

It was possible that *omb*-GAL4 driver is not strong enough to induce a phenotype in the haltere disc. We therefore employed *Ubx*-GAL4 driver to over-express Akt. *Ubx*-GAL4, also being an allele of *Ubx*, provides a sensitized background for inducing changes in haltere development (Pallavi and Shashidhara, 2003). Indeed, *Ubx*-GAL4/UAS-GFP third instar haltere imaginal discs had increased, although marginally, cell size as compared to wild type haltere imaginal discs (Fig. 3.6E). However, we did not observe any difference in cell size in UAS-Akt;*Ubx*-GAL4 haltere discs (Fig. 3.6E), suggesting that haltere cells are resistant to any growth-promoting activity of Akt. This could be due to Ubx-mediated down regulation of multiple steps downstream of Akt in this pathway.

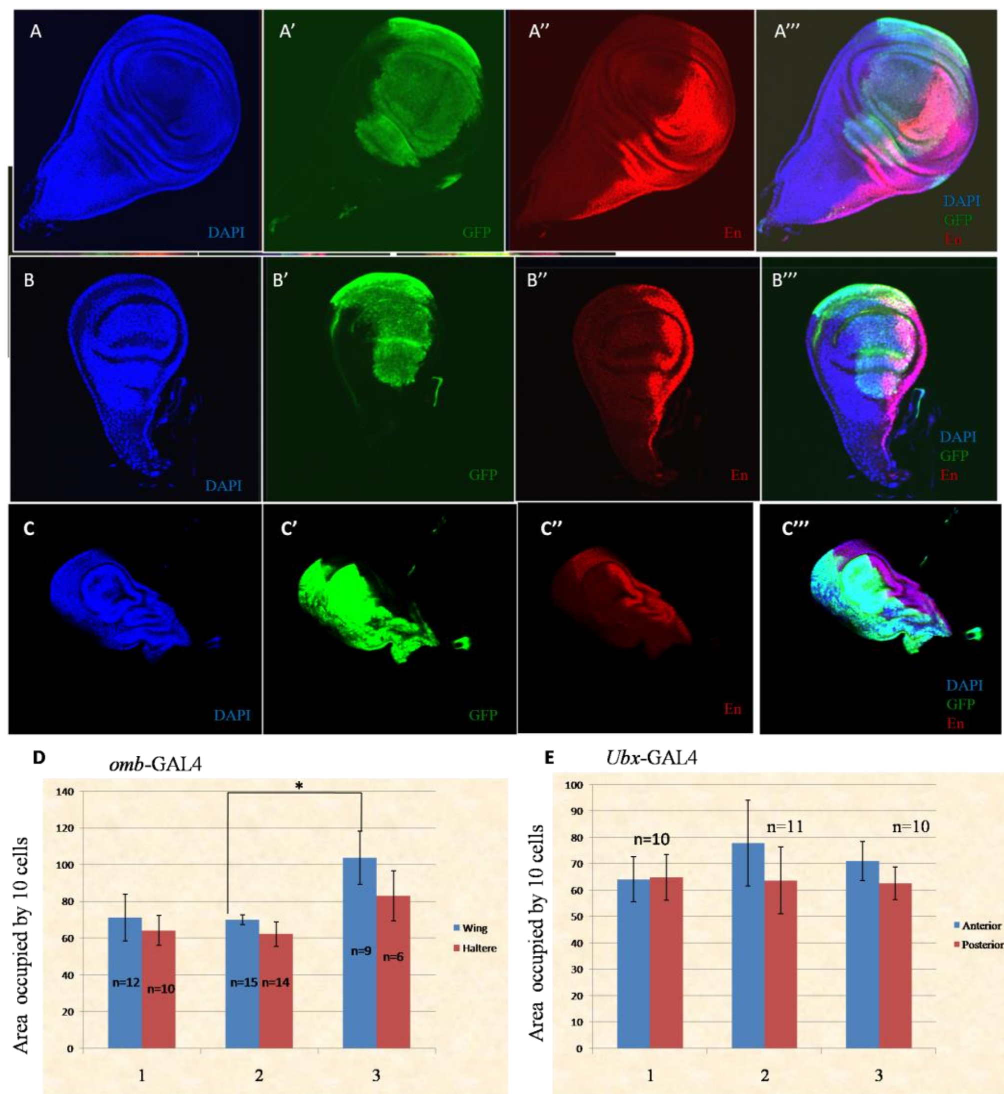


Figure 3.6 Effect of over-expression of Akt

(A-A''') Expression pattern of *omb*-GAL4/+;UAS-GFP in wing and (B-B''') haltere imaginal discs. (C-C''') expression pattern of *Ubx*-GAL4/UAS-GFP in the haltere imaginal disc. All discs in A-C are stained with DAPI (blue), En (red), GFP (green).

(D) Effect of *omb*-GAL4 driven Akt over-expression in wing and haltere discs. Genotypes are: 1. CS, 2. *omb*-GAL4/+;UAS-GFP, and 3.*omb*-GAL4/+;UAS-Akt. Note increase in cell size (determined by measuring the area of occupied by 10 cells within the *omb*-GAL4 expressing region) in wing discs over-expressing Akt, while haltere discs did not induce this phenotype. . Confocal images were processed using Image J for measurements.

(E) Genotypes are: 1. CS, 2 *Ubx*-GAL4/UAS-GFP, 3 UAS-Akt; *Ubx*-GAL4. Over-expression of Akt using *Ubx*-GAL4 driver cause no effect on cell size in haltere discs. *P =< 0.001

3.2.3 Effect of over-expression of components Akt pathway on compartmental size

In wing discs, anterior and posterior compartments are of similar size. Posterior compartment of haltere is highly reduced in size compared to the anterior compartment. Modulation of Dpp and its pathway component by Ubx is responsible for this phenomenon (Crickmore and Mann, 2006; de Navas et al., 2007; Makhijani et al., 2007). Miss-expression of *dally* and mother of thick-vein (*mtv)*, components downstream pathway of Dpp, using posterior specific GAL4 drivers induce moderate increase in size of the posterior compartment (de Navas et al., 2007; Makhijani et al., 2007), but neither of them is able to restore the A/P ratio to approximately 1:1.

Modulation of components of IIS signaling pathway is known to cause changes in compartment size in an autonomous manner without altering the size of other compartment (Garofalo, 2002). We, therefore, examined the effect of over-expression of various components of IIS pathway on the ratio of the size of anterior and posterior (A/P) compartments. We used the posterior-specific GAL4 drivers, *en*-GAL4 and *hh*-GAL4. The A/P ratio for *en*-GAL4/+ and *hh*-GAL4/+ wing discs is 1.18:1 and 1.14:1. The anterior compartment of wing disc was found to be slightly larger in size compared to its posterior compartment. A/P ratio for *en*-GAL4/+ and *hh*-GAL4/+ haltere discs is 2.4:1 and 2.45:1. Expression of UAS-Akt in third instar wing and haltere imaginal discs using *en*-GAL4s caused moderate decrease in A/P ratio (0.99:1 wing and 2:1 haltere), while there was no phenotype with *hh*-GAL4. UAS-Rheb and UAS-*tsc1*^{RNAi} with *en*-GAL4 and *hh*-GAL4 drivers resulted in stronger effect on the A/P ratio in haltere discs (*en*-GAL4 ;UAS-Rheb, 1.64:1, *en*-GAL4;UAS-*tsc1*^{RNAi} 1.68 and *hh*-GAL4;UAS-Rheb, 1.67:1 and *hh*-GAL4;UAS-*tsc1*^{RNAi} ,1.88:1) compared to wing discs (*en*-GAL4;UAS-Rheb, 1.04:1, *en*-GAL4;UAS-*tsc1*^{RNAi} 1.11:1 and *hh*-GAL4;UAS-Rheb,1.06:1, *hh*-GAL4;UAS-*tsc1*^{RNAi,}1.11:1) (Fig. 3.7). All the results were normalized with *en*-GAL4/+ or *hh*-GAL4/+, as appropriate, as control. Thus, posterior compartment of haltere discs causes stronger growth response to the

modulation of expression of components downstream to Akt in the IIS pathway.

As TSC1 is known to affect cell size, we measured the effect of down regulation of *tsc1* on cell size. Expression of UAS-*tsc1*^{RNAi} with *en*-GAL4 resulted in larger haltere cells compared to wing cells (Fig. 3.8). It is therefore likely that the reason for poor response by haltere cells to the over-expression of Akt is due to the effect of Ubx on components downstream of Akt and upstream of TSC1.

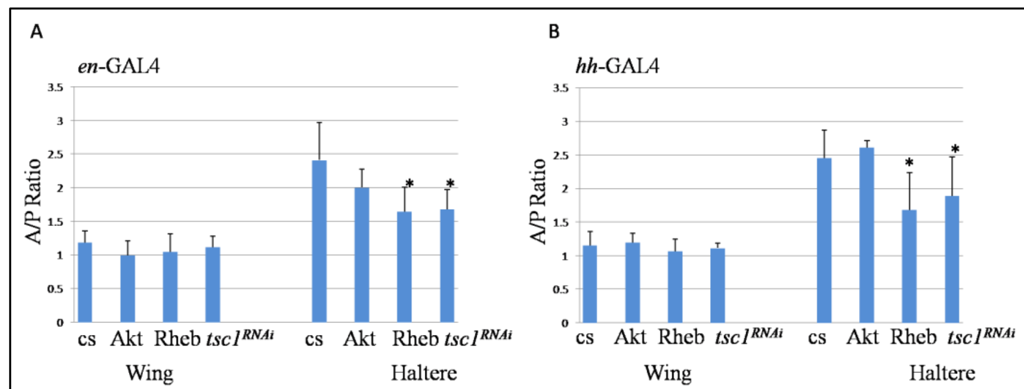


Figure 3.7 Effect of modulation of expression of various components of IIS pathway on the ratio of A/P compartment

Y-axis of both the Graphs represents anterior/posterior (A/P) compartment ratio. w=wing and h=haltere, *en*-GAL4 and *hh*-GAL4 drives expression of UAS transgenes in the Posterior compartment.

A) A/P compartment ratio of third instar wing and haltere imaginal discs of genotype: *en*-GAL4/+ (w,n=16; h,n=8) as control, *en*-GAL4/UAS-Akt (w,n=6; h,n=6), *en*-GAL4;UAS-Rheb(w,n=6; h,n=6) ,*en*-GAL4;UAS-*tsc1*^{RNAi} (w,n=8; h,n=4). A moderate decrease in A/P ratio is observed with *en*-GAL4/UAS-Akt in wing, while *en*-GAL4/UAS-Rheb, *en*-GAL4;UAS-*tsc1*^{RNAi} show significant decrease in A/P ratio only in haltere discs.

B) A/P compartment ratio of third instar wing and haltere imaginal discs of *hh*-GAL4/+(w,n=7; h,n=6) as control, UAS-Akt;*hh*-GAL4 (w,n=3; h,n=3),*hh*-GAL4/UAS-Rheb (w,n=8; h,n=10), *hh*-GAL4/UAS-*tsc1*^{RNAi}(w,n=6; h,n=7). No change in A/P ratio was observed in both wing and haltere discs when Akt was over-expressed, while *en*-GAL4; UAS-Rheb, *en*-GAL4/UAS-*tsc1*^{RNAi} have significant decrease in A/P ratio only in haltere discs. *P =< 0.005

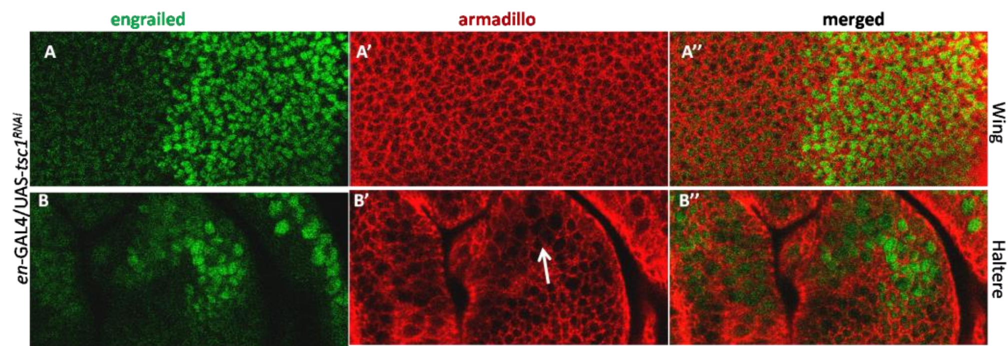


Figure 3.8 Increase in cell size in haltere discs in response to the modulation of the expression of various components of IIS pathway

(A-B) *en*-GAL4/UAS-*tsc1*^{RNAi} wing (A-A'') and haltere (B-B'') imaginal discs. The discs stained with Engrailed (Green) to mark the posterior compartment and armadillo (red) to mark cell outline. Note arrow in B' shows increase in size of cells compared to its anterior compartment. No such phenotype was observed in wing discs.

3.2.4 Effect of modulation of expression of components of IIS pathway on adult haltere

As shown above, expression of UAS-*tsc1*^{RNAi} and UAS-Rheb, but not Akt, induced considerable growth phenotype in haltere discs. Next, we examined the effect at the adult level. Over-expression of Akt with *omb*-GAL4 did not cause any growth phenotype or any transformation of haltere capitellum. We also used *Ubx*-GAL4, a sensitized background for Ubx as a driver (Pallavi and Shashidhara 2003). *Ubx*-GAL4 capitellum is slightly larger in size with 4 to 6 wing-type sensory bristles. Over-expression of Akt using *Ubx*-GAL4 too did not induce any growth phenotype in the adult haltere capitellum (Fig. 3.9B, G). Down regulation of *tsc1* or over-expression of Rheb in haltere using *Ubx*-GAL4, however, resulted in moderate increase in the size of the adult haltere capitellum (Fig. 3.9C, D and G). As Ubx likely to regulate Akt pathway at multiple levels, we modulated the expression of more than one gene at a time. UAS-Akt;UAS-*tsc1*^{RNAi} and UAS-Akt;UAS-Rheb with *Ubx*-GAL4 showed phenotypes similar to *tsc1*^{RNAi} or UAS-Rheb alone (Fig. 3.9E,F and G). In all these experiments, we did not observe any change in the number of sensory bristles (Fig. 3.9).

Expression of Upstream components of IIS pathway such as UAS-*PI3K*^{RNAi} and UAS-Dp110, which regulate both cell size and number, did not cause any increase in the size of the adult haltere capitellum (data not shown). Unfortunately, unlike wing trichomes where a single hair represents a single cell, trichomes on haltere blade are not placed uniformly, making it difficult to score the size of individual cells at adult stage. Taken together, it may be inferred that IIS pathway is tightly regulated by Ubx during haltere development to specify haltere size.

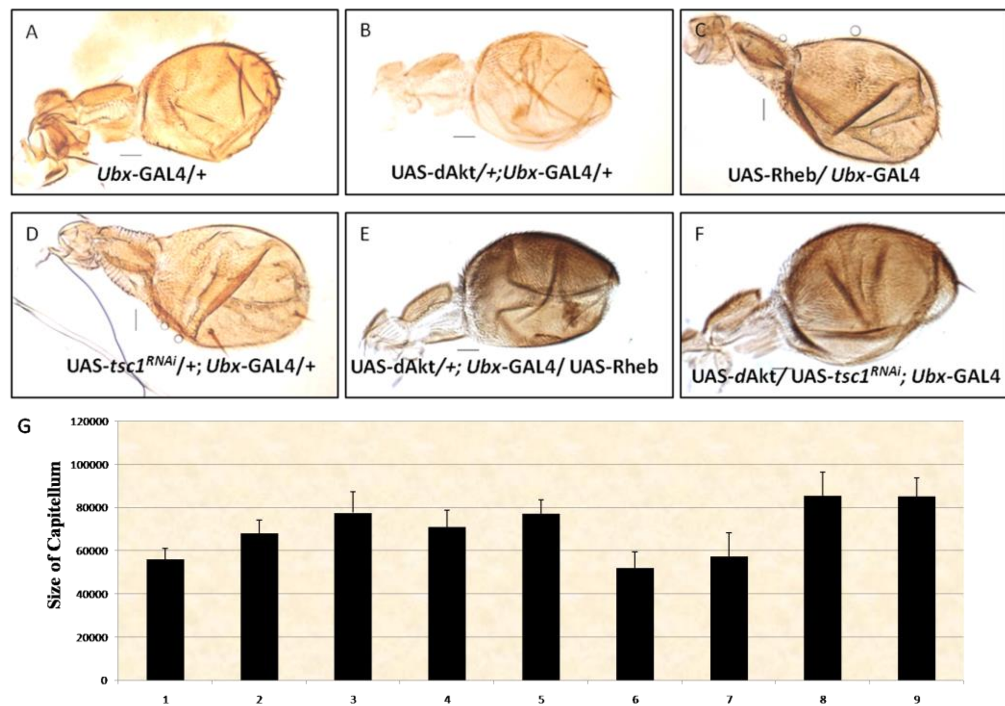


Figure 3.9 Phenotypes at the levels of adult haltere in response to the change in the expression of components of IIS pathway

(A, B, C, D, E, F) Representative halters of genotype as shown on images. All Images are of similar magnification. Note visible increase in haltere size in D, E and F. (G) Graph showing the size of the adult haltere of capitellum of genotypes: 1. *Ubx*-GAL4/+ (n= 20) as control, 2. UAS-Akt*;Ubx*-GAL4, (n=11), 3. *Ubx*-GAL4/UAS-Rheb, (n=11), 4. *Ubx*-GAL4/UAS-*tsc1*^{RNAi} (line1) (n=11), 5. *Ubx*-GAL4/UAS-*tsc1*^{RNAi} (line2) (n=11), 6. *Ubx*-GAL4/UAS-foxo^{RNAi}(N=11) 7.*Ubx*-GAL4/UAS-Thor (N=7), 8. UAS-Akt*;Ubx*-GAL4/UAS-Rheb (n=20), 9. UAS-Akt*;Ubx*-GAL4/UAS-*tsc1*^{RNAi} (n=20). Area of the capitellum was measured using Image J software and was normalized against *Ubx*-GAL, which was used as control.

3.2.5 Targets of IIS pathway are not differentially expressed between wing and haltere

Components downstream to Akt such as *rheb* and *s6k* are potential targets of Ubx. As described above, over-expression of Rheb induces increased growth in the haltere disc. Antibody staining for Rheb, 4EBP and S6k, targets of IIS/Akt pathway, did not show any differential expression between wing and haltere imaginal discs (data not shown). We also carefully examined the expression pattern of those components inducing $Ubx^{6.28}/Ubx^{6.28}$ null clones in haltere. Expression of Rheb, 4EBP and S6k was not different in cells within the $Ubx^{6.28}/Ubx^{6.28}$clones and outside the clones (Fig. 3.10). This suggests that while Akt itself is regulated by Ubx and may be a limiting factor, Ubx may be regulating other downstream components of the IIS pathway, which are not studied here.

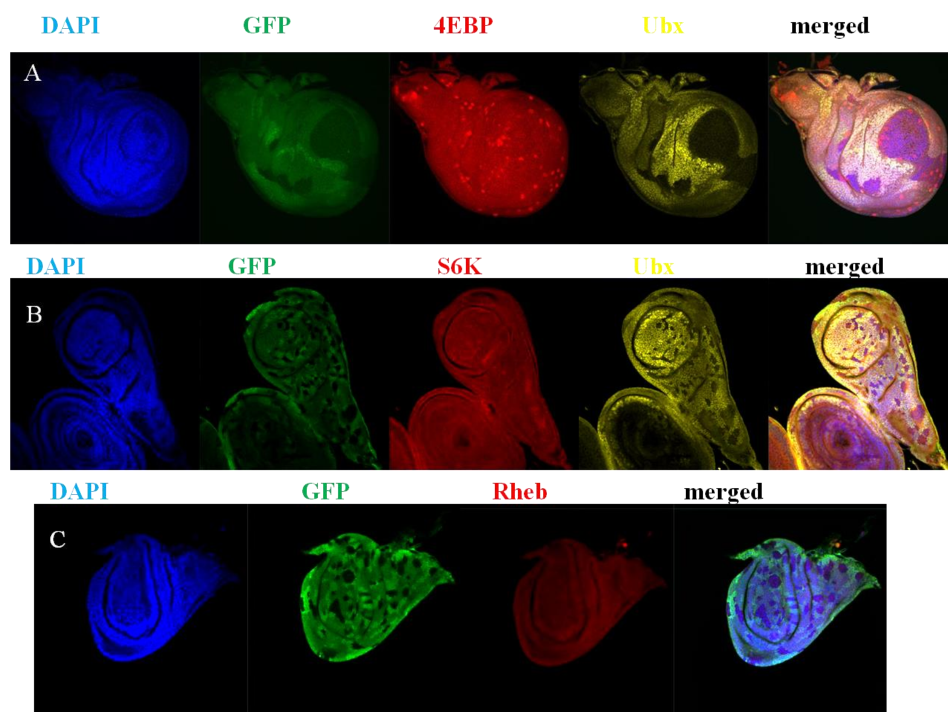


Figure 3.10 Ubx does not regulate the expression of Rheb, 4EBP and S6k
Confocal images of third instar haltere imaginal discs with $Ubx^{6.28}/Ubx^{6.28}$clones showing no difference in expression pattern of 4EBP (A), S6K (B), Rheb (C) between *Ubx* null clones to their respective twin spots and wild type cells.

3.3 Discussion

IIS regulates the size of organ by promoting both cell growth and proliferation. Components of IIS signaling pathways are known to regulate growth in a cell autonomous manner (Garofalo et al., 2002). Here we examined to what extent this pathways mediates the function of Ubx to specify the size of haltere.

Akt is the central component of the pathway, which is differentially expressed between wing and haltere imaginal discs (Fig. 3.5). Its expression in the haltere pouch is lower compared to that of wing discs (Fig. 3.5). We therefore studied the effect of over-expression of Akt on the growth of wing and haltere discs. We observed increase in cell size in wing discs, but not in haltere discs (Fig. 3.6). This suggests that haltere is resistant to any changes in growth patterns, probably due to Ubx-mediated regulation of additional components of IIS pathway. Consistent with this, when we down regulated *tsc1*, we observed increase in cell size in haltere discs (Fig. 3.8). More interestingly, we observed better response by haltere discs at the level of ratio of A/P compartment size compared to wing discs (Fig. 3.7). This indicates that wing disc may have reached saturation in terms of its size (and relative sizes of the two compartments).

Reason for absence of any haltere phenotype when Akt is over-expressed could be due to the fact that Ubx down regulates downstream components. However, S6K, Rheb and 4EBP are not regulated by Ubx during haltere development, although over-expression of Rheb causes increased cell size in the haltere. Phosphorylation of TSC1 and TSC2 by Akt is not required by Akt to drive tissue growth in *Drosophila* (Dong and Pan, 2004; Schleich and Teleman, 2009). Furthermore, IIS does not activate TORC1 in most tissue under normal physiological conditions in *Drosophila* (Radimerski et al., 2002; Pallares-Cartes et al., 2012). Thus, it is possible that some other targets of Akt responsible for its function vis-à-vis growth are under the regulation of Ubx.

Wing and haltere imaginal disc have same division rate during the last two larval instars and the difference in number of cells are because of differences in number of founder cells at early stages of development. They have similar growth parameters in terms of cell size and their proliferation rate

at third instar. Presence of both wing and haltere territories (by inducing mitotic clones for a null allele of *Ubx*) in the same imaginal disc induces a mutual interferences in cell proliferation between wing and haltere disc (Gonzalez-Gaitan et al., 1990). However, no difference in size of cells has been observed between wing and haltere territories. This suggests that size of haltere disc is predominantly determined at the level of cell number and not cell size. However, the differences in cell number between the two organs (5 times) do not fully represent the differences in their adult size (more than 8 times). This is explained by the fact that adult wing cells are more flattened with larger surface area compared to adult haltere cells. It is therefore, pertinent to study how Ubx specifies size of adult haltere during pupal development (further discussed in next chapters).

Cell proliferation and cell growth are co-ordinated events. TSC1 has a role in controlling both cell size and number (Potter et al., 2001). Relative increase in the size of cells by expression of UAS-$tsc1^{RNAi}$ was more in haltere discs as compared to wing discs (Fig. 3.8). As we did not observe corresponding increase in the size of the capitellum (Fig. 3.9), it is possible that there is reduction in cell number, as expected when *tsc1* is down regulated. In other words, increase in cell size could be due to reduction in cell number. It can be further inferred that down regulation of *tsc1* has more severe effect on cell proliferation in haltere discs than in wing discs. This could be due to the fact that wing disc has already attained a size, which is controlled by the IIS and other pathways. Any change to this size may need more drastic alternation to the controlling mechanism. Haltere, however, may not be subjected to that kind of organ size-controlling molecular mechanism due to Ubx and hence is relatively more sensitive. However, in absolute terms, haltere too is resistant to changes in growth control due to regulation by Ubx at multiple levels. Difference between wing and haltere cells surface area becomes evident at pupal stages but most of the growth and patterning event required to show these differences are laid before puparium formation. Therefore small events like differential growth response by IIS components can provide haltere a differential growth response at pupal stage.

Nevertheless, above data indicates that Ubx regulates a distinct mechanism by which IIS is regulated differentially between wing and haltere (Fig. 3.11). IIS/Akt signaling drives its cell proliferation via Hippo pathway (Straßburger et al., 2012; Ye et al., 2012). Decoupling of growth and proliferation observed in haltere by IIS signaling can in-part be explained by down regulation of Hippo pathway (discussed in next chapters).

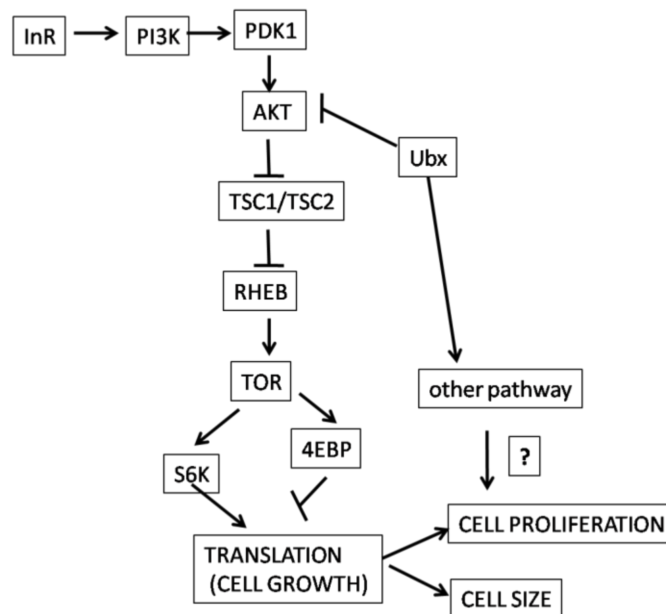


Figure 3.11 Schematic diagram illustrating the regulation and function of IIS pathway in haltere

Chapter 4

Chapter 4

Ultrabithorax, Hippo Pathway and Haltere Specification

Summary

Recent studies have shown that Hippo pathway has multiple roles in cell proliferation, cell size, apoptosis, regeneration, differentiation and determining the size of an organ. While multiple components of this pathway are regulated in tissue/organ-specific manner, sub-cellular localization of Yorkie (Yki) is key to determine if the pathway is in activated or repressed state. In the presence of active components of Hippo pathway, Yki is cytoplasmic. Yki becomes nuclear when one or more components of the pathway are repressed. When localized to the nucleus, Yki functions as a transcriptional co-activator and activates many downstream effectors to determine the output of the pathway. Studies to identify targets of (Ultrabithorax) Ubx in haltere have reported that multiple components of Hippo pathway are potential direct or indirect targets of Ubx. In this context, our aim was to understand how this pathway is differentially regulated between wing and haltere and the implication of the same for the development and specification of the haltere. Here we show that RNAi-mediated knock-down of tumour suppressor genes of the pathway (*fat, hippo, expanded* and *ds*) and over-expression of Yki cause increased growth in the haltere. Interestingly, nuclear levels of Yki are much higher in haltere discs than in wing discs. Furthermore, over-expression of Yki enhanced Ubx-induced wing-to-haltere transformations. These results suggest that, regulation of Yki in haltere discs may have a different mechanism than in wing discs and Yki may cooperate with Ubx to specify haltere fate.

4.1 Introduction

4.1.1 The Hippo pathway

The Hippo pathway is one of the major organ-size-controlling pathways. Recent studies have shown roles of this pathway in growth, apoptosis, proliferation, differentiation and regeneration (For reviews, see Liu et al., 2012; Hiemer and Varelas, 2013). Hippo pathway also plays a crucial role in stem cell self-renewal and in the maintenance of genomic stability. In addition, this pathway has unique capacity to sense various aspects of tissue architecture, such as cell polarity and mechanical tensions imposed by the surrounding microenvironment, and there by control cell size and shape. Many components of this pathway are tumour suppressors and are known to be deregulated in many types of cancer. (For reviews, see Pan, 2010; Bao et al., 2011; Harvey et al., 2013; Nishio et al., 2013).

Hippo pathway is known to be regulated in an organ specific manner. They differ in tissues in terms of which of the pathway components are required in a given tissue/organ and the biological outcome of the pathway activity. In *Drosophila*, for example, requirement of Hippo components differ between imaginal discs and ovarian follicle cells (Polesello and Tapon, 2007). Components of this pathway are also regulated in developmental stage-specific manner (Maitra et al., 2006; Fang and Adler, 2010; Reddy and Irvine, 2011).

Most of the Hippo pathway components are highly conserved across species and can be categorized into the central kinase cascade, upstream regulatory inputs and multiple transcriptional outputs (For reviews, See Halder and Johnson, 2011; Staley and Irvine, 2012; Boggiano and Fehon, 2012; Schroeder and Halder, 2012;Yu and Guan, 2013).

4.1.2 The central kinase cascade

The central Kinase cascade components of Hippo pathway consists of (i) Ste20-like kinase Hippo (Hpo), (ii) nuclear Dbf2-related (NDR) family Kinase, Warts (Wts), (iii) WW domain containing adaptor protein Savador (Sav) (iv) mob as tumour suppressor (Mats) and (v) Tao1. All these components are tumour suppressors and were first identified in *Drosophila* by

genetic screens. Hpo kinase once activated binds to Sav, which in turn phosphorylates and activates the Wts kinase. Wts along with Mats phosphorylates the downstream transcriptional co-activator Yorkie (Yki). Recently, one more component Tao1 has been added to kinase cascade and functions together with Hpo to regulate the Wts activity. Through these sequential phosphorylation events, Yki is sequestered to the cytoplasm and therefore, would be unavailable for its normal function as a transcriptional co-activator (Justice et al., 1995; Xu et al., 1995; Tapon et al., 2002; Harvey et al., 2003; Jia et al., 2003; Lai et al., 2005; Poon et al., 2011).

4.1.3 Multiple upstream regulators

Hippo pathway is regulated by multiple upstream regulatory branches such as the Merlin (Mer)-Expanded (Ex)-Kibra complex, Fat (Ft) and Dachous (Ds), Crumbs (Crb), and Echinoid (Ed).

The first upstream regulators to be linked to core Hippo kinase are two Ezrin-Radixin-Moesin (ERM) family members, Ex and Mer. *ex* and *mer* were independently identified as tumour suppressors and are partially redundant, as mutations in either gene cause tissue growth through increased Yki activation, but together they show a stronger phenotype (Boedigheimer and Laughon, 1993; McCartney et al., 2000; Maitra et al., 2006). The role of Ex and Mer varies between different tissues, as mutation of *ex* alone shows stronger phenotype than mutation of *mer* alone during wing development, but not in other tissues such as ovary (McCartney et al., 2000; Polesello and Tapon, 2007).Their requirement also differ between larval and pupal stages (Milton et al., 2010). Kibra the other partner of this complex shows additive genetic interactions with Ex and Mer (Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010). Mer-Ex-Kibra proteins co-localize and they physically associate with each other. Multiple physical associations between members of Mer-Ex-Kibra complex and the Hpo kinase cascade have been detected such as Sav -Mer-Kibra and Hpo-Ex-Wts-Kibra (Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010). The Phosphorylation of Hpo and Wts by this complex has been detected in cell culture assays (Hamaratoglu et al., 2006; Yu

et al., 2010). Ex also regulates Yki by directly binding to it in the cytoplasm (Badouel et al., 2009).

Crb, a transmembrane protein, also binds to Ex and regulates its localization and loss of Crb is associated with overgrowth phenotype (Chen et al., 2010; Ling et al., 2010; Robinson et al., 2010). Ed is an upstream regulator of pathway and interacts with Sav at adherens junctions (Yue et al., 2012).

Ft encodes a large transmembrane protein with 34 cadherin repeats in its extracellular domain. Ft has a role in both Hippo signaling and planar cell polarity pathway (reviewed in Reddy and Irvine, 2008).The Ft branch of Hippo signalling pathway consists of an atypical cadherin, Dachsous (Ds), which is a ligand for Ft; Discs overgrown (Dco), a Golgi-localized kinase that phosphorylates Ft and Ds to modulate binding between them and few other less studied partners such as Dachs (D), Approximated (APP), Lowfat (Lft) and Zyx102 (Zyx). Recently, two more components, the WD40 repeat protein Riquiqui (Riq) and the DYRK-family kinase Minibrain (Mnb) have been added to this pathway (Degoutin et al., 2013).

4.1.4 Yorkie (Yki)

The transcriptional output of Hippo signaling pathway is mediated by the downstream transcriptional co-activator protein Yki. It is a non-DNA binding transcriptional co-activator, which promotes growth and inhibits apoptosis in a cell type specific manner. Yki is regulated multiple ways: phosphorylation at multiple sites, sub-cellular localization, direct binding to some of the upstream components like Ex (Oh and Irvine, 2010). In quiescent cells, the kinase cascade through Wts phosphorylates Yki and thereby it is retained in the cytoplasm. If phosphorylation of Yki is inhibited and the unphosphorylated form of Yki enters the nucleus, interacts with transcription factors such as Scalloped (Sd), Homothorax (Hth),Teashirt (Tsh), and Mothers against dpp (Mad) to regulate transcription of downstream target genes in cell type specific manner and induce cell proliferation (Halder and Johnson, 2011).

4.2 Objectives

Genome wide studies to identify targets of Ubx have identified many components of Hippo pathway as potential targets (Mohit et al., 2006; Hersh et al.,2007; Pavlopoulos and Akam, 2011; Slattery et al., 2011; Choo et al., 2011; Agrawal et al., 2011). This indicated probable role of Hippo pathway during haltere development.

Specific objectives of this study in this context were,

1. To examine the expression patterns of various components of the Hippo pathway and determine components of this pathway that is differentially regulated between wing and haltere.
2. Implication of Ubx-mediated regulation of Hippo pathway in specifying haltere size.
3. Role of Hippo pathway in cell fate determination during haltere development.

4.3 Results

4.3.1 Differential regulation of Yki in wing and haltere discs

Yki is the downstream effecter of the Hippo pathway. To investigate whether Yki is differentially regulated in wing and haltere, we examined its expression and localization at third instar imaginal discs. Immunohistochemistry for Yki indicated differential localization in wing and haltere (Fig. 4.1A-B). Yki was found to be both cytoplasmic and nuclear in wing as well as haltere, nuclear levels of Yki was higher in haltere discs as compared to wing discs (Fig. 4.1C-D).

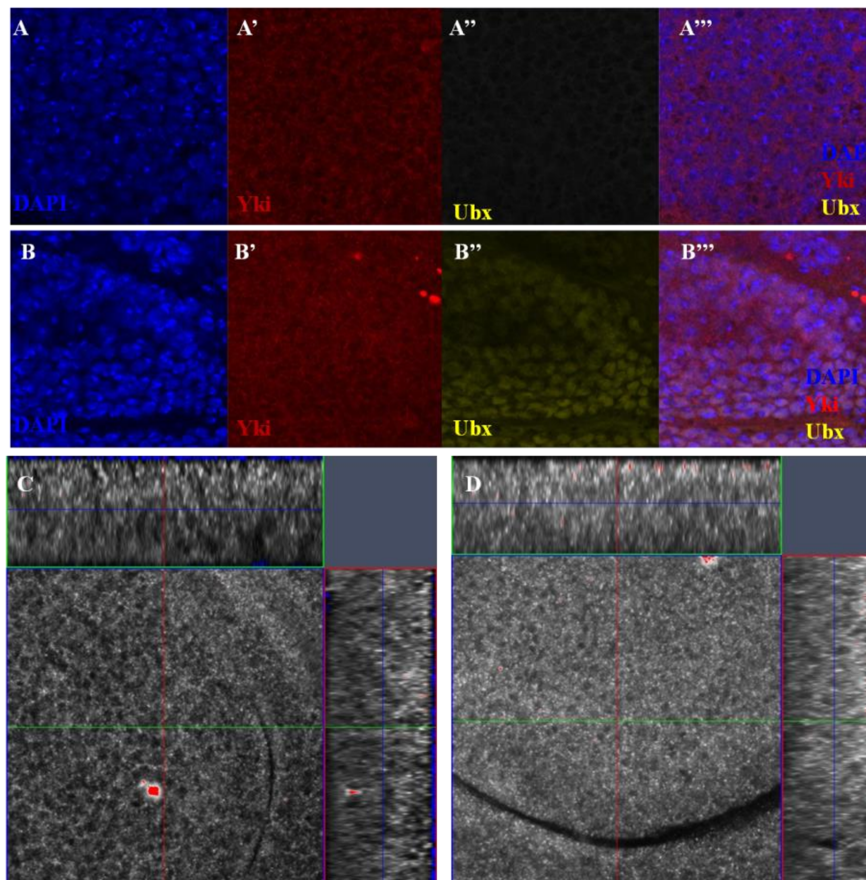


Figure 4.1 Differential regulation of Yki

(A-A’’’) Wild type third instar wing imaginal disc stained with DAPI (blue), Yki (red) and Ubx (yellow). Yki is present in both nucleus and the cytoplasm.

(B-B’’’) Wild type haltere imaginal disc stained with DAPI (blue), Yki (red) and Ubx (yellow). Similar to wing, Yki is present in both nucleus and the cytoplasm but levels of nuclear Yki is higher in haltere compared to wing.

(C and D) A X-Z section of a third instar wing (C) and haltere (D) imaginal disc stained with Yki showing higher levels of nuclear Yki in haltere disc compared to the wing disc.

4.3.2 Cell autonomous regulation of Yki by Ubx

We further examined regulation Yki by Ubx by making somatic clones for a null allele of *Ubx* in haltere using FLP/FRT system. Somatic clones of *Ubx*$^{6.28}$/*Ubx*$^{6.28}$ in haltere discs showed lower levels Yki expression compared to their wild type counterparts (Fig. 4.2A), particularly; nuclear Yki was significantly reduced in *Ubx*$^{-}$ cells (Fig. 4.2B). This suggests that Yki is cell autonomously regulated by Ubx in the haltere. However, it is not known if *yki* is a direct target of Ubx.

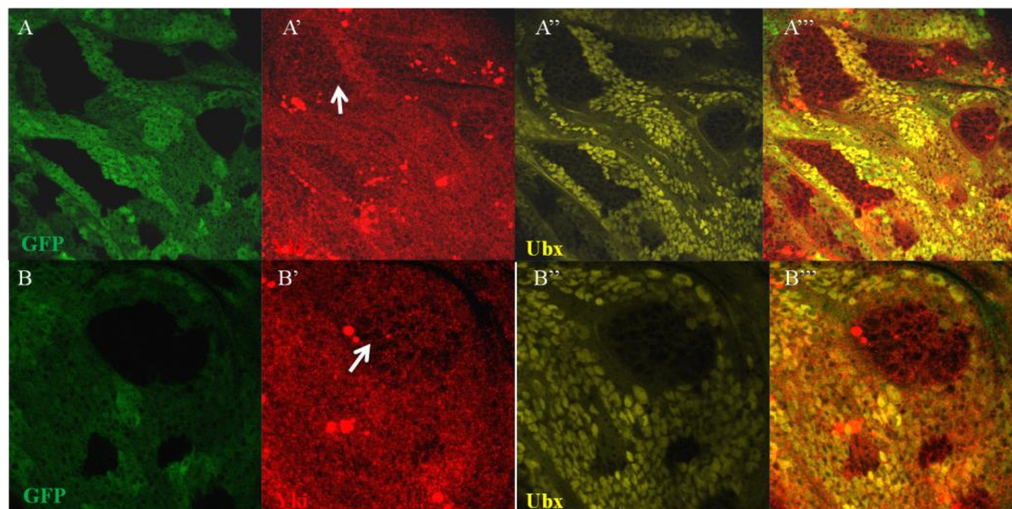
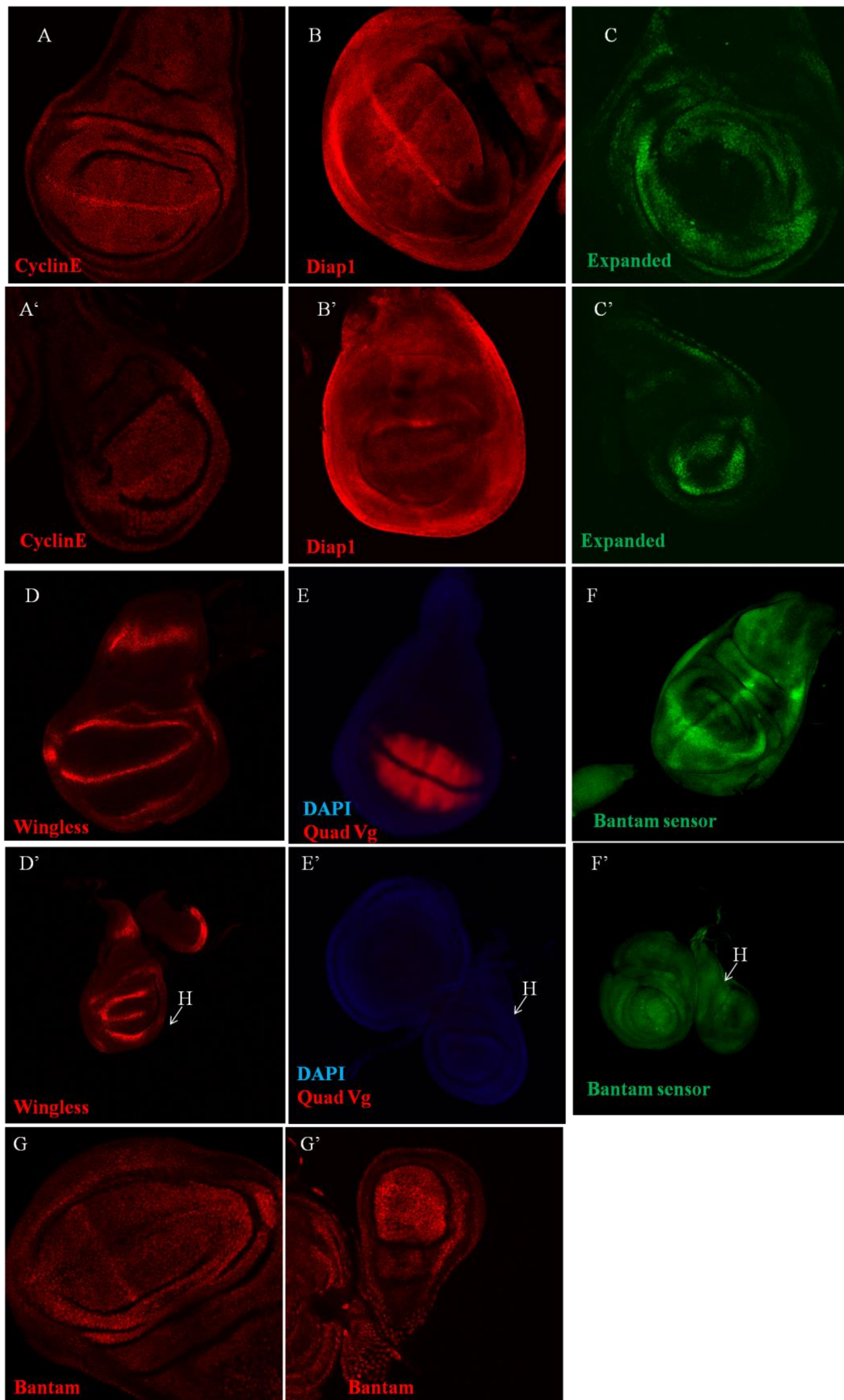


Figure 4.2 Cell autonomous regulation of Yki by Ubx

(A-A'') Third instar haltere imaginal disc stained with GFP (green), Yki (red), and Ubx (Yellow). Mitotic clones of *Ubx*$^{6.28}$ were induced in haltere using FLP/FRT system.*Ubx*$^{6.28}$/*Ubx*$^{6.28}$ clones show no expression of GFP or Ubx. Twin spots are marked with higher expression of GFP and Ubx compared to wild type cells. *Ubx*$^{6.28}$/*Ubx*$^{6.28}$ null clones (arrow) show reduced levels Yki compared to Ubx expressing wild type cells. (B-B''') Higher magnification image of the same haltere imaginal disc as in (A). Yki levels are significantly lower in nuclei of the *Ubx*$^{6.28}$/*Ubx*$^{6.28}$cells (arrow).

4.3.3 Targets of Yki are differentially regulated between wing and haltere

In light of the differential regulation of Yki in haltere, we next examined the expression patterns of targets of Yki. Microarray and ChIP based studies suggest that some of the upstream components of Hippo pathway such as *ex, four-jointed (fj) and crb* are targets of Ubx in haltere (Mohit et al., 2006; Hersh et al.,2007; Pavlopoulos and Akam, 2011; Choo et al., 20011; Slattery et al., 2011; Agrawal et al., 2011) . Interestingly, these are also downstream targets of Yki. Previous reports indicate that vein (vn), Wingless (Wg), Dally-like and Vestigial (Vg; its quadrant enhancer), which are downstream of Yki, are also regulated by Ubx (Pan, 2010; Makhijani et al., 2007; Pallavi et al 2006; Mohit et al., 2003, 2006; Shashidhara et al., 1999). We further examined the expression levels of Cyclin E (CycE), Death-associated inhibitor of apoptosis 1(DIAP1), *bantam (ban)* micro RNA (using a Bantam-sensor) and observed that all these genes are differentially regulated between wing and haltere discs. While CycE (Fig. 4.3A), DIAP1 (Fig. 4.3B), Ex (Fig. 4.3C) Wg (Fig. 4.3D) and Quadrant Vg (Fig. 4.3E) are down regulated, *ban* miRNA (as detected by *ban*-lacZ) levels are much higher in the haltere pouch compared to wing discs (Fig. 4.3F).Consistent with the increased levels of *ban*-lacZ, the *ban* sensor is down regulated in haltere discs(Fig. 4.3G). As *fj, ex, diap1* are potential direct targets of Ubx, it appears that Yki and Ubx share a pool of common targets, which may have functional significance in the canalization of the haltere fate.



A
CyclinE
B
Diap1
C
Expanded
A'
CyclinE
B'
Diap1
C'
Expanded
D
Wingless
E
DAPI
Quad Vg
F
Bantam sensor
D'
H
Wingless
E'
H
DAPI
Quad Vg
F'
H
Bantam sensor
G
Bantam
G'
Bantam

Figure 4.3 Several targets of Yki are differentially expressed between wing and haltere discs

(A-A') Third instar wing (A) and haltere (A') imaginal discs showing expression pattern of *cyclinE*-lacZ, which is predominantly expressed in the Dorsal/Ventral (D/V) boundary cells of wing disc, while *cyclinE*-lacZ expression is at much lower levels in haltere discs. This is more pronounced in the posterior compartment of the haltere disc.

(B-B') Third instar wing (B) and haltere (B') imaginal discs stained for *diap1*-lacZ. DIAP1 is expressed predominantly in the D/V boundary of wing imaginal disc while *diap1*-lacZ expression in much lower in the haltere disc.

(C-C') Third instar wing (C) and haltere (C') imaginal discs stained for *ex*-lacZ. Compared to *ex*-lacZ expression in the wing disc, Ex levels are lower in the outer hinge region and in the pouch of the haltere disc.

(D-D') Third instar wing (D) and haltere (D') imaginal discs stained for *quadrant vg*-lacZ. *quadrant vg*-lacZ expressed in the non-D/V cells of wing pouch, *quadrant vg*-lacZ expression is completely absent in the haltere disc.

(E-E') Third instar wing (E) and haltere (E') imaginal discs stained for Wg. Wg is expressed predominantly in D/V boundary of wing imaginal disc, while it is down regulated in posterior compartment of haltere imaginal disc.

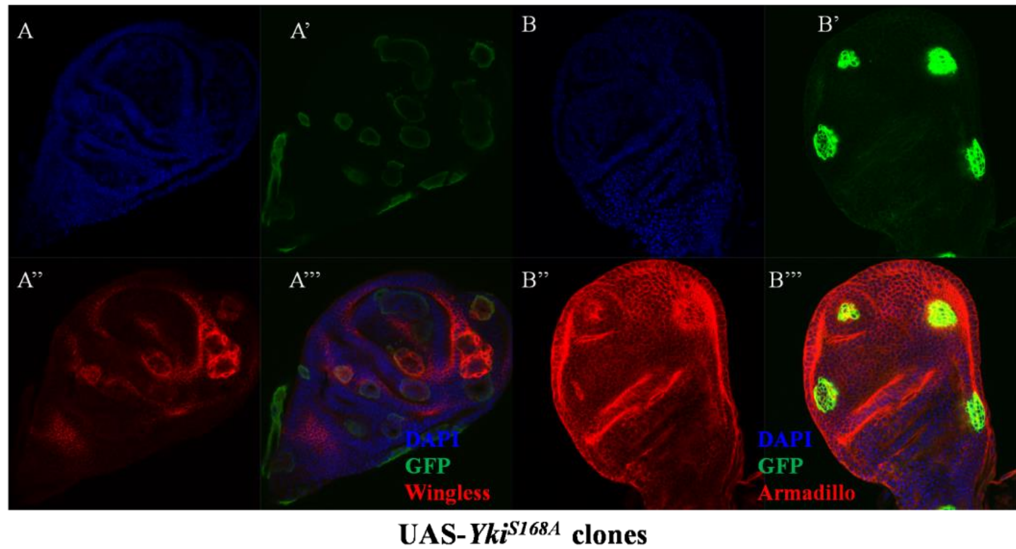
(F-F') Third instar wing (F) and haltere (F') imaginal discs stained for a GFP-tagged *ban* sensor. Presence of GFP is an indication of absence of *ban* and vice-versa. In wing imaginal disc, *ban* is mostly absent in the pouch (except in Anterior/Posterior (A/P) cells), and hence the disc shows higher levels of the sensor (and lower levels in A/P cells). Please note very low levels of *ban* sensor in haltere imaginal disc confirming higher levels of *ban* in both D/V and non-D/V cells.

(G-G') Third instar wing (G) and haltere (G') imaginal discs stained for *ban*-lacZ. Note high levels of *ban* in the haltere pouch, while wing discs show relatively lower levels. The A/P boundary cells of wing disc shows higher levels compared to the rest of the pouch.

4.3.4 Effect of over-expression of constitutively activated form of Yki

When Yki is phosphorylated by the Hippo pathway, it is normally cytoplasmic and when not phosphorylated it is nuclear (Oh and Irvine, 2010). In this scenario, Yki should be more active in haltere discs. However, many of the targets of Yki are down regulated in wing discs suggesting that Yki is not in the activated form, even if it is nuclear localized.

To validate this, we over-expressed a constitutively activated form of Yki. A serine-to-alanine mutation in Yki phosphorylation site (Yki^{S168A}) makes Yki to lose its ability to interact with Wts, thus resulting in enhanced nuclear localization and activity (Ren et al., 2010). This constitutively active form of Yki was over-expressed in haltere using *Ay*-GAL4. Not only over-expression of Yki^{S168A} induced overgrowth in both wing and haltere imaginal discs, it also resulted in the activation of Wg and Armadillo (Arm) in the pouch region of both wing (data not shown) and haltere discs (Fig. 4.4). This suggests that nuclear localized Yki in haltere discs is not an activated form and it is either phosphorylation-independent or phosphorylated by a different kinase than Wts. It also suggests that Ubx may interfere with both up stream regulators and downstream effectors of Yki during haltere specification.



UAS-*Yki*^{S168A} clones

Figure 4.4 Over-expression of constitutively activated form of Yki results in the activation of Wg signaling in haltere discs

Third instar haltere discs of genotype Ay-GAL4;UAS-GFP/UAS-Yki^{S168A}. Flp out clones are marked with GFP. All clones are overgrown and are isolated from rest of the disc. (A-A''') Haltere imaginal disc stained with DAPI (blue), GFP (green) and Wg (red). Note *Yki*^{S168A}-expressing clones, specifically those present outside the pouch region of haltere, show activation of Wg. (B-B''') Haltere imaginal disc stained with DAPI (blue), GFP (green) and Arm (Red). Note Yki^{S168A}-expressing clones show activation of Arm.

4.3.5 Down regulation of Hippo pathway results in increase in the haltere size

Hippo pathway components such as Ex, Ft, Ds, Hpo are primarily tumour suppressors, which control the growth of organ by inhibiting the nuclear function of Yki. To understand to what extent they are involved in Ubx-mediated specification of haltere size, we down regulated the expression of *ex, ft, ds, and hpo* and over-expressed Yki in developing haltere using two pouch-specific GAL4 drivers, *omb*-GAL4 and *Ubx*-GAL4. RNAi-mediated down regulation of *ex, ft, ds* and *hpo* and over-expression of Yki resulted in increased size of haltere capitellum. As *Ubx*-GAL4 is also a null allele of *Ubx*, as expected, we observed significantly enhanced haltere size when this GAL4 was used compared to when *omb*-GAL4 was used (Fig. 4.5).

This is further validated as comparable enhanced growth was also observed when the UAS lines were crossed to *omb*-GAL4 driver in a genetic background that is heterozygous for Ubx^1, a null allele of *Ubx* (Fig. 4.5). Growth response observed by the manipulation of the Hippo pathway was more prominent compared to even over-expression of Wg, Decapentaplegic (Dpp), epidermal growth factor receptor (EGFR) etc. (Prasad et al., 2003; Pallavi et al., 2006; Makhijani et al., 2007).

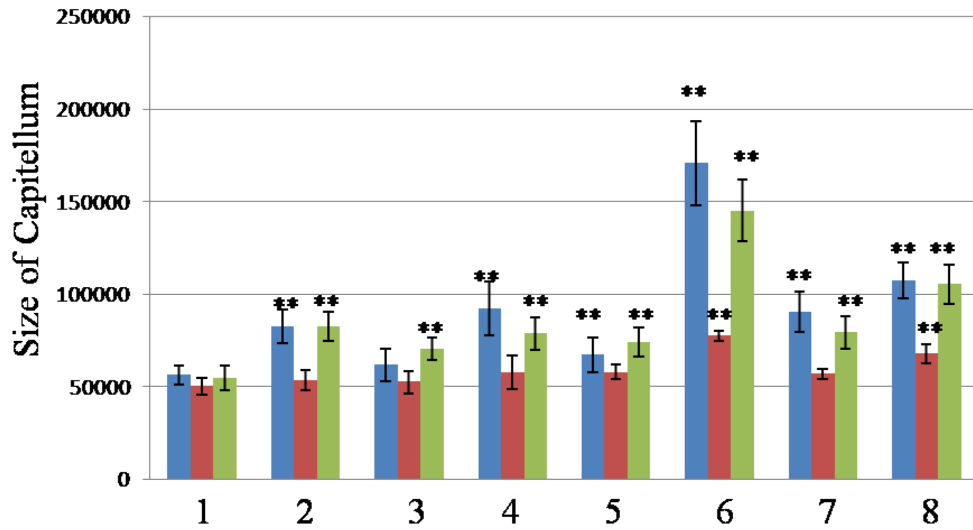


Figure 4.5 Effect of over-expression or down regulation of positive and negative components, respectively of Hippo pathway on the size of the haltere capitellum

Blue bar: Expression of Hippo pathway components using *Ubx*-GAL4 driver. The genotypes are: 1.*Ubx*-GAL4, 2.*Ubx*-GAL4/UAS-Yki, 3.*Ubx*-GAL4/UAS-*wts*^{RNAi}, 4.*Ubx*-GAL4/UAS-*hpo*^{RNAi}, 5.*Ubx*-GAL4/UAS-*mer*^{RNAi}, 6.*Ubx*-GAL4/UAS-*ex*^{RNAi}, 7.*Ubx*-GAL4/UAS-*ft*^{RNAi}, 8.*Ubx*-GAL4/UAS-*ds*^{RNAi}. N=20 for all crosses.

Red bar: Expression of Hippo pathway components using *omb*-GAL4 driver. Genotypes are: 1.Wildtype (n=10), 2.*omb*-GAL4;UAS-Yki(n=18), 3.*omb*-GAL4;UAS-*wts*^{RNAi}(n=15), 4.*omb*-GAL4;UAS-*hpo*^{RNAi}(n=12), 5.*omb*-GAL4;UAS-*mer*^{RNAi}(n=17), 6. *omb*-GAL4;UAS-*ex*^{RNAi}(n=09), 7.*omb*-GAL4;UAS-*ft*^{RNAi}(n=9), 8. *omb*-GAL4;UAS-*ds*^{RNAi}(n=13).

Green bar: Expression of Hippo pathway components using *omb*-GAL4 driver in *Ubx*^{1} heterozygous background. Genotypes are: 1.*omb*-GAL4;*Ubx*^{1}(n=15), 2.*omb*-GAL4;UAS-Yki/*Ubx*^{1}(n=9), 3.*omb*-GAL4;UAS-*wts*^{RNAi}/*Ubx*^{1} (n=17), 4.*omb*-GAL4;UAS-*hpo*^{RNAi}/*Ubx*^{1}(n=11), 5.*omb*-GAL4;UAS-*mer*^{RNAi}/*Ubx*^{1}(n=10), 6.*omb*-GAL4;UAS-*ex*^{RNAi};/*Ubx*^{1}(n=15), 7.*omb*-GAL4;UAS-*ft*^{RNAi}/*Ubx*^{1} (n=15), 8.*omb*-GAL4;UAS-*ds*^{RNAi}/*Ubx*^{1} (n=20).

Error bar represents Standard Deviation and $^{**}P < 0.001$. Y-axis represents area of capitellum as measured using Image J software.

4.3.6 Haltere-to-wing Homeotic transformations induced by changing the expression profile of Hippo pathway components

A Homeotic transformation of haltere-to-wing is marked by the appearance of ectopic sensory bristles (predominantly bristles of the wing-margin) on haltere. Expression of UAS-*ex*^{RNAi}, UAS-*ds*^{RNAi} and UAS-Yki with *Ubx*-GAL4 caused increase in number of ectopic sensory bristles present on *Ubx*-GAL4 capitellum (Fig. 4.6). These sensory bristles were arranged in two rows in the same way as seen on the wing margin indicating that both growth and patterning events in the haltere are specified by Ubx by regulating the components of the Hippo pathway.

Next, we asked the question whether increase in the number of sensory bristles is a result of cell proliferation or change in cell fate. Assuming similar fold increase in the cell division of sensory bristles and the epithelial cells, we compared the fold change in the size of the haltere capitellum to the increase in number of sensory bristles. The capitellum size and the number of sensory bristles on *Ubx*-GAL4 haltere were taken as basal level. UAS-*ex*^{RNAi}, UAS-*ds*^{RNAi}, UAS-Yki had approximately 3.04, 1.77 and 1.47 fold increase in the size of haltere capitellum, respectively and 4.8, 2.4 and 2.58 fold increase in the number of ectopic sensory bristles, respectively.. Thus, increase in the number of sensory bristles appears to be not due to mere proliferation of sensory bristles present in the *Ubx*-GAL4 itself.

Furthermore, expression of *ex*^{RNAi} using *MS1096*-GAL4 and *omb*-GAL4 drivers also induced, albeit a single, ectopic bristle on the haltere capitellum (Fig. 4.7). Interestingly, although *ft*^{RNAi} and *hpo*^{RNAi} cause increase in the growth of capitellum, they had decrease in the number of sensory bristles. Nevertheless, these results suggest that components of Hippo pathway may be involved in specifying the cell fate in the haltere.

Genotype	No of wing type bristles on adult haltere		
	Range	Average	Standard Deviation
Ubx-GAL4	4-8	5.95	1.468
Ubx-GAL4/UAS-Yki	10-24	17	3.825
Ubx-GAL4/UAS-*wts*^{RNAi}	4-8	5.55	1.099
Ubx-GAL4/UAS-*hpo*^{RNAi}	0-5	1.35	1.53
Ubx-GAL4/UAS-*mer*^{RNAi}	1-7	4.95	0.998
Ubx-GAL4/UAS-*exp*^{RNAi}	15-40	28.65	7.028
Ubx-GAL4/UAS-*ft*^{RNAi}	0-5	2.3	1.838
Ubx-GAL4/UAS-*ds*^{RNAi}	11-21	14.85	2.58
omb-GAL4;*Ubx*^{1}	1-11	6.55	2.584
omb-GAL4;*Ubx*^{1}/UAS-Yki	5-10	6.65	1.268
omb-GAL4;*Ubx*^{1}/UAS-*wts*^{RNAi}	0-4	2.75	1.446
omb-GAL4;*Ubx*^{1}/UAS-*hpo*^{RNAi}	0-2	0.6	0.82
omb-GAL4;*Ubx*^{1}/UAS-*mer*^{RNAi}	0-4	1.947	1.22
omb-GAL4;*Ubx*^{1}/UAS-*exp*^{RNAi}	14-26	18.45	3.017
omb-GAL4;*Ubx*^{1}/UAS-*ft*^{RNAi}	0-2	0.45	0.686
omb-GAL4;*Ubx*^{1}/UAS-*ds*^{RNAi}	5-12	7.46	2.29

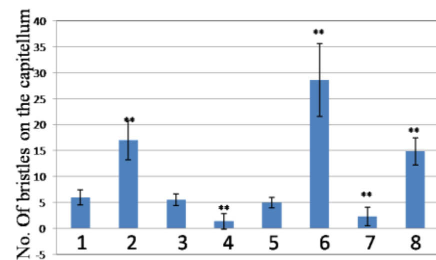


Figure 4.6 Ectopic bristles on haltere are not because of proliferation of existing bristles on haltere capitellum

(A) **Table 4.1 Change in number of wing type bristles on haltere capitellum**. Over-expression of the positive component, Yki and down regulation of the negative components of Hippo pathway results in change in the number of sensory bristles on haltere capitellum; normally seen in *Ubx*-Gal4 driver alone. n=20 in all crosses, except for *omb*-GAL4/UAS-*ds*^{RNAi}; *Ubx*^{1} wherein n=15.

(B) bar diagram showing number of sensory bristles of genotypes: 1. *Ubx*-GAL4, 2.*Ubx*-GAL4;UAS-Yki,3.*Ubx*-GAL4;UAS-*wts*^{RNAi},4.*Ubx*-GAL4;UAS-*hpo*^{RNAi},5.*Ubx*-GAL4;UAS-*mer*^{RNAi}, 6.*Ubx*-GAL4;UAS-*ex*^{RNAi}, 7.*Ubx*-GAL4;UAS-*ft*^{RNAi} and 8.*Ubx*-GAL4;UAS-*ds*^{RNAi}. n=20 for all genotypes. **$P < 0.001$

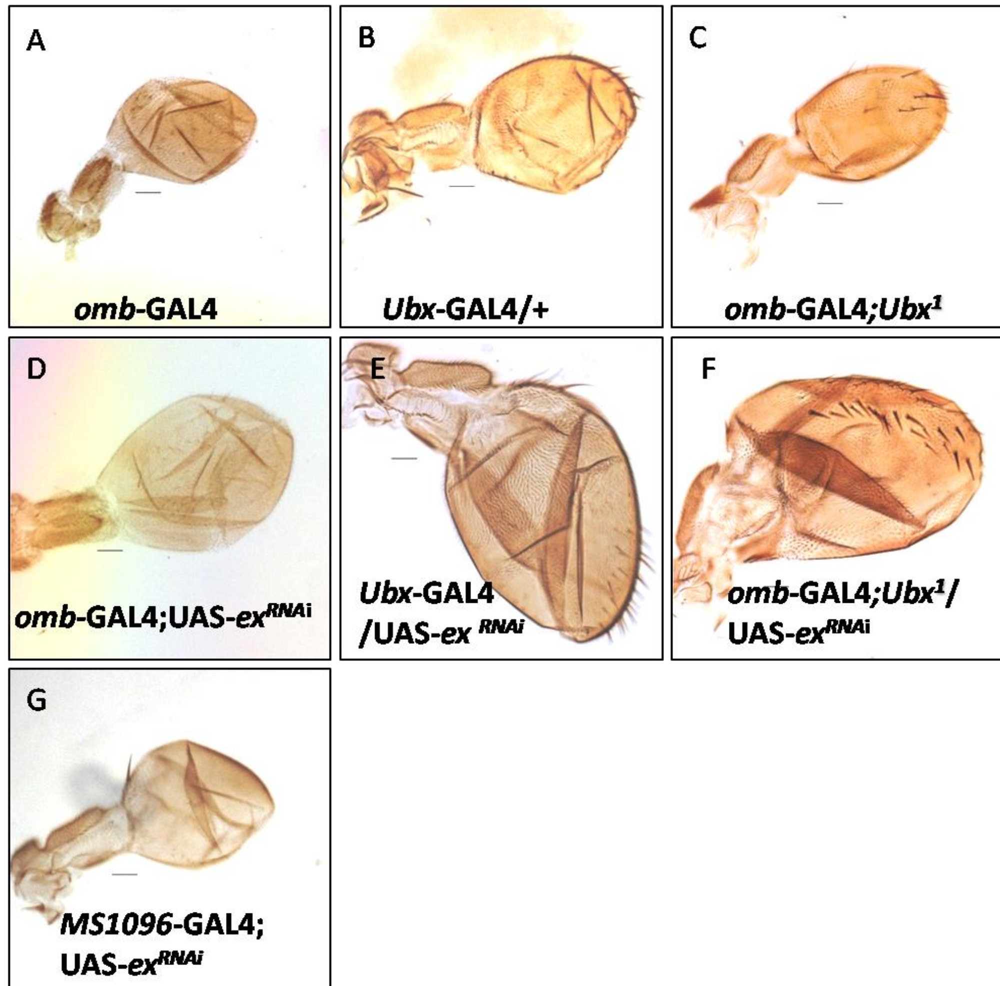


Figure 4.7 Partial transformation of haltere-to-wing caused by the down regulation of *ex*

(A) *omb*-GAL4, haltere is identical to that of wild type,(B) *Ubx*-GAL4 shows homeotic transformation marked by slight increase in the size of haltere capitellum and presence of ectopic sensory bristles, (C) *omb*-GAL4;Ubx^1 haltere is similar to that of *Ubx*-GAL4, (D) *omb*-GAL4;UAS-ex^{RNAi} induced increase in the capitellum size and some halteres showed presence of a single ectopic sensory bristle on the capitellum (not shown here), (E)*Ubx*-GAL4/*UAS*-ex^{RNAi} induced dramatic increase in the size of haltere capitellum and in the number of ectopic sensory bristles, which are arranged in two rows, (F) *omb*-GAL4/UAS-ex^{RNAi} haltere is similar to that of *Ubx*-GAL4;*UAS*-ex^{RNAi} and (G) *MS1096*-GAL4;UAS-ex^{RNAi} haltere capitellum with one ectopic sensory bristles at the base of the capitellum

4.3.7 Effect of Hippo pathway on the shape of haltere capitellum

Varied degree of change in the haltere shape was observed when Hippo pathway components were over-expressed or down regulated using *Ubx*-GAL4 driver. *Ubx*-GAL4; *UAS-ex*^{RNAi} haltere was apple shaped, while *Ubx*-GAL4;UAS-*hpo*^{RNAi} was round shaped and *Ubx*-GAL4;UAS-Yki haltere was elongated. Some of the *Ubx*-GAL4; *UAS-ds*^{RNAi} halteres were round, while some were elongated in shape (Fig. 4.8). The difference in shapes of haltere may represent differences in the degree of growth in different compartments in either D/V and/or A/P axis.

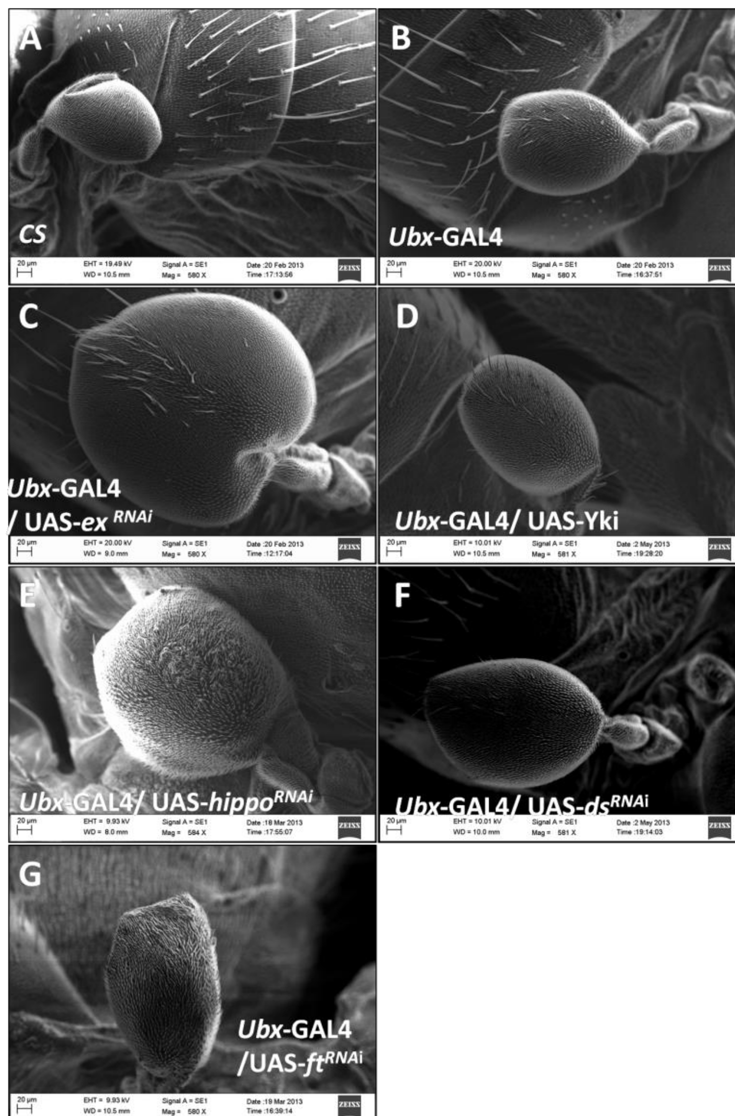


Figure 4.8 Scanning electron microscopy (SEM) images showing increase in the size and morphology of various genotypes

(A) Wild type, (B) *Ubx*-GAL4.Note presence of 6 bristles and increased size of haltere capitellum, (C) *Ubx*-GAL4/UAS-*ex*^{RNAi} Note dramatic increase in the

capitellum size; in the number of sensory bristles; apple-shaped haltere, (D) *Ubx*-GAL4/UAS-Yki Note moderate increase in the capitellum size; in the number of sensory bristles; shape of haltere is more elongated. (E) *Ubx*-GAL4/UAS-*hpo*^{RNAi}Note moderate increase in the capitellum size; loss of ectopic bristles of the *Ubx*-GAL4capitellum haltere is round in shape. (F) *Ubx*-GAL4/UAS-*ds*$^{RNAi.}$ Note marginal increase in the capitellum size; in the number of sensory bristles; shape of haltere is similar to *Ubx*-GAL4 haltere. (G)*Ubx*-GAL4/UAS-*ft*$^{RNAi.}$ Note moderate increase in the capitellum size loss of ectopic bristles of the *Ubx*-GAL4 capitellum

4.3.8 Effect of Hippo pathway on the morphology of haltere trichomes

Each wing cell has a single long hair (trichome), while haltere cells have 2, 3 or 4 hairs, which are reduced in size. Compared to wing blade, haltere trichomes are more densely arranged and flat at the base while the haltere trichomes are bulbous in nature (Fig. 4.9A, B). In *Ubx*-GAL4;UAS-*ex*^{RNAi} and in *Ubx*-GAL4;UAS-*ds*^{RNAi} halteres, trichomes were less densely arranged, were longer in length as well as their base was flatter compared to that in *Ubx*-GAL4 (Fig. 4.9C, D and H). Degree of trichome density was found to be varied within the haltere. The proximal region had less densely arranged trichome as compared to the distal parts. *Ubx*-GAL4;UAS-*ft*^{RNAi} and *Ubx*-GAL4;UAS-Yki halteres did not show any change in trichome morphology or density (Fig. 4.9E and I). Surprisingly, *Ubx*-GAL4; UAS-*hpo*^{RNAi} halteres caused change in polarity of trichomes and many hairs were arranged in bundles (Fig. 4.9 F and G). Morphology of trichomes depends upon the architecture of actin cytoskeleton. Various upstream components of Hippo pathway such as Ex and Hippo are known to regulate actin cytoskeleton (Fernandez et al., 2011).Wing and haltere cells differ in their actin cytoskeleton architecture, starting from the pupal stages (Roch and Akam, 2000). Thus, Ubx-mediated regulation of Hippo pathway could be critical for determination of trichome morphology and density in developing haltere. It is likely that the size and shape of the haltere is a product of the effect of Hippo pathway on trichome morphology and density.

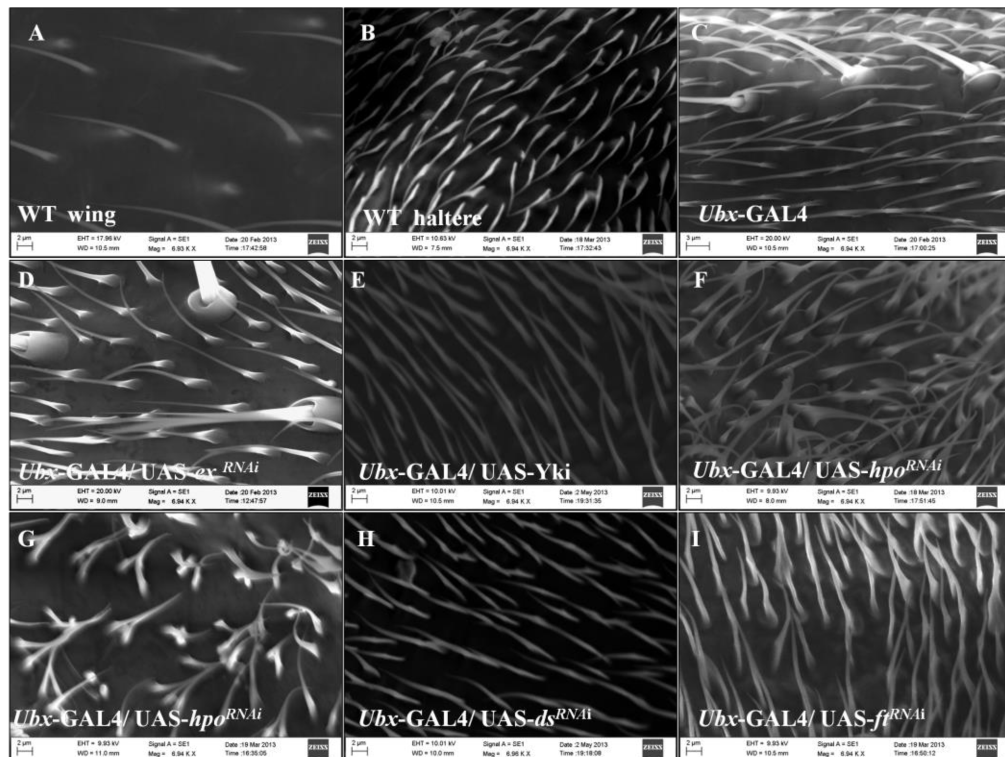


Figure 4.9 Changes in the morphology of trichomes due to the manipulation in the expression of different components of Hippo pathway

All are SEM images of wing and haltere are from female flies. (A) Dorsal surface of the adult wing of a wild type fly showing trichomes of the intervein region. Note the less densely arranged trichomes, with flat base. Small dots are trichomes of ventral side of the wing blade, indication of the fact that the dorsal and ventral sides are very closely adhered to each other. (B) Adult haltere of a wild type fly with densely arranged trichomes, which are shorter in length. (C) Adult haltere of an *Ubx*-GAL fly. Compared to wild type, these trichomes are less densely arranged, are slightly longer and the base of the trichomes are somewhat flattened. (D) *Ubx*-GAL4/UAS-ex^{RNAi} haltere. While the density of trichomes is similar to that of C, they are much longer with significantly flattened base. (D) *Ubx*-GAL4/UAS-Yki haltere. There is no change in the phenotype compared to Ubx-GAL4halteres. (E) and (F) *Ubx*-GAL4/UAS-hpo^{RNAi} halteres showing loss of polarity and disorganized trichomes. They are in bundles of 3 to 4 trichomes. (G) *Ubx*-GAL4/UAS-ds^{RNAi} and (H) *Ubx*-GAL4/UAS-ft^{RNAi} halteres. There is no change in the phenotype compared to Ubx-GAL4 halteres.

4.3.9 Over-expression of *bantam* mimics the effect of over-expression of Yki

As described above, down regulation of *ex* or over-expression of Yki resulted in haltere-to-wing transformations at the levels of haltere size, number of sensory bristles on the capitellum and trichome morphology. We further examined if over-expression of any of the downstream effectors of Yki induces similar phenotypes. We over-expressed Cyc-E, DIAP1 and *bantam* in developing haltere. No phenotype was observed with Cyc-E or DIAP1 with either *omb*-GAL4 or *Ubx*–GAL4 driver (Fig. 4.10B, C). However, over-expression of *bantam* using *omb*-GAL4 caused prominent increase in size of the capitellum and 4 to 6 sensory bristles. *bantam* over-expression with *Ubx*-GAL4 was early larval lethal.

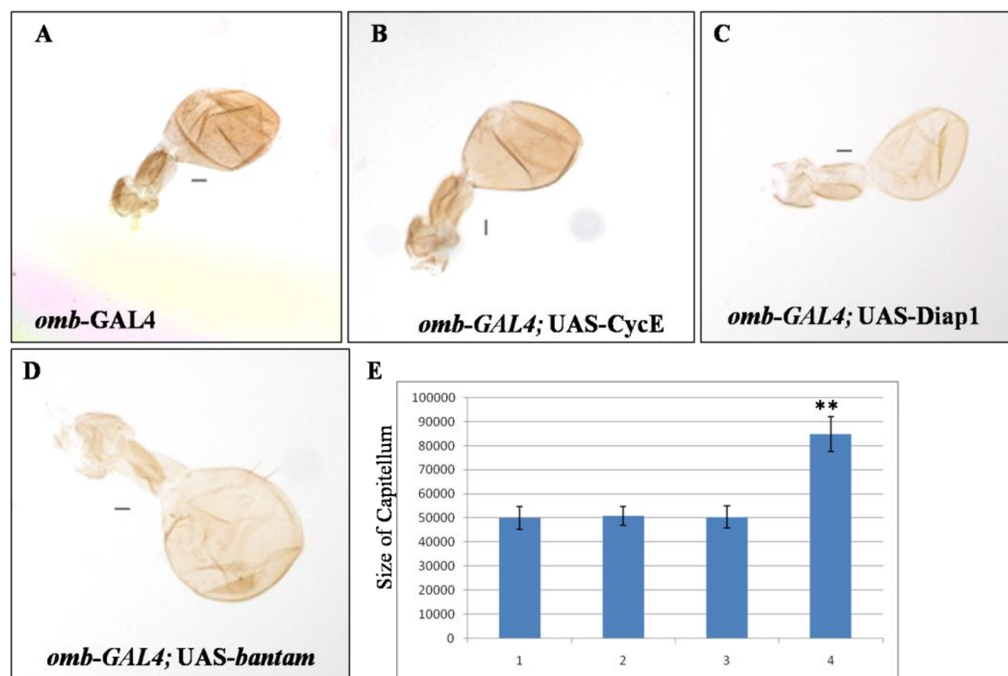


Figure 4.10 Homeotic transformations by bantam

(A) *omb*-GAL4 haltere, identical to the wildtype. (B) *omb*-GAL4;UAS-CycE and (C) *omb*-GAL4;UAS-Diap1 halteres. Both are similar to the wild type haltere. (D) *omb*-GAL4;UAS-*ban* haltere showing increased size of the capitellum with ectopic sensory bristles suggesting partial transformation to wing type. (E) Graph comparing the size of haltere capitellum of genotypes: 1. Wild type (n=10), 2.*omb*-GAL4;UAS-CycE(n=7), 3.*omb*-GAL4;UAS-Diap1(n=7), 4.*omb*-GAL4;UAS-*ban* (n=13). Note, increase in the size of the capitellum due to over-expression of *ban*. **P < 0.001

4.3.10 Epistasis interactions between Ubx and Yki

Ubx and Yki together have many common targets that are differentially regulated between wing and haltere. In addition, Yki itself is regulated by Ubx. To determine the functional relationship between Ubx and Yki in specifying haltere fate, they were expressed either alone or together in developing wing using *omb*-GAL4. Ectopic expression of Ubx in wing imaginal disc caused reduction in the *omb*-GAL4 domain (Fig. 4.11B), reduced adult wing blade (Fig. 4.11D) and bulged cells (Fig. 4.11G), an indication of wing-to-haltere transformations. Over-expression of Yki alone had no phenotype (Fig. 4.11F, H). Contrary to the expectations, we observed enhanced phenotype induced by the over-expression of Ubx, when Yki was co-expressed (Fig. 4.11I, J).This suggests that Ubx may require Yki to induce haltere fate. Higher levels of nuclear Yki in haltere discs compared to wing discs could be an indication of this requirement.

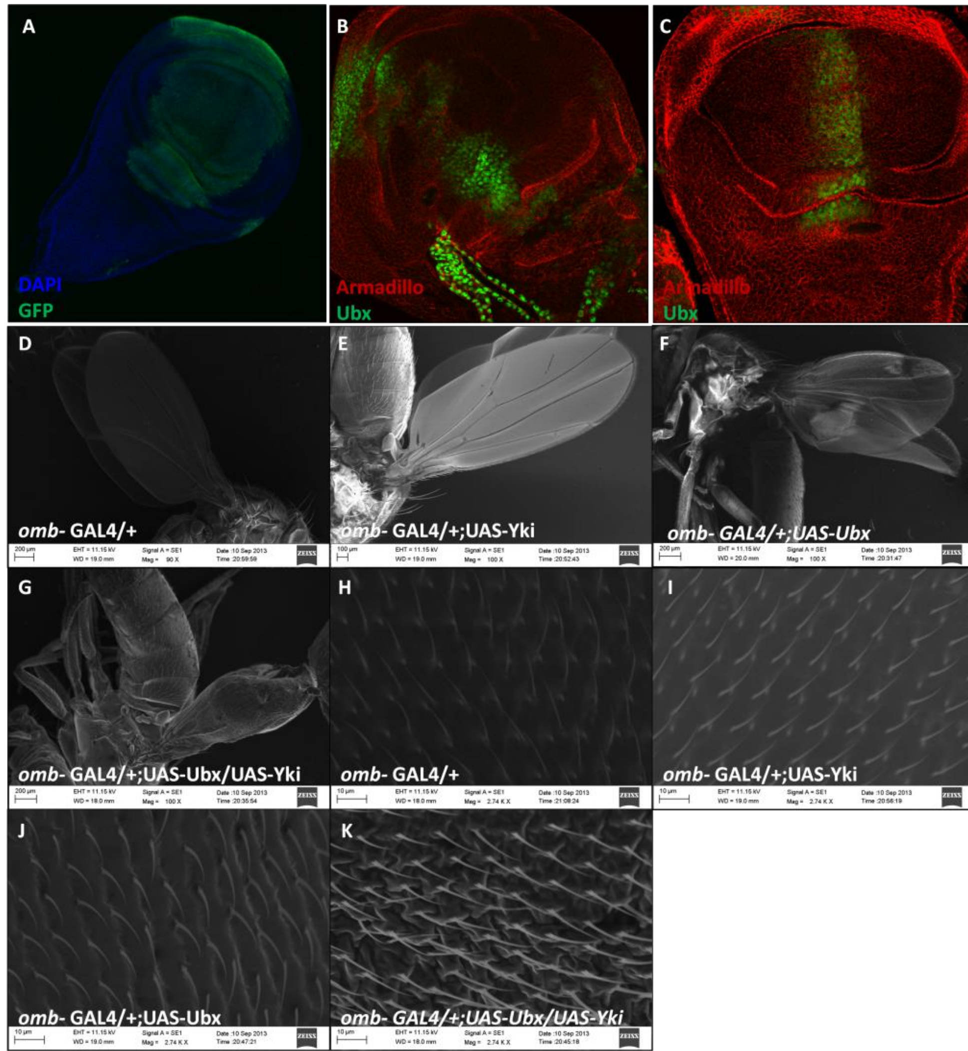


Figure 4.11 Yki enhances Ubx-induced wing-to-haltere transformations

(A) *omb*-GAL4;UAS-GFP wing imaginal disc stained for GFP (green) and DAPI (blue) to show *omb*-GAL4 domain. (B-C) *omb*-GAL4;UAS-Ubx (B) and *omb*-GAL4; UAS-Ubx/UAS-Yki (C) wing imaginal discs stained with Arm (red) and Ubx (green).Note reduction in *omb*-GAL4 domain in both the discs. (D-G) SEM images of adult females of genotype *omb*-GAL4/+ (D) *omb*-GAL4;UAS-Ubx (E), *omb*-GAL4;UAS-Yki (F) and *omb*-GAL4; UAS-Ubx/UAS-Yki (G). Note reduced wing size and separation of dorsal and ventral layers in E, which is enhanced in G. (H-K) Higher magnification SEM images of adult wing blades of genotype *omb*-GAL4 (H), *omb*-GAL4;UAS-Ubx (I), *omb*-GAL4;UAS-Yki (J) and *omb*-GAL4; UAS-Ubx; UAS-Yki (K). Note bulged individual wing cells due to over-expression of Ubx (I), which is further enhanced when Yki is co-expressed (K).

4.4 Discussion

Hippo pathway is a major organ size controlling pathway, which is also known to have diverse functions. Here we report the regulation of Hippo pathway by Ubx and function during haltere specification.

4.4.1 Regulation of Yki in haltere

Hippo signaling pathway mediates its downstream effect through a non-DNA binding transcriptional co-activator protein Yki, which is kept under the tight control by several independently acting upstream components of the pathway. Recent studies have shown multiple modes of Yki regulation, this includes Yki regulation by multiple phosphorylation sites, phosphorylation-independent regulation, effects on expression and stability and additional DNA-binding partner proteins etc. (Oh and Irvine, 2010). In wild type haltere discs, we observed increased nuclear Yki than in wing discs (Fig. 4.1). Loss of function clones of *Ubx* in haltere discs showed decreased levels of nuclear Yki, suggesting that localization of Yki is regulated by Ubx (Fig. 4.2).

Wts-mediated phosphorylation of Yki is required to block Yki entry to the nucleus (Ren et al., 2010). However, when Wts-independent form of Yki (Yki^{S168A}) was over-expressed in haltere discs, we observed activation of Wg and Arm. Yki^{S168A}-expressing cells also had increased growth (Fig. 4.4). This suggests that Ubx-mediated nuclear localization of Yki in haltere cells is not the same form as Yki that is not phosphorylated by Wts in wing disc (and because of which it gets localized to the nucleus). Either phosphorylated (mediated by Wts) form of Yki itself somehow (due Ubx) gets localized to the nucleus or this Yki is post-translationally modified in haltere cells in entirely different way.

Yki itself does not have any DNA-binding potential and requires other DNA binding transcription factors to show its activity. When Hippo pathway is not active, Yki translocate into the nucleus and binds to its DNA binding partners such as Sd, Hth, Tsh, and Mad, to activate expression of its target genes for regulating cell proliferation and apoptosis (Pan, 2010; Schroeder and Halder, 2012; Staley and Irvine., 2012; Yu et al., 2013). Recently it has been shown that Yki regulates the normal growth in wing pouch by relieving the

Sd-mediated default repression (Guo et al., 2013). All of the known DNA-binding targets like, Hth, Tsh, Mad are down regulated in haltere. Although Sd expression in haltere is not known, it is a potential target according to ChIP studies. This is further reflected in the fact that identified target of Yki, viz. *diap1, cycE, akt, crb ,fj ,ex ,vg ,vn ,wg , arm*, are down regulated in haltere discs (Mohit et al.,2006 ; Slattery et al., 2011and Fig. 4.3). Interestingly, all of these targets are also direct targets of Ubx.

As co-expression of Yki enhanced Ubx-induced phenotypes in wing cells, it is possible that Yki has a more positive role to play in haltere specification. The form of Yki that is present in the nuclei of haltere cells could have a specific function along with Ubx to up-regulate or down regulate certain common targets during haltere development. Understanding of the precise nature of interactions between Yki and Ubx needs additional investigations.

4.4.2 Regulation of haltere growth and patterning by Hippo pathway

Over-expression of some of the well-known growth promoting molecules such as Dpp, EGFR, Wg and IIS signaling induce moderate growth response in developing haltere. However, we observed dramatic increase in the size of haltere capitellum when some of the components of Hippo pathway were down regulated or when Yki was up regulated (Fig. 4.8). This was associated with change in trichome morphology towards wing type and ectopic sensory bristles (Fig. 4.9). This is the first report of such high degree of haltere-to-wing transformations by altering a component downstream of Ubx. As down regulation of *ex* caused more intense phenotypes than in any other combinations, it is likely to have a critical role in the suppression of wing fate and the specification of haltere fate.

4.4.3 Ubx, Hippo pathway and actin cytoskeleton

Ubx expression in haltere causes its cells to have reduced size, different shape, and different cellular affinities in comparison to wing cells. Ubx is expressed throughout the development of haltere, but most of the events required for such morphological differences are determined before puparium formation. Wing and haltere imaginal discs are identical during larval and early pupal

stages. At around 32-36 hrs after puparium formation (APF), wing and haltere epithelia undergo major cytoskeleton reorganization (Roch and Akam, 2000). Wing cell develop a single hair, while haltere cells have multiple foci and multiple hair. Furthermore, wing cells become flat and star-shaped and haltere cells are cuboidal (Roch and Akam, 2000). Previous reports suggest that increase in the levels of F-actin in haltere cells coincides with the appearance of these morphological differences (Roch and Akam, 2000).

Interestingly, regulation of actin cytoskeleton by the Hippo pathway has been reported, although the biological significance of reveres regulation is not fully understood (Matsui et al., 2013). Down regulation of *ex* in haltere induced differentiation of trichomes towards wing type, suggesting possible down regulation of F-actin in haltere cells.

Differential regulation of F-actin at different developmental stages has been observed in wing disc. Over-expression of Yki in larval wing disc does not affect the F-actin levels, while its over-expression in pupal stages cause increase in F-actin levels (Fang et al., 2010; Fernández et al., 2011). It is therefore possible that the Yki that is present in the nuclei of haltere cells may induce increased F-actin levels to specify haltere fate, although this Yki is not able to induce growth the way it does in other contexts. This is further examined in the next Chapter.

Chapter 5

Chapter: 5

Integration of Hippo and Other Signaling Pathways in Haltere

Summary

One of the remarkable features of Hippo signaling pathway is integration with many other signaling pathways. In the previous chapter, we have shown that Hippo pathway is a major growth regulating pathway in haltere compared to other pathways studied so far (viz. epidermal growth factor receptor (EGFR), Wingless (Wg), Decapentaplegic (Dpp) etc.). Here, we show that integration of Hippo pathway with other pathways such as EGFR and Insulin/insulin-like growth factor signaling IIS provides a higher growth response in haltere. Vein a component of EGFR pathway and Akt, a component of IIS pathway are able to de-repress the growth response in haltere when expressed in a background of down regulated *expanded*, a component of Hippo pathway. Therefore, Hippo pathway appears to be at upstream in the hierarchical requirement of signaling pathways. Furthermore, increased Akt and down regulated *expanded* are together required to show differentiation of haltere trichomes to wing type. Thus, we have been able to identify genetic factors that are required in the larval stages to specify differentiation patterns in the adult.

5.1 Introduction

Developmental fate of every cell is determined by multiple signaling pathways. These pathways interact with each other and finally integrate together to provide a contextual response. Furthermore, these pathways operate in a hierarchal manner to form a network. Understanding the molecular links between these networks is important to solve some questions of organogenesis. Ultrabithorax (Ubx) modulates several signaling pathways to specify haltere. Genome wide studies have shown that many signaling pathways are targeted by Ubx to provide haltere its diverse morphological traits when compared to the wing (Chapter1, Table 1.1). How these signaling pathways integrate together in haltere is very important to understand the mechanism of Ubx mediated organogenesis.

As shown in previous Chapter (Chapter 4), modulation of Hippo pathway components cause increased growth of haltere capitellum. The growth response observed in the haltere is much higher compared to other signaling pathways examined so far (such as Wg, Dpp, EGFR, and IIS).

Recent studies have shown that one of the remarkable features of Hippo signaling is its multiple levels of cross-talk with other signaling pathways (reviewed in Irvine, 2012). In *Drosophila*, multiple links between Hippo and EGFR, IIS, Vestigial (Vg), Dpp, Notch, Jun kinase (Jnk) pathways have been identified (Fig. 5.1). EGFR activates Yorkie (Yki) through its EGFR-RAS-MAPK signaling by promoting the phosphorylation of Ajuba family protein WTIP (Reddy and Irvine, 2013). Yorkie/Yap (mammalian Yki homolog) regulates the expression of ligands for EGFR signaling (Zhang et al., 2009). EGFR can also regulate a key Yki target *bantam* through repression of the transcriptional repressor protein Capicua, which is itself a target of *bantam* (Herranz et al., 2012). Merlin, a component of Hippo signaling is also known to be an inhibitor of EGFR signaling (Yi and Kissil, 2010). IIS pathway is known to activate Yki signaling and vice-versa (Straßburge et al., 2012). Hippo signaling negatively regulates the IIS signaling pathway to inhibit

cellular growth. Akt, a major component of IIS signaling, is negatively regulated by Hippo signaling (Ye et al., 2012).

In the wing, Hippo pathway activities are dependent on Four-jointed (Fj) and Dachous (Ds) gradients, which are influenced by Dpp, Wg and Notch signaling (Rogulja et al 2008). Role of Vg has been studied in detailed to establish Fj and Ds gradient (Zecca and Struhl, 2010). Glypicans, which play a prominent role in morphogen signaling, are regulated by Hippo signaling (Baena-Lopez et al., 2008).

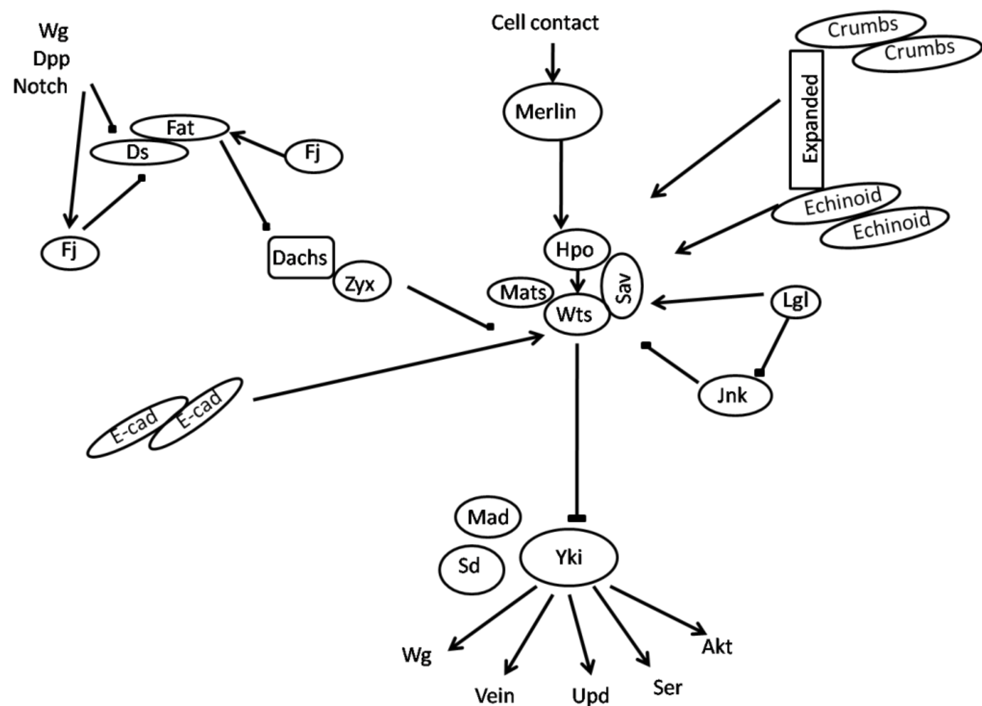


Figure 5.1 Cross-talk between Hippo signaling and other pathways (Modified from Irvine, 2012)

5.1.1 Objectives:

All the studies on haltere growth are by individually manipulating various signaling pathways. As most of these pathways may be integrated through Hippo pathway, which is targeted by Ubx to control haltere size, we examined here the effect of manipulating more than one pathway.

Objective 1: To examine, whether integration of theses pathways in haltere have any role in morphogenesis of haltere, and to further understand the hierarchical requirement of the pathways.

Hippo signaling is regulated by multiple upstream regulators including several components of cell-cell junctions, regulators of cell polarity, cellular stress, F-actin accumulation and mechanical tension (reviewed in Schroeder and Halder, 2012). Wing and haltere have different morphologies at cellular, tissue and organ levels (Chapter3 Fig. 3.1, 3.2 and 3.3). Cellular morphologies and cytoskeleton organizations, which are differentially manifested in wing and haltere (Roch and Akam, 2000), regulate the Hippo pathway and vice-versa. We therefore examined the regulation of cell size, shape and trichome morphology in adult haltere when multiple pathways are manipulated.

Objective 2: To explore the role of integrations of these pathways in providing different morphologies at cellular, tissue and organ levels.

Ubx is expressed throughout the development of haltere and regulates its target gene in developmental stage specific manner. Interestingly, it has been observed that some of the cellular processes, which are evident at later stages of development such as cell shape and vein/inter-vein differentiation are affected by ectopic Ubx throughout the stages of wing development. Whereas others, such as cell proliferation, marginal bristle formation, and adhesion, are more sensitive to ectopic Ubx during the larval and prepupal stages (Pavlopoulos and Akam, 2011).

Objective 3: Therefore, our next objective was to determine the spatial-temporal regulation of these pathways in haltere.

5.2 Results

5.2.1 Enhanced growth of haltere capitellum on co-expression of different pathways

Previously, we have reported that regulation at the level of Yki and Expanded (Ex) is critical in haltere (chapter 4).We co-expressed some of the targets of Ubx such as vein (vn), Akt, Dpp, Vg in the background of ex^{RNAi} and over-expressed Yki using *omb*-GAL4 drive. Dpp expression on its cause moderate increase in haltere size (Makhijani et al., 2007), while with over-expressed Yki or down regulated *ex* it resulted in pupal lethality. While there was no in growth response in haltere when Akt and vn are individually over-expressed, their co-expression with ex^{RNAi} resulted in much increased growth in the haltere. UAS-vein with UAS-ex^{RNAi} resulted in highest growth response amongst all the combinations tested (Fig. 5.2A, B). Such dramatic growth response has never been reported for the ectopic expression (or removal of the expression) of any downstream targets of Ubx. Interestingly, the growth response observed on co-expression of UAS-Yki and UAS-ex^{RNAi} was not as prominent as co-expression of two different pathway components (Fig. 5.2 A). Co-expression of other pathway components with Yki caused less growth of haltere compared to UAS-ex^{RNAi}(Fig. 5.2 A). Taken together these results suggest that modulating more than one pathway at a time has more severe phenotypic effect than one pathway at a time. This is reflective of Ubx regulating multiple pathways to control size of the haltere.

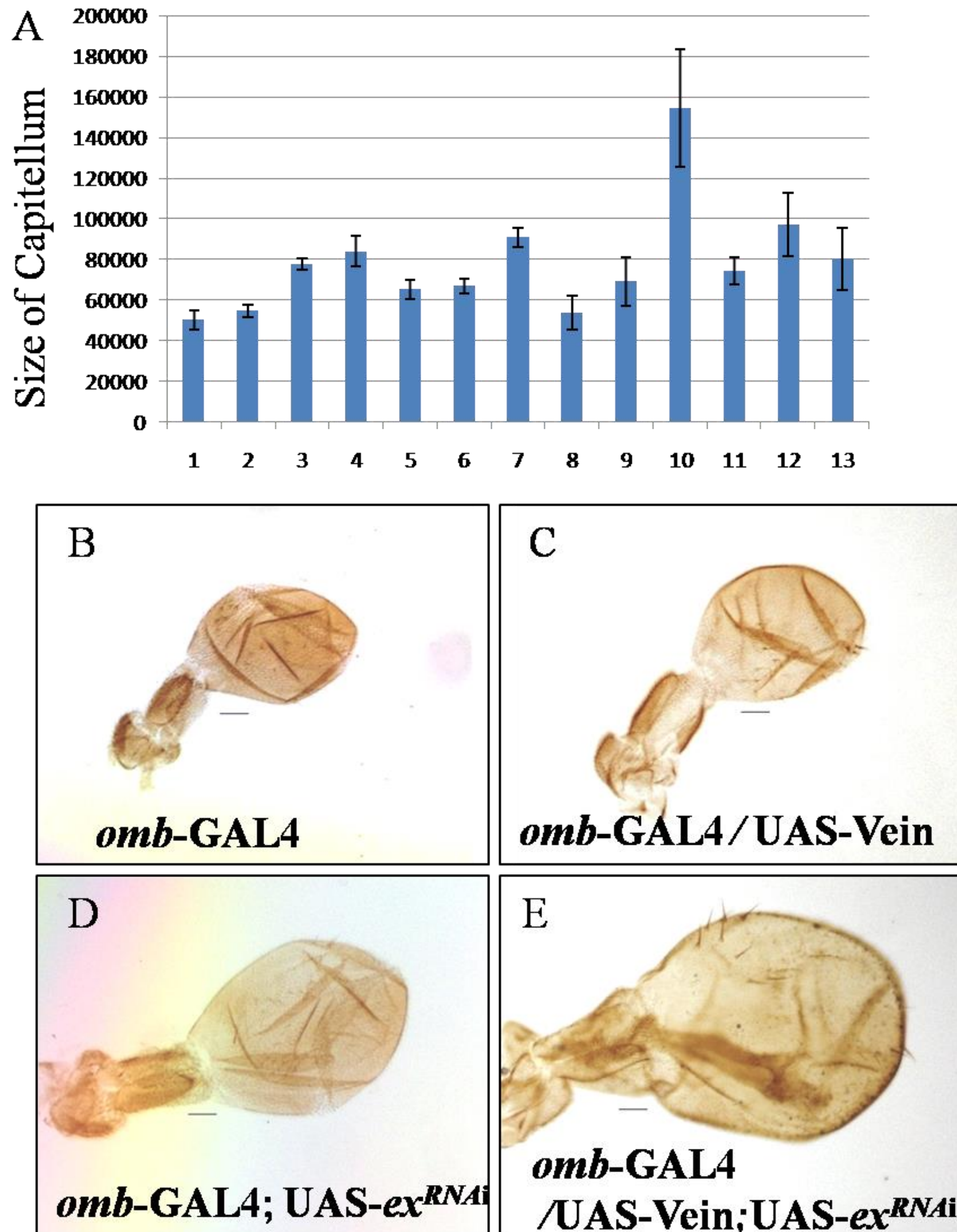


Figure 5.2 Growth phenotype in the haltere capitellum on co-expression of Hippo with other pathways.

(A)Graph represents increase in area of haltere capitellum. The genotypes are: 1. Wild type (n=10), 2.*omb*-GAL4;UAS-Yki (n=11), 3.*omb*-GAL4;UAS-ex^{RNAi}(n=9), 4.*omb*-GAL4;UAS-Yki/UAS-ex^{RNAi} (n=10) 5.*omb*-GAL4;UAS-Akt(n=10), 6.*omb*-GAL4;UAS-Akt;UAS-Yki (n=10),7.*omb*-GAL4;UAS-Akt;UAS-ex^{RNAi}*(n=10) 8.omb*-GAL4/UAS-Vein (n=10), 9.*omb*-GAL4/UAS-Vein;UAS-Yki (n=9), 10.*omb*-GAL4/UAS-Vein; UAS-ex^{RNAi}*(n=11), 11.omb*-GAL4;UAS-Vg (n=13), 12.*omb*-GAL4;UAS-Vg/UAS-ex^{RNAi}(n=12),13.*omb*-GAL4;UAS-Yki/UAS-Vg(n=9).

Expression of Vein in ex^{RNAi} background causes highest growth of haltere capitellum. Fold increase in the size of haltere capitellum was measured using NIH Image J software. (B) An adult *omb*-GAL4 haltere as control, (C) Expression of Vein using

omb-GAL4 shows presence of 2 to 3 bristles with no increased growth of capitellum. (D) *omb*-GAL4/UAS-Vein;UAS-Yki haltere. (E) *omb*-GAL4/UAS-Vein;UAS-ex^{RNAi} haltere. Note increased in size with 6 to 7 ectopic bristles. Error bar represents SD.

5.2.2 Haltere capitellum shows dramatic growth response on expression of Akt in ex^{RNAi} background using *Ubx*-GAL4 driver

We further examined the combined effect on haltere growth by Akt and Hippo pathways in haltere using *Ubx*-GAL4 driver. As mentioned in the previous chapter, over-expression of Akt with *Ubx*-GAL4 driver did not show any growth phenotype in the haltere capitellum (Chapter 3 and Fig. 5.3B). UAS-Akt was combined with either UAS-Yki or UAS-ex^{RNAi} and crossed to the *Ubx*-GAL4 driver. UAS-Akt with UAS-Yki resulted in more than additive increase in size of haltere capitellum without any change in number of ectopic bristles present on the haltere when UAS-Yki alone was crossed to the GAL4 driver (Fig. 5.3D, C). UAS-ex^{RNAi} along with UAS-Akt caused synergistic growth effect with no change in number of ectopic bristles compared to UAS-ex^{RNAi} alone (Fig. 5.3 F, E). These results imply that Akt along with Hippo pathway regulates the growth of haltere without affecting the patterning. Therefore, it is likely that when both Hippo pathway and Akt are activated simultaneously, haltere achieves increase in both cell number and size.

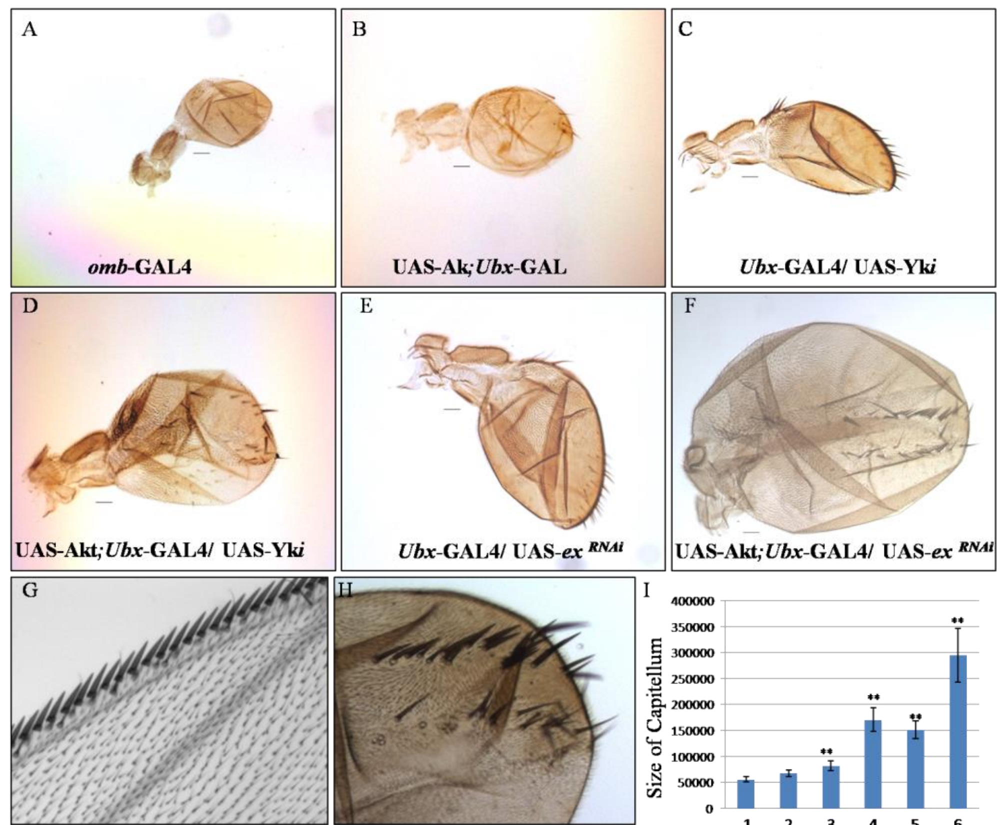


Figure 5.3 De-repression of both Akt and Hippo pathways is required to deregulate growth of haltere capitellum

Adult halteres of (A) *Ubx*-GAL4, (B) UAS-Akt;*Ubx*-GAL4, (C) *Ubx*-GAL/UAS-Yki, (D)UAS-Akt; *Ubx*-GAL4/UAS-Yki,(E) *Ubx*-GAL4/UAS-ex^{RNAi} and (F) UAS-Akt;*Ubx*-GAL4/UAS-ex^{RNAi}. (G) An adult wing with two rows of marginal bristles. (H)UAS-Akt;*Ubx*-GAL4/UAS-ex^{RNAi} bristles nearly as wing type. (I) graph represents size of haltere capitellum of genotype: 1. *Ubx*-GAL (n=20), 2. UAS-Akt;*Ubx*-GAL4 (n=11), 3. *Ubx*-GAL4/UAS-Yki (n=21), 4. *Ubx*-GAL4/UAS-ex^{RNAi}(n=20), 5. UAS-Akt;*Ubx*-GAL4/UAS-Yki(n=9), 6.UAS-Akt;*Ubx*-GAL4/UAS-ex^{RNAi}(n=15). Error bar represents SD and **$P < 0.001$. Please note increase in the size of the haltere capitellum is highest when Akt is over-expressed in the background of knock-down of *ex*.

5.2.3 Akt is activated by Yki in haltere

Akt is known to be activated by Yki in wing discs (Ye et al., 2012), while it is a direct target of Ubx in the haltere. We examined if over-expression of Yki can override the effect of Ubx and activate Akt. We over-expressed constitutively active form of Yki, Yki^{S168A}, using *Ay-GAL4* and observed levels of Akt in both wing and haltere discs (Fig. 5.4). We observed increased levels of Akt in both wing and haltere discs. It is likely that the growth phenotype observed in adult haltere on combining Akt and Yki (or *ex*^{RNAi}) is because of the compounded effect of Yki.

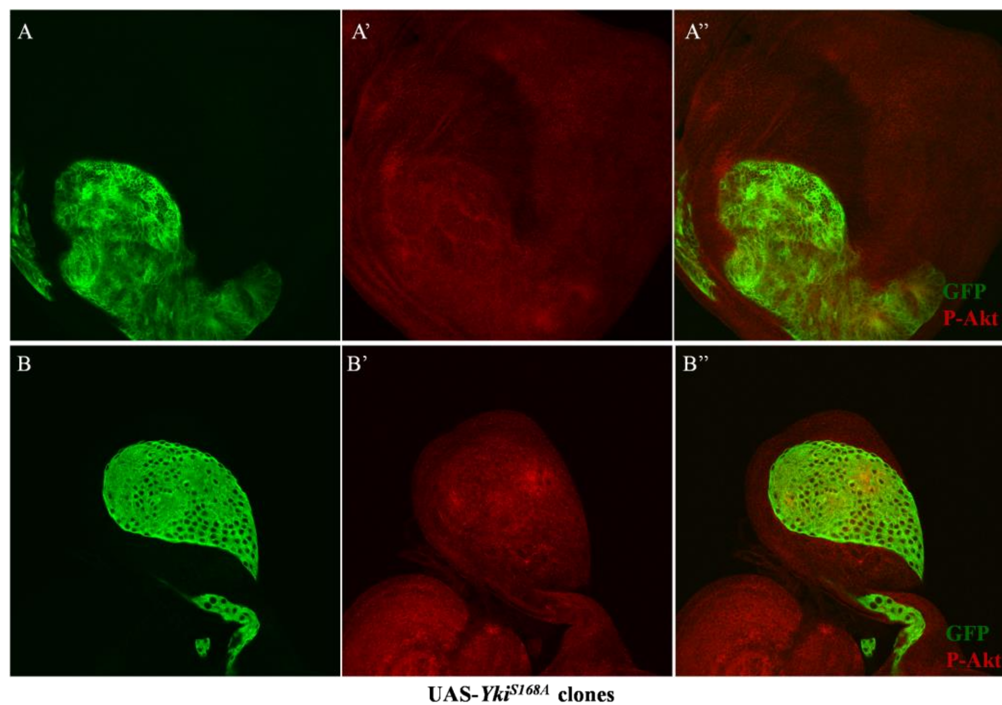


Figure 5.4 Activation of Akt by over-expressed Yki in haltere discs
(A-A'')Third instar Ay-GAL4/UAS-*Yki*^{S168A}; UAS-GFP wing and (B-B'') haltere imaginal discs stained with GFP (green) and P-Akt (red). Clones of *Yki*^{S168A}was generated by crossing with *AyGAL4.* Akt expression is increased in GFP expressing clones in both wing and haltere discs.

5.2.4 *bantam* regulation in haltere

bantam microRNA, a known regulator of both proliferation and apoptosis, is essential for Yki induced over-proliferation (Nolo et al., 2006, Thompson and Cohen, 2006). As shown in the previous chapter, compared to all the tested targets of Hippo pathway using *omb*-GAL4 driver, over-expression of *bantam* caused homeotic transformation of haltere-to-wing, albeit partial, marked with prominent growth of haltere capitellum and wing-like ectopic sensory bristles (Chapter 4). Moreover, *bantam* is differentially expressed in wing and haltere. While it is expressed in the whole pouch of haltere, it is down-regulated in the D/V boundary of wing pouch (Chapter 4). Recently, *bantam* has been shown to be a common effecter of Hippo and other signaling pathways such as Notch, EGFR, IIS and Dpp (Herranz, et al., 2008; Oh and Irvine, 2011; Chen et al., 2011; Herranz et al., 2012).

To further understand the regulation of *bantam* expression in haltere, we examined its expression pattern with the help of enhancer *br-C12* Lac-Z, which is dependent on both Yki and Mad (Oh and Irvine, 2011). Expression pattern of *br-C12* Lac-Z is similar in wing and haltere discs (Fig 5.5), suggesting that differential regulation of signaling pathways has no role in regulating *bantam* activity in haltere. We had shown in the previous chapter that haltere discs expresses higher levels of *bantam* than wing discs even while Hippo pathway is down regulated. It is therefore likely that over-expression of Yki will have compounded effect in haltere due to prior activation of *bantam* by Ubx.

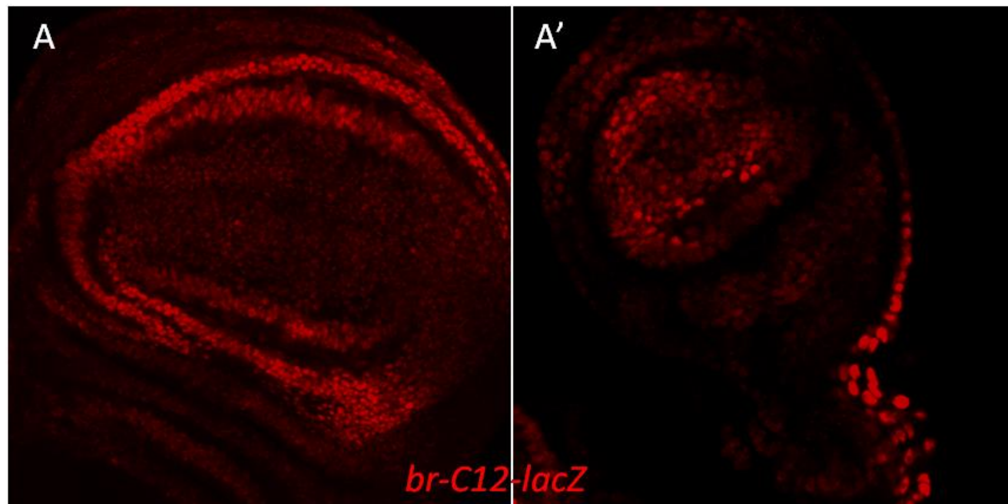


Figure 5.5 bantam regulation in haltere disc is independent of Yki-Mad binding on itsbr-C12 regulatory region

(A and B) Third instar wing and haltere imaginal discs stained with Lac-Z antibody showing no differential expression of br-C12 Lac-Z.

5.2.5 Down-regulation of *ex* and activation of Akt together induces wing-type cellular differentiation in haltere

Previously we have shown that loss of *ex* using *Ubx*-GAL4 caused partial change in morphology of haltere trichomes towards wing type (Chapter 4). One can speculate here that increased haltere growth is responsible for less densely arranged trichomes. In order to investigate the above speculation, we looked at morphology of overgrown UAS-Akt;UAS-Yki and UAS-Akt;UAS-ex^{RNAi} halteres. SEM images of the UAS-Akt;*Ubx*-GAL4/UAS-Yki trichomes showed no change in morphology, while trichomes of overgrown halteres of UAS-Akt;*Ubx*-GAL4/UAS-ex^{RNAi} were modified to wing type (Fig 5.6 G, H and I). They were longer and less densely arranged as compared to *Ubx*-GAL4/UAS-ex^{RNAi} alone. No change in trichome morphology was observed in UAS-Akt;*Ubx*-GAL4 haltere (Fig. 5.6F). Although down regulation of *ex* and not up-regulation of Yki along with over-expressed Akt in Ubx heterozygous background caused transformation of trichome morphology, it is likely that Hippo pathways plays a major role in regulating both growth and differentiation during wing development. Perhaps, this pathway connects the two seemingly different events during development.

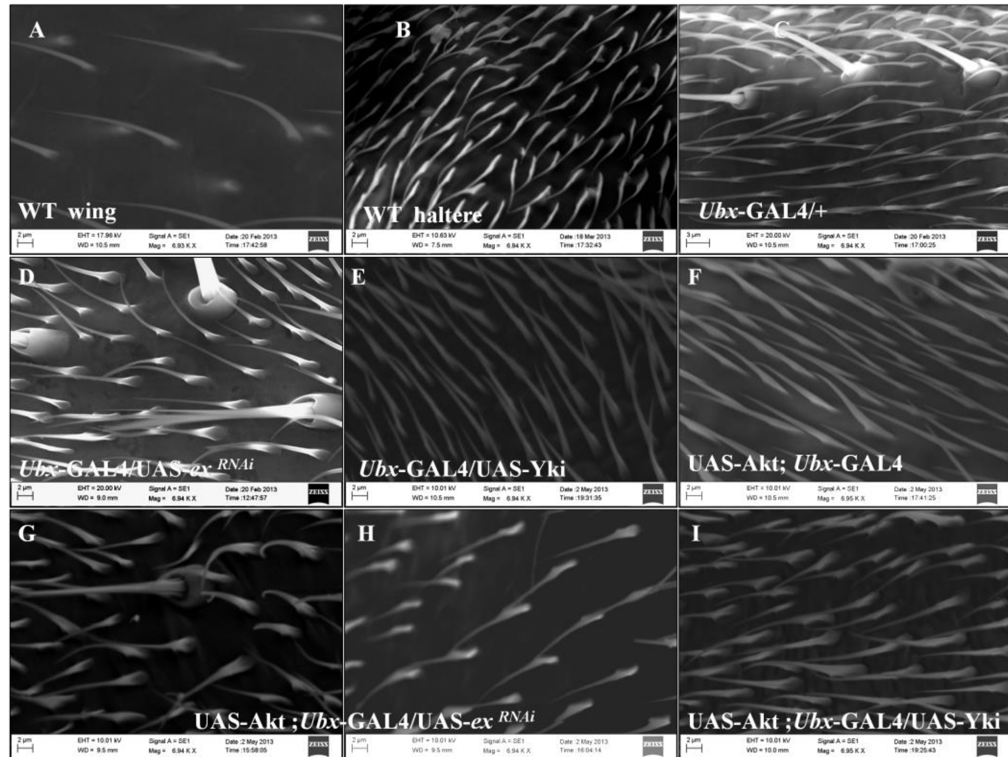


Figure 5.6 Haltere-to-wing homeotic transformations at the level of trichome morphology

SEM images of adult female wing and halteres of genotypes as indicated. (A) Wild type wing blade showing trichomes of intervein region, which are arranged less densely. Small dots represent trichome of other side of the wing blade reflecting the fact that wing blade is a very thin organ. (B) Wild type haltere with highly dense trichomes, which are shorter in length with flat base. (C) *Ubx*-GAL4 haltere. Trichomes are less densely arranged and are slightly longer. (D) *Ubx*-GAL4/UAS-*ex*^{RNAi} haltere. Trichomes are even less sparely arranged compared to C, although they are shorter and thicker like in B. (E) *Ubx*-GAL4/UAS-Yki and (F) UAS-Akt ;*Ubx*-GAL4 halteres. Trichomes are similar to *Ubx*-GAL4. (G) and (H) UAS-Akt:*Ubx*-GAL4/UAS-*ex*^{RNAi} halteres showing images of two different haltere regions. Note trichome arrangement is almost like in the wing blade and morphology too is more wing-type. (I) UAS-Akt; *Ubx*-GAL4/UAS-Yki haltere. The trichome arrangement and morphology is similar to that of *Ubx*-Gal4.

5.2.6 Haltere discs show higher growth response to loss of *ex* than wing discs

We next examined the growth response at compartment levels.The posterior compartment was stained with anti-en antibodies and A/P compartment ratio was measured. A/P compartment ratio was 1.18 for wild type wing disc and 2.41 for haltere disc (Fig. 5.7E).UAS-*ex*^{RNAi} was expressed in the posterior compartment using posterior-specific *en*-GAL4 (Fig. 5.7A-D). Haltere showed higher increase in area of the posterior compartment as compared to wing with A/P compartment ratio changing to 0.894 in wing and1.378 in haltere (Fig. 5.7E). It is likely that expression of UAS-*ex*^{RNAi} in haltere disc results in a change in A/P compartment towards wing type.

Ubx-GAL4 is expressed in the anterior compartment of haltere imaginal discs. We examined the effect of expressing UAS-Akt, UAS-Vein, UAS-*ex*^{RNAi} and UAS-Akt;UAS-*ex*^{RNAi} in the anterior compartment using *Ubx*-GAL4 driver on the ratio of A/P compartment size. Expression of UAS-Akt and UAS-vein alone in haltere had no significant effect on A/P ratio (Fig 5.7F). In contrast to this, expression of UAS-*ex*^{RNAi} and UAS-Akt;UAS-*ex*^{RNAi} caused significant increase in A/P compartment ratio (Fig 5.7F).

Next, we examined the effect activating different signalling pathways at cellular level using clonal approach. Wing and haltere imaginal primordial are specified in the late embryo. After four days of larval growth, the mature wing disc contains approx. 50,000 cells, while the haltere consists of approx. 10,000 cells. We generated clones by crossing *Ay-GAL4* to UAS-lacZ, UAS-*ex*^{RNAi}, UAS-Yki, and UAS-vein. Heat shock was given at two time points' viz. 37-49 hrs and 65-77 hrs after egg laying (AEL) and growth or any change in the size of clones was quantified by measuring surface area of clones. Clone size was identical in wing and haltere Lac-Z control clones, implying that the difference in number of cells between wing and haltere is irrespective of their proliferation rates (Morata and Garcia-Bellido, 1976). At 65-77 hrs AEL of heat shock, UAS-Yki andUAS-*ex*^{RNAi} clones showed increased growth compared to control Lac-Z clones (Fig 5.8). This we observed in both wing and haltere discs. UAS-vein clones, however, did not show any increase size compared to Lac-Z clones (Fig 5.8). The fold change in UAS-*ex*^{RNAi} clone

size, when compared to the control Lac-Z clones, was higher in haltere discs than in wing discs (Fig 5.8).

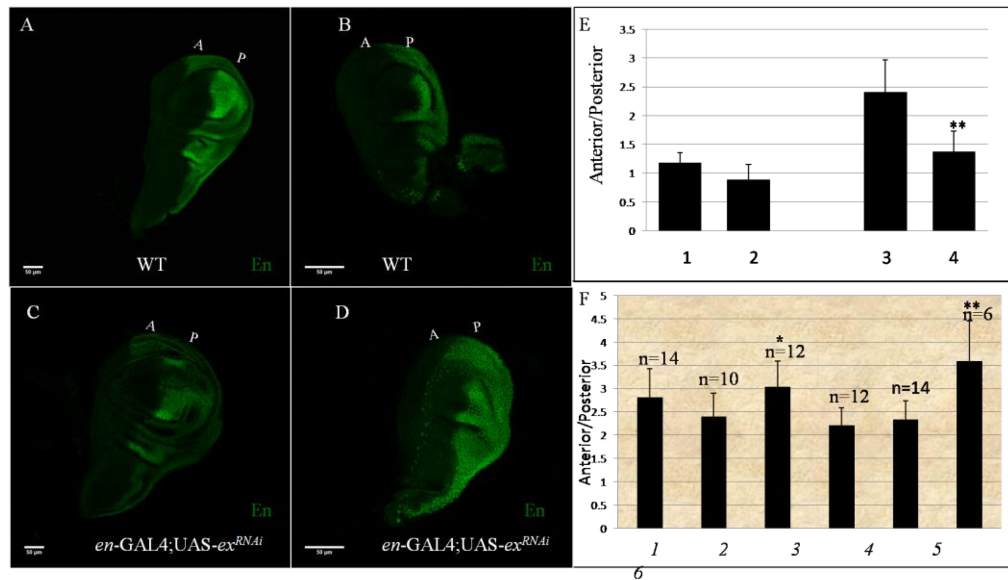


Figure 5.7 Higher growth responses by posterior compartment of haltere disc than that of wing disc on down-regulation of *ex*

(A, B) Wild type wing (A) and (B) haltere imaginal discs stained for En. (C, D) *en*-GAL4;UAS-*ex*^{RNAi}wing (C) and haltere (D) imaginal discs. (E) Graph showing change in A/P compartment ratio due to decease in the levels of Ex. The genotypes in the graph are 1.*en*-GAL4 wing (n=16), 2. *en*-GAL4;UAS-*ex*^{RNAi} wing (n=10), 3.*en*-GAL4 haltere (n=8), 4. *en*-GAL4;UAS-*ex*^{RNAi} haltere (n=5). Note more drastic change in the A/P ratio in the haltere compared to wing in response to knock-down of *ex*. (F) Graph showing change in A/P ratio on expresssion of various components using *Ubx*-GAL4 driver which is expreseed only in the anterior compartment of haltere imaginal discs. The genotypes are: 1. Wild type, 2.*Ubx*-GAL4/UAS-GFP, 3.*Ubx*-GAL4/UAS-*ex*^{RNAi}, 4.UAS-Akt;*Ubx*-GAL4, 5.UAS-vein;*Ubx*-GAL4, 6.UAS-Akt;*Ubx*-GAL4/UAS-*ex*^{RNAi}. No difference is observed in A/P ratio between wild tpe and *Ubx*-GAL4/UAS-GFP discs. Note, significant increase in A/P ratio in *Ubx*-GAL4/UAS-*ex*^{RNAi} *and* UAS-Akt;*Ubx*-GAL4/UAS-*ex*^{RNAi} compared to*Ubx*-GAL4/UAS-GFP. A(anterior) and P (posterior). Error bar represents SD. *$P < 0.05$, **$P < 0.01$.

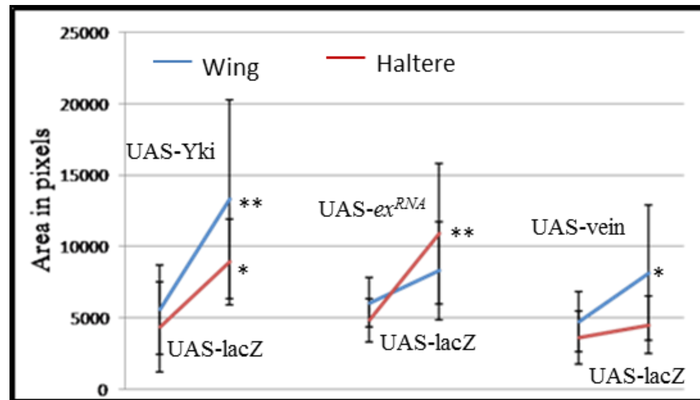


Figure 5.8 Relative sizes of clones with increased Yki activity

AyGAL4 was crossed to UAS-*ex*^{RNAi}, UAS-Yki, UAS-vein and UAS-lacZ as control. Graph represents size of clones induced after 65 -77hrs AEL. Blue line wing (w); Red line haltere (h); N=number of discs; n= number of clones. {UAS-Yki, w(,N=11,n=17), h(N=11,n=15) UAS-lacZ, w(N=8,n=13), h(N=3,n=6) }, {UAS-*ex*^{RNAi} w(N=12, n=13), h(N=5,n=5); UAS-lacZ, w(N=9,n=12), h(N=6,n=9)}, {UAS-Vein, w(,N=7,n=8), h(N=6,n=10) UAS-lacZ, w(N=10,n=17), h(N=5,n=12) } . There was no difference in the size of LacZ clones between wing and haltere discs (In lines lower points represent control and upper points are test). Clones of UAS-Yki and UAS-*ex*^{RNAi} show steep increase in the size of the clones in haltere discs compared to wing discs. UAS-vein clones, however, are of the larger size compared to the control only in wing discs. UAS-*ex*^{RNAi} clones are larger haltere discs compared to wing suggesting that wing discs have additional growth control mechanisms, which haltere discs lack. Error bars represents SD; ** p =<0.001; * p=<0.006.

5.2.7 Combination of Akt over-expression and down regulation of *ex* results in increase in haltere cell size

Based on growth response of haltere at adult and compartment levels, we next examined response at cellular level. Surface area occupied by 10 cells was measured. The wing and haltere cells are similar in size and shape at the third instar disc stage. Expression of UAS-ex^{RNAi} in wing and haltere, and UAS-vein in wing using *omb*-GAL4 caused increased disc size with no change in cell size (Fig. 5.9D, I and C), indicating that the growth is because of change in number of cells. Expression of UAS-Akt with *omb*-GAL4 resulted in increase in cell size in wings (Fig. 5.10, B). But, haltere cells did not differ in size (Fig. 5.9, G). However, co-expression of UAS-Akt and UAS-ex^{RNAi} resulted in cell size increase in both wing and haltere discs (Fig. 5.9E and J) and cells were rounder in appearance. Interestingly, surface area of cells increased to similar extent in both wing and haltere discs (Fig. 5.9K). Additionally, we looked at effect on cell growth using *Ubx-GAL4* driver which is expressed mostly in the anterior compartment of haltere discs, and posterior compartment of the same discs was used as control. UAS-Akt, UAS-ex^{RNAi}, and UAS-vein did not show any effect on surface area of cells (Fig 5.10, G). UAS-Akt*;Ubx*-GAL4/ UAS-ex^{RNAi} expressing haltere cells showed dramatic increase in cell size (Fig 5.10, E and F). Surprisingly, along with autonomous increase in the size of anterior compartment cells, posterior compartment cells also increased in their size (Fig 5.10, G).

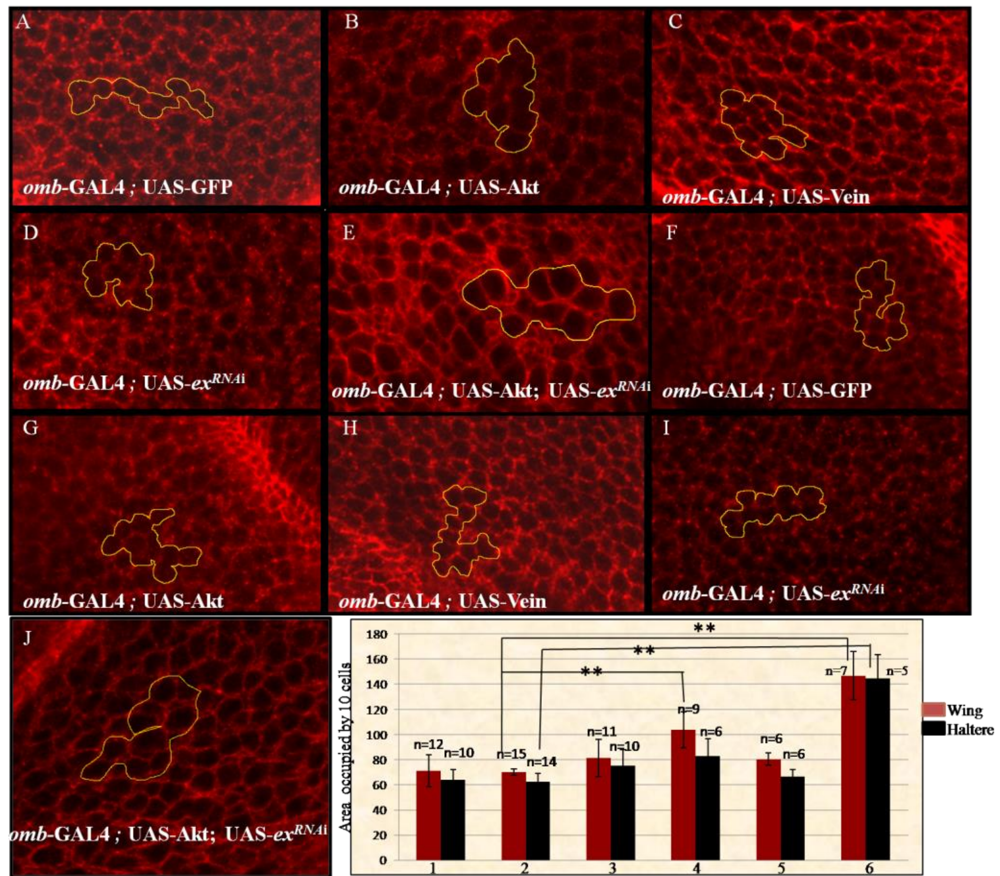


Figure 5.9 Upregulation of Akt in the background of down-regulation of *ex* causes increase in the size of haltere cells

Confocal sections of anterior compartment region of third instar wing (A-E) and haltere (F-J) imaginal discs stained with armadillo (red) of genotypes as written on the images. All discs show equivalent region in the anterior compartment and all are imaged at identical magnification (63X) and adjusted to similar magnification. (K) Graph showingeffect on the area occupied by 10 cells of genotype: 1. Wild type, 2.*omb*-GAL4;UAS-GFP, 3. *omb*-GAL4;UAS-ex^{RNAi}, 4. *omb*-GAL4;UAS-Akt, 5. *omb*-GAL4;UAS-vein, and 6. UAS-Akt*;omb*-GAL4/UAS-ex^{RNAi}. Error bar represents SD. **P < 0.001. Size of *omb*-GAL4;UAS-Akt (B, 4) wing cells are marginally higher compared to *omb*-GAL4;UAS-GFP (A, 1), which is used as control. UAS-Akt;*Ubx*-GAL4/UAS-ex^{RNAi} wing cells (E, 6) show dramatic increase in size with charecterstics of highly dividing round cells. *omb*-GAL4/UAS-Akt haltere cells (G, 4) show no growth of cells while UAS-Akt;*Ubx* GAL4/UAS-ex^{RNAi}(J, 6) cells show increased size, to the same extent as wing cells.

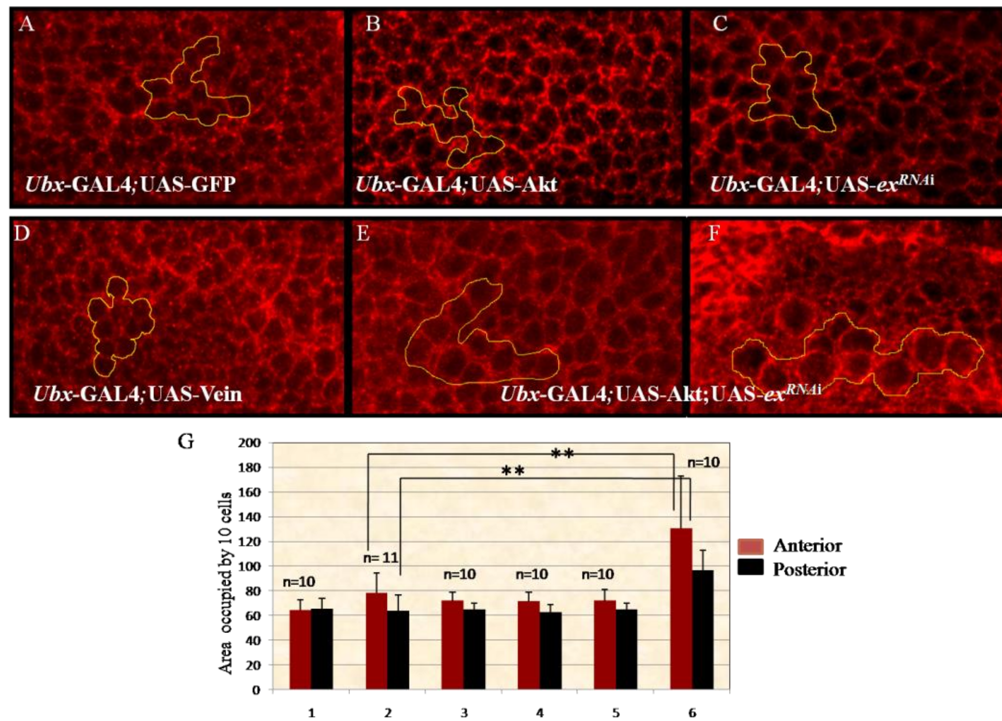


Figure 5.10 Upregulation of Akt in the background of down-regulation of *ex* causes non-cell autonomous growth

Confocal sections of anterior compartment of third instar haltere imaginal dsics stained with armadillo (red) to outline cells of genotypes as written on the images. Sizeof cells expressing Akt in the background of reduced *ex* is higher (E and F) than those of control discs (A) also the cells are round in appearence, features of highly dividing cells . All images are taken at identical magnification (63X) and adjusted to similar magnification.. G) Graph representing effect onarea occupied by 10 cells of genotype from selected regions of anterior and posterior compartments: 1. Wild type, 2.*Ubx*-GAL4;UAS-GFP, 3. *Ubx*-GAL4/UAS-*ex*^{RNAi}, 4. UAS-Akt;*Ubx*-GAL4, 5. UAS-vein;*Ubx*-GAL4, 6. UAS-Akt;*Ubx*-GAL4/UAS-*ex*^{RNAi}. Note, increase in cell size in the posterior compartment in UAS-Akt;*Ubx*-GAL4/UAS-*ex*^{RNAi} haltere discs. Error bar represents SD. *P < 0.05 ,**P < 0.005.

5.2.8 Temporal regulation of pathway components in haltere

Expression of various genes construct (causing over-expression or RNAi-mediated knock-down) with *Ubx*-GAL4 driver had visible effect on growth at the level of adult halteres compared to other drivers (Fig. 5.3). Interestingly, the severity of growth response at adult stage in *Ubx*-GAL4/UAS-ex^{RNAi} and UAS-Akt;*Ubx*-GAL4/UAS-ex^{RNAi} (Fig 5.3) is not demonstrated by the third instar larval discs of those genotypes (Fig 5.11, D-D'). To further figure out the stage at which knock-down of *ex* is required for such dramatic growth response, we temporally controlled the expression of UAS-ex^{RNAi} using *Ubx*-GAL4/*tub*-$Gal80^{ts}$.*Ubx*-GAL4 expression starts at early stages of development and remains throughout the pupal stage (Pallavi and Shashidhara, 2003). We restricted the activity of Ex by incubating embryos at 19^{0}C, and transferring them to 29°C at different stages of development to activate the GAL4 protein.The flies showed transformation only when *ex* was down regulated from embryonic to early larval stages. Down-regulation of *ex* at post wandering third instar larval stages did not show any phenotype. This experiment suggests that Hippo pathway regulates haltere growth at early larval stages.

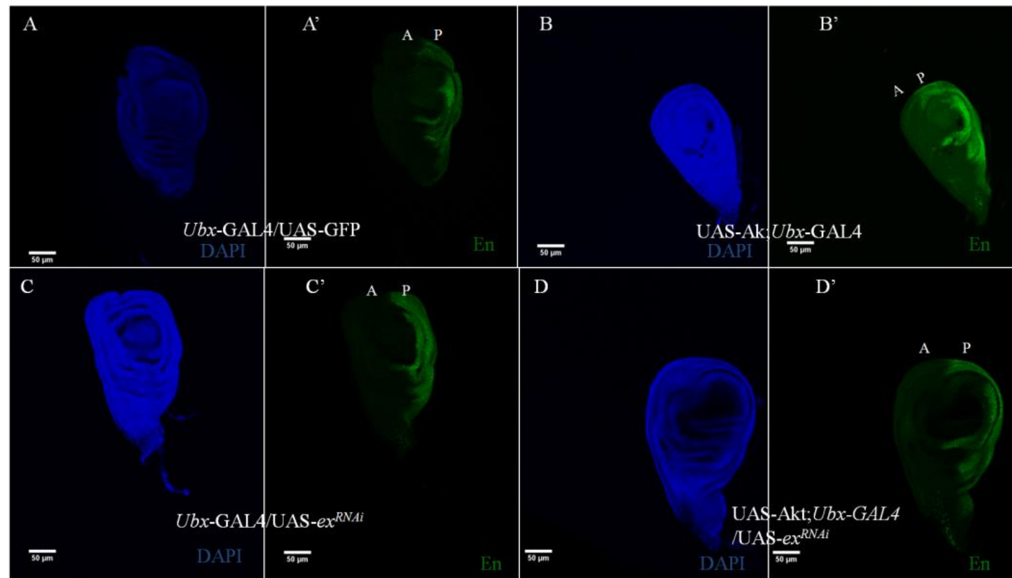


Figure 5.11 Haltere imaginal disc is more sensitive to changes in Hippo/Akt pathways at early larval stages

Confocal imagesof third instar haltere imaginal disc stained with DAPI(blue) and En(green) of genotpyes as shown on the images. Note haltere capitellum of genotype *Ubx*-GAL4/UAS-*ex*^{RNAi}*,* UAS-Akt*;Ubx*-GAL4/UAS-*ex*^{RNAi},which showed significant growth of haltere capitellum at adult stage (Fig 5.3), show only marginal increase in the size of the disc (comapred to *Ubx*-GAL4/UAS-GFP, which is used as control). A (anterior) and P (posterior).

6.3 Discussion

6.3.1 Hippo and other signaling pathways in haltere

Ubx regulates various pathways at multiple levels to specify the haltere and makes it difficult to reverse the fate to wing type by mutations in a single gene other than in *Ubx* itself. Co-expression of Vg and Dpp, major components of two different pathways involved in growth regulation, while induced homeotic transformations at the levels of trichomes and sensory bristles, induced only marginal increase in the size of the haltere capitellum (Mohit et al., 2006; Makhijani et al., 2007). As Hippo pathway appears to be a critical target of Ubx, we explored the effect of de-repressing this signaling pathway in combination with one or more other pathways.

All the components of various pathways tested here, such as Akt, vein, and Vg are common targets of both Ubx and Yki and are down regulated in haltere discs. Consistent with this, Vg is activated in haltere discs when Yki is over-expressed (Goulev et al., 2008). Similarly, Akt is activated by the over-expressed Yki in haltere discs (Fig. 5.4). Expression of Vg and Dpp with Hippo pathway components resulted in pupal lethality and couldn't be investigated further. We observed dramatic overgrowth phenotype when Akt is over-expressed in the background of loss of *ex*. Higher growth response observed with Akt and ex^{RNAi} could be due to additional activation of Akt when ex is down regulated. Interestingly, co-expression of vein a ligand of EGFR pathway in the background of ex^{RNAi} resulted in even stronger enhancement of size of the haltere capitellum (Fig. 5.2). This suggests that EGFR and Hippo pathway interactions are very critical during growth regulation. Unpublished data from the laboratory of S Cohen suggests that when Hippo is down regulated in the background of over-expressed EGFR, wing discs become highly tumorous, which is consistent with our observations in the haltere.

A recent study to identify the Hippo pathway interactome in Drosophila has identified 153 high confidence interacting proteins out of which 102 of them affect the transcriptional activity of Yki (Kwon et al.,

2013). This protein-protein network connects the Hippo pathway to proteins involved in other signaling pathways and cellular processes such as interactions with cytoskeletal components in the regulation of cell division, orientation and positioning anaphase spindle and astral microtubules, spindle organization, endocytosis and vesicle trafficking complexes. Also out of 153high confidence interacting proteins, many of them are also potential targets of Ubx (some are listed below Table 5.1). This further indicates possible role of partners of Hippo signaling pathway and their crosstalk in haltere as an important step regulated by Ubx. These, however, needs to be tested experimentally.

Sd	*Dp1*	*Eif4G*
14-3-3zeta	*ena*	*Aly*
tai	*Lasp*	*ft*
ex	*Myo61F*	*fj*
CG4674	*pod1*	
CG10741	*Rm62*	
Wts	*kst*	
Fax	*RpII215*	
Ote	*cora*	
Ds	*CG3226*	

Table 5.1 Genes common between protein-protein interactome data of Hippo pathway (Kwon et al., 2013) and targets of Ubx (Agrawal et al., 2011; Choo et al., 2011).

6.3.2 IIS and Hippo signaling pathway in haltere

In chapter 3, we have reported that over-expression of Akt, a central component of the IIS pathway, has no haltere phenotype at cellular, compartment or organ levels. In this chapter we have shown that over-expressed Akt in the background of down regulated *ex* or over-expressed Yki does induce significant changes in the growth of haltere at cellular, compartmental and organ levels. At adult level, UAS-Akt;UAS-ex^{RNAi} and UAS-Akt;UAS-Yki with both *omb*-GAL4 and *Ubx*-GAL4 were able to induce these phenotypes in the haltere capitellum (Fig. 5.2 and Fig. 5.3). Size of the adult haltere capitellum of *Ubx*-GAL4; UAS-Akt;UAS-ex^{RNAi} is the largest so

far obtained by manipulating downstream targets of Ubx in wild type or *Ubx* heterozygous backgrounds. As these halteres did not show change in the number of sensory bristles, it is likely that the increase in size is not directly associated with cell proliferation. When examined in more detail, we observed that trichomes organization and morphology in these halteres are similar to those of wing blade. Thus, the increase in the size of the capitellum could be due to more flattened cells, which are also arranged more sparsely in contrast with normal haltere trichomes, which are stouter and densely packed. This observation provides a new experimental tool to address a decades old question in developmental biology, how growth and pattern formation (or differentiation) is linked.

One line of investigation could be: does Hippo pathway by activating Akt change, cell number, size, morphology and thereby organ size and morphology. If so, what is the precise molecular mechanism? This could be by acting on the cytoskeletal organization of the epithelial cells. Roch and Akam (2000) have shown that wing cells have lower levels of F-actin than haltere cells and this is evident by 36h after puparium formation (APF). Haltere cells for reasons of more F-actin may become more rigid and thus may resist any other cellular processes that make them flattened. This may lead to their denser organization. Such cellular shape and organization when combined with reduced cell proliferation may make haltere much smaller and bulbous. This could be the reason why we frequently observed phenotype at the levels of the adult haltere and not at the larval disc level, even when the critical stage of requirement of Hippo pathways is early larval stages. Validation of this needs further investigation of actin cytoskeleton in haltere cells during pupal development.

Chapter 6

6. Summary and Future Work

6.1 Summary

The Homeotic/*Hox* genes are highly conserved throughout metazoan evolution and code for homeodomain containing transcription factors that control specific developmental pathways. The Hox gene, *Ultrabithorax (Ubx)* is expressed in third thoracic segment of *Drosophila* and regulates many wing patterning genes to form haltere. Loss of *Ubx* from developing haltere and ectopic expression of Ubx in wing causes haltere-to-wing, wing-to-haltere transformations, respectively. Genome wide and transcriptome studies identified many genes/pathways involved in wing development as targets of Ubx in haltere. Ubx regulates cellular processes such cell division, cell proliferation, cell differentiation, cell size, and cell affinity in developmental stage specific manner to provide haltere a distinct morphology. As compared to wing, haltere is highly reduced in size, they lack wing type marginal bristles, vein/inter-vein, have reduced cell size, different cell shape and densely arranged trichomes. However the functionality of genes and pathways required by Ubx to provide identity to haltere are not completely known. Functional relevance of only few Pathways like Wingless (Wg), Decapentaplegic (Dpp), and epidermal growth factor receptor (EGFR) has been studied previously. Here, we have addressed how differences in cell size and shape influence organ size and shape.

We have explained the role of two major organ size determining pathways in haltere i.e. Insulin/Insulin like signaling (IIS) pathway and Hippo pathway. Akt a central component of IIS pathway is down-regulated in haltere. Hippo pathway component Yorkie (Yki), a transcriptional co-activator protein is differentially regulated in wing and haltere. Also, regulation of upstream component like Expanded (Ex) and down-stream component like *bantam* microRNA is critical in haltere. Interestingly, while both over-expression of Yki and knock-down of ex caused significantly increased haltere size, degree of increase were much higher when *ex* expression is down regulated. This, suggests that de-regulation of both Yki-dependent and Yki

independent components of Hippo pathway is critical for liberating Ubx mediated regulation of haltere identity. While over-expression of Akt of IIS pathway or Vein of EGFR pathway did not affect haltere growth, when over-expressed in the background of down regulation of *ex* caused dramatic increase in haltere growth and differentiation of cell shape and organization to wing type. We conclude that regulation of Hippo pathway by Ubx is central to the modification of wing identity to that of haltere.

6.2 Future perspective

6.2.1 Regulation of *bantam* and Ex in haltere

bantam is up-regulated in haltere and over-expression of *bantam* causes Homeotic-transformation of haltere-to-wing (chapter 4). We observed no such transformation on down regulation of *bantam* in haltere. Ectopic expression of Ubx in wing causes reduction in size of wing. Further we co-expressed UAS-Ubx with UAS-*ban*, UAS-ex^{RNAi} in wing. Knock-down of *ex* was able to rescue the size of wing while no such rescue was observed with over-expression of *ban*. Suggesting that Ex is down-stream to Ubx and requires further validation. Further studies are required to understand the regulation of *ban* and its down-stream targets in haltere by Ubx. Also, further repetition of this result is required with more quantitative measurement of degree of growth responses.

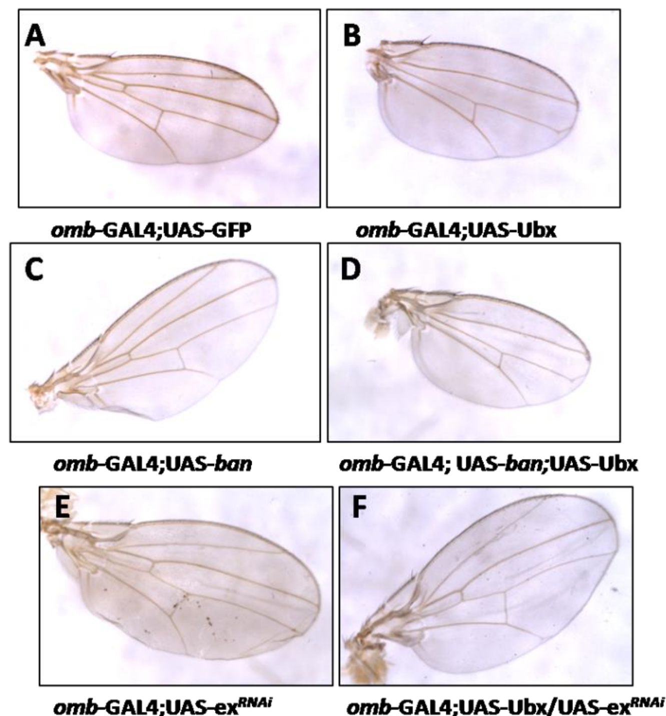


Figure 6.1 Regulation of *bantam* and Ex by Ubx
Genotypes are represented on figures (A) control adult wing (B) ectopic expression of Ubx, showing reduced size of wing (C) over-expression of *ban* causes growth of wing (D) no change in size of wing on expression of UAS-Ubx and UAS-*ban*. (D) Increase in size of wing on known-down of *ex* (E) knock-down of *ex* is able to induce growth of wing in presence of ectopic expression of Ubx.

6.2.2 Understanding relationship between Yki and Ubx

Genetic experiment shows that Yki and Ubx together are required in haltere. They share a large number of target genes. Yki is a transcriptional co-activator protein which requires other DNA binding partners to show its activity. Ubx is known to have both activator and repressor function and it directly binds to DNA. Understanding the relationship between Yki and Ubx requires further investigation. In addition, further investigation needed to understand how Ubx regulates Yki expression and translocation needs.

6.3.3 Link between Differential regulation of cytoskeleton organization in wing and haltere and Hippo pathway

Elucidating the mechanisms that regulates the growth to provide organ a specific organ size and shape is a fundamental question for Developmental biology. Recent studies indicate that the Hippo pathway is unique in that it is regulated by cellular architecture and the mechanical properties of the environment Wing and haltere are homologous organs and differs greatly in organ size and shape. Differences in morphology of trichomes, cell shape and size reflect differences in organization of cytoskeleton. F-actin cytoskeleton is known to be regulated differentially in wing and haltere. During our studies, we have observed that by modifying the expression of components of Hippo and other pathways, we could change organ size, cell shape, cell morphology etc. Further studies are required to understand the role of Hippo pathway in regulating the cytoskeleton of epithelial cells.

6.4.4 Haltere and Wing as a model system study cellular function of Hippo pathway

Hippo pathway is a tumour suppressor pathway. Although lot of studies have been done to understand the role of Hippo pathway, what is its role during development, particularly in specifying organ size, is not clear. Deregulation of various components Hippo pathway is known to be associated with different types of cancers. Here we observed that de-regulation of Hippo pathway in conjunction with one more signaling pathway leads to differentiation of haltere cells to Wing type. It is therefore possible that wing and haltere may be used as a model system to understand how various signaling pathways integrate together in normal and cancerous conditions.

6.5.5 Identifying cross-talk between Hippo and other signaling pathways

Compared to wing, haltere is dramatically reduced in size and has potential to reach to a size of wing. It has been shown here and earlier that roles of multiple signaling pathways are integrated to specify size and shape of the haltere. However, precise molecular nature of the cross-talk between Hippo and other signaling pathways needs further investigation.

6.5.6 Model for Regulation of Fat/Hippo pathway at multiple levels in the Haltere.

During our studies we found Fat/Hippo pathway as critical target of Ubx in haltere. Ubx regulates this pathway at multiple levels- by regulating Yki as well as by regulating upstream and downstream components of the pathway. Yki is present at higher level inside the nuclei and is present in non-activated form. However, the mechanism for differential regulation of Yki by Ubx in haltere requires further study in the following direction:

1. Upstream components of Fat/Hippo pathway like Ex, Hpo, and Wts can directly bind to Yki and thereby prevents its nuclear localization. Ubx can down regulate upstream components of this pathway causing Yki to be available to translocate inside nuclei.
2. Ubx can manipulate targets (potential targets of Ubx like HipK, Leash, MASK, Leash) that have role in Yki transcriptional activity, stability and translocation.
3. Ubx can down regulate DNA binding partners of Yki.
4. Yki is regulated by F-actin at different stages of development. F-actin levels are increased in clones over expressing Yki at pupal stage of development. Also F-actin regulates translocation of Yki inside the nuclei (Fang and Adler, 2010; Fernández et al., 2011). Further experiments at pupal stage of development are required to understand this reverse regulation in haltere.

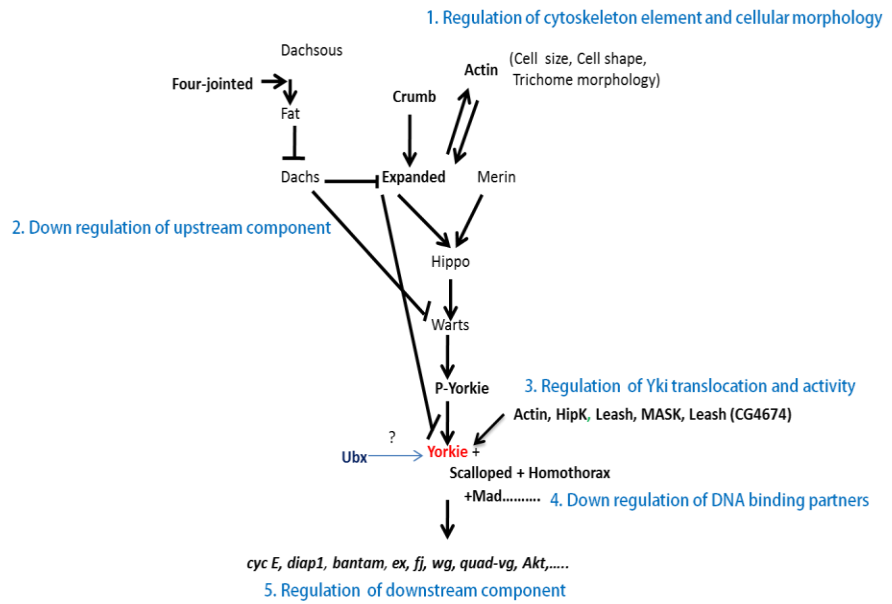


Figure 6.2 Ubx regulates Fat/Hippo pathway at multiple level.

Annexure:

Publication

Critical role for Fat/Hippo and IIS/Akt pathways downstream of Ultrabithorax during haltere specification in *Drosophila*

Savita Singh, Ernesto Sánchez-Herrero and L S Shashidhara

Mech Dev. 2015 Aug 20. pii: S0925 4773(15)30008-3. doi: 10.1016/j.mod.2015.07.017. [Epub ahead of print]

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