CHAPTER 1

INTRODUCTION

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Chapter 1

Introduction

One wonders at the diversity in shape and size of the body plan of organisms during the course of evolution. Discovery of homeotic transformations and Homeotic genes (or Hox genes) have revolutionized the field of Developmental Biology by empowering biologists to redesign and shape animal morphology and thereby study underlying morphological mechanisms. Fascination to unravel many more genes and developmental mechanisms that set up simple construction rules behind these body structures is ever increasing since the days of discovery of Hox genes.

1.1. Model organisms

Depending on number of factors such as - researcher’s interest of exploring a particular biological event, feasibility of culturing and manipulating them in accordance with experimental requirements, particular species of organisms or model organisms are chosen. There are number of model organisms, including viruses, prokaryotes such as Escherichia coli, Pseudomonas fluorescens etc and eukaryotes such as protists, fungi (baker yeast Saccharomyces cerevisiae, Neurospora crassa), plants (Arabidopsis thaliana, Oryza sativa) and animals. Among animals, invertebrates such as Drosophila melanogaster, Caenorhabditis elegans, Hydra etc and vertebrates such as Mouse (Mus musculus), Chick (Gallus gallus domesticus), African clawed frog (Xenopus laevis), Guinea pig (Cavia porcellus), Zebrafish (Danio rerio) etc are widely used. Other than these organisms, number of cell lines are cultured and used to meet research requirements. Extensive conservation of metabolic and developmental pathways and gene sequences aid researchers to extrapolate and provide insights into other organisms including human. For example, mouse disease models are used to investigate potential causes and develop novel treatments to cure human diseases. In the present study Drosophila melanogaster has been used as the model organism.
1.2. *Drosophila* as a Model organism

*Drosophila* is a tiny microscopic organism that gets attracted to the smell of fruits and hence popularly called fruit flies. Thomas Hunt Morgan introduced this model organism for cytogenetic and genetic studies and Ed Lewis, Christiane Nusslein-Volhard, Eric Wieschaus and many others made it a choicest organism among developmental biologists. It is one among the well-known genetic model organisms that have short generation time and that can easily be cultured and maintained in a very small place. They breed in large numbers with a 12-day life-cycle (at 25°C) and thus, follow-up of genetic changes over generations is very easy and very less time consuming unlike other model organisms. In addition to the simpler genome organization, flexibility of manipulating them in requirement to all genetic analyses and ease of generating mutations and mapping them has made it a popular organism. Numerous advanced techniques such as GAL4-UAS system for target expression (Brand and Perrimon, 1993) and FLP-FRT system for somatic recombination (Xu and Rubin, 1993) and transgenic RNAi lines for knock-down studies have helped drosophilists to advance quickly and throw insight into numerous biological phenomena. Furthermore, very high similarity between the fly and the human genome has made scientists to use this organism to study human diseases and in drug industry.

1.3. Life cycle of *Drosophila*

*Drosophila*, a dipteran, belongs to a holometabolous group of insect, wherein, the immature wingless larval stage transforms itself into a winged adult stage via a pupal stage. The fertilized egg undergoes an embryonic stage that lasts for 24h. Larvae hatch from the egg and undergo three moulting at 24h intervals. Larval stages, called instar, is entirely different from their adults both in morphology and behavior. Larvae generally feed profusely. There is an intermediate pupal stage (that lasts for about 5 days), between the larval stage and the adult stage. Pupal stage is a quiescent and immobile phase with a thickened puparium or covering around them. During this pupation, the larva undergoes striking changes to eclose into an adult form. Adults, male and female can be morphologically distinguished from each other based on the size, pigmentation on the
dorsal abdominal tergite plates, tapering/blunt abdomen ends, sex combs and so on. It
generally takes 12 days (at 25 °C) to complete one round of development from fertilized
egg to an adult stage (Fig 1.1).

1.4. Patterning

Dynamic patterning that occurs right from the fertilized egg to the adult stage is a very
interesting phenomenon to study. There are two stages of patterning in *Drosophila*
*melanogaster*, namely patterning during embryonic stages and patterning during larval
stages that form the blue-prints of the adult development. Patterning that takes place
along the Anterior-Posterior (A/P) axis and Dorso-Ventral (D/V) axis of the embryo is
known as primary patterning while the patterning that takes in larval stages to determine
adult structures such as leg, wing is known as secondary patterning (Williams *et al.*, 1993).

1.4.1 Patterning within primary fields (Segmentation)

Unique feature of all arthropods, particularly of insects, is the subdivision of the body
into repeating segmental units. Bateson (1894) defined segmentation as a repetition of
pattern elements along the major body axis. In insects, this segmental pattern is distinct
and clearly visible in the external structures of both the larva and adult. An insect egg is
generally large and has a central yolk mass that accommodates the nuclei that undergo
early divisions. Initially, there are only nuclear divisions and no associated cytokinesis.
This leads to the formation of syncytial blastoderm. As nuclei move to the peripheral
cytoplasm, transformation of syncytial form to cellular blastoderm takes place. In
*Drosophila*, most of the blastoderm contributes to the embryo and segments form early
and simultaneously along the anterior posterior axis. One of the earliest events in the
development of the fruit fly is the establishment of a segmental pattern of gene
expression along the anterior-posterior axis of the embryo. Most of the molecular
mechanisms underlying the process of segmentation are well understood from studies on
*Drosophila* by Nusslein-Volhard, Weischaus and co-workers (1980, 1994) as well as
Lewis (1978).
Fig. 1.1. Life cycle of *Drosophila melanogaster*. It includes an embryonic stage, three larval instars, the pupal stage and an adult stage. It takes 12 days (at 25°C) to complete the life cycle.
Segmentation of the *Drosophila* early embryo is accomplished by position information provided by an interacting group of regulatory genes. A cascade of gene regulation, initiated by maternally active coordinate genes that specify the polarity of the egg, to zygotically active gap, pair-rule, and segment polarity genes are required for the development of the segmented body plan of the animal (Nusslein-Volhard and Wieschaus, 1980). The Homeotic genes eventually convert the equivalent segmental units by providing identities to them (Lewis, 1978). Although these regulatory genes are not directly involved in differentiation, they specify the embryonic developmental pathways (Garcia-Bellido, 1975). In the A/P axis of *Drosophila*, this series of regulation describe a sequence of developmental events from the beginning of axis formation upto the differentiation of cells at the end of embryogenesis (Fig 1.2)

1.4.1.a. Egg polarity genes/Maternal Genes

In *Drosophila*, pattern formation starts during oogenesis with the expression of maternal gene and the localisation of transcripts *bicoid* and *nanos* at the anterior and posterior ends of the oocytes, respectively. Translation and diffusion of Bicoid and Nanos proteins on either side result in their gradients in the early egg. These proteins in turn, repress the translation of other maternal transcripts such as *caudal* and *hunchback*, respectively, thereby generating reciprocal gradients of those proteins. The gradients are generated to provide positional cues which directly regulate a hierarchy of gap, pair-rule and segment polarity genes, the localisation of which further progressively subdivides the embryo into segments (St Johnston and Nusslein-Volhard, 1992; Rivera-Pomar and Jackie 1996).

1.4.1.b. Gap Genes

All gap genes (*hunchback, Kruppel, knirps* etc) that encode transcription factors, are the first of the segmentation hierarchy genes to be transcribed in the zygote. Anteriorly localized Bicoid activates *hunchback* and later on other gap genes are expressed at the ends of the embryo. These gap proteins too diffuse and form overlapping gradient in the syncytial embryo. High levels of Bicoid and low levels of Hunchback activate *Kruppel* expression throughout most of its region. However, the expression of *Kruppel* is
Maternal morphogen gradients e.g. nanos bicoid

Gap genes e.g. Kruppel

Pair-rule genes e.g. even-skipped

Segment polarity genes e.g. engrailed wingless

Hox genes e.g. Ultrabithorax abdominal A

Fig. 1.2. Patterning within the primary fields (Segmentation). In the A/P axis of *Drosophila*, series of regulation by maternal, gap, pair-rule, segment polarity and homeotic genes regulate the sequence of developmental events from the beginning of axis formation upto the differentiation of cells at the end of embryogenesis.

Source: Sanson, 2001
repressed on the anterior and posterior sides by high levels of Hunchback and Knirps, respectively. Thus, these genes subdivide the embryo into broad domains, such as a central Caudal band and a posterior Hunchback band, encompassing the progenitors of several contiguous segments.

1.4.1.c. Pair-rule Genes

Not only the gap genes regulate each other’s expression but also the next phase of genes in the hierarchy, namely the pair-rule genes (*even-skipped, hairy, fushi-tarazu*, etc.). Pair-rule genes that encode for transcriptional factors are transcribed in 7 broad stripes of cells corresponding to every other segment. Within pair-rule genes, a hierarchy, namely primary pair-rule genes and secondary pair-rule genes is maintained. Different combination of Gap protein concentration and presence of individual enhancers controls the expression of each pair-rule gene. The gap genes directly influence the primary pair-rule genes (*even-skipped, hairy, and runt*). Bicoid and Hunchback activate *even-skipped* stripe 2 expression. While anteriorly, the gap gene *giant* represses the expression of *even-skipped* stripe 2, *Kruppel* represses the same in posterior segments (Small et al., 1991). On the other hand, interaction among primary-pair rule genes regulate and refine the expression of the secondary pair-rule genes (*fushi-tarazu, odd-skipped, odd-paired, paired*, and so on). For example, *even-skipped* (*eve*) represses the expression of *fushi-tarazu* (*ftz*) leading to the expression of the two genes in complementary graded patterns of expression in alternating stripes. Thus, modulation of pair-rule expression among each other, pattern the seven striped embryo into fourteen narrow segmental stripes and determine the position of the parasegments (reviewed in Akam, 1987; Ingham, 1988). The embryo cellularizes and undergoes gastrulation after activation of the pair-rule genes.

1.4.1.d. Segment Polarity Genes

The pair-rule gene that express in a periodic and overlapping pattern establish the expression patterns of first phase of segment polarity genes (*wingless, hedgehog, engrailed*, etc.) within every parasegment. The segment polarity genes *engrailed* (*en*) and *wingless* (*wg*) are established through the positive and negative transcriptional regulation
by the pair-rule genes. For example, in a parasegment, Ftz and Eve activate the expression of en while repress the expression of wg. The second phase of regulation is by cell-cell signaling and hence segment polarity genes not only encode transcriptional factors but also other signaling factors. For example, en encodes a homeodomain protein, while wg encodes a secreted protein. Early during embryogenesis (stage 9-10), the stripe of en transcription marks the posterior compartment of each segment (or anterior compartment of each parasegment) and wg is transcribed solely in a row of cells immediately posterior (but these posterior cells of the parasegments would constitute the anterior cells of the segments) to cells where en is transcribed. Wg secreted into adjacent cells activates En in those cells, which in turn activates another secretory protein Hedgehog (Hh) that maintains Wg in the adjacent posterior cells. At the end of stage 10, when en expression becomes independent of Wg, Wg and Hh initiate intrasegmental patterning. Consequently, the Wg and Hh domains form a bipartite organizer that sets up the parasegment (PS) boundary and patterns cells on both sides. The PS boundary is visible in stage 10-11 embryos as a transient groove at the interface between Wg- and En/Hh-expressing cells. Later during the stage 12, a segmental groove (S) forms at the posterior edge of the En/Hh stripe and marks the deep segmental grooves evident in the fly larva (Hidalgo and Ingham, 1990; Angelini and Kaufman 2005).

1.4.1.e. Homeotic Genes

Once the segmental boundaries are setup, the segmental identity is provided by the activation of a set of genes known as Homeotic selector genes (Lewis, 1978). They are commonly known as Hox genes. The pair-rule genes activate both the segment polarity genes and the Hox genes. Gap genes also influence Hox genes directly. In Drosophila, although Hox genes do not have a direct role in segmentation, it functions to provide unique identities to individual segments. In the fly genome, there are two Homeotic gene complexes, namely, Antennapedia complex and Bithorax complex (Lewis, 1978; Kaufmann et al., 1980) located on two regions of the third chromosome. While the Antennapedia complex that codes for the Homeotic genes proboscipedia (pb), labial (lab), deformed (dfd), sex comb reduced (scr) and Antennapedia (Antp) regulate the anterior region of the embryo, the Bithorax complex (Lewis, 1978) including the
homeotic genes Ultrabithorax (Ubx), abdominal A (AbdA) and abdominal B (AbdB) specify the posterior region. While the lab and dfd gene products specify the head segment, scr and Antp contribute to thoracic segmentation. In the bithorax complex, Ultrabithorax (Ubx) is required for the identity of the third thoracic segment while abdominal A (AbdA) and abdominal B (AbdB) gene products are required for the specification of abdominal segments (Sanchez-Herrero et al., 1985).

In addition, the Hox genes and segment polarity genes are involved in specifying the denticle belts and ventral epidermis of the larval abdomen. Both segment polarity genes and Hox genes function synergistically to control the differentiation of each segment of the larval stage. During the stage 11 of embryogenesis, they activate a single stripe of Ser expression within each abdominal parasegment. Although the initiation of Ser expression in broad domain is under the control of the Hox genes, wg and hh refine the boundaries of the Ser domain by inactivating its expression in a subset of cells in each parasegment. At stage 12, Hh activates rhomboid (rho) that codes for a transmembrane protein required for the activation of EGFR ligand Spitz. At the end of stage 12, the PS boundaries gradually disappear, while the segmental grooves are deepened immediately posterior to the En cells. At the end of embryogenesis, the posterior row of En cells and Rho and Ser-expressing cells secrete denticles that make up the ventral denticle belts of the larval abdomen. Wg signaling specifies smooth cuticle in asymmetric fashion. The Ser-expressing cells secrete rows 5 and 6 of the denticle belts (Sanson, 2001).

1.4.2 Patterning within secondary fields (appendages)

Appendage refers to body outgrowths irrespective of mechanism of origin, position, or patterning. Insects, like all other arthropods, possess a series of appendage-bearing segments (Minelli 2003). Insects are the most successful organisms, mainly due to their flexibility in their articulated appendages that enabled them to possess a wide range of feeding behavior, movement and behavior. Synapomorphy of appendage segmentation is one among the features that distinguish arthropods from allied phyla. Robert Evan Snodgrass (1935), an entomologist and arthropod physiologist, termed the segments of arthropod limbs as “podomeres”. For example, the limbs of the phyla Onychpora,
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belonging to lophopodous animal, is a close relative of arthropods, have a muscle attachment that extend only from the cuticle of the appendage to the bodywall, while the arthropods have muscle attachments that interconnect the individual distal podomeres. This allows flexure at the joints of the limb segments and thus a greater range of motion in the appendages.

Generally, homology between structures is inferred by comparing them in ontogeny, composition, and position of segments. For example, the homology of insect wings is ascertained by comparing the similarities in venation and articulation, wherein, the wings of all insects can be derived from the same basic pattern of ground plan (de Celis 2003). Similarly, the tetrapod limbs are homologous to the paired fins of the bony fishes, elytra of beetle correspond to the membranous fore wings of other insects and fly halteres are related to membranous hind wings of other insects and so on.

Serial homologs refer to related structures on different segments of an individual insect. Although the cephalic appendages such as antennae and mouthparts and legs appear to be very dissimilar in appearance from those of thoracic (legs) and abdominal segments (genitalia and cerci) (Flower, 1964; Kukalova'-Peck and Richardson, 1983), they are otherwise serial homologs at molecular level (Averof and Cohen, 1997). Hence during evolution diversification of appendages could have occurred either along the body axis of the individual species or between species. Addressing the developmental and molecular variation is very essential for understanding the evolutionary diversification of these appendages.

There are two types of appendages, namely, ventral appendages (such as legs, antennae and mouthparts) and dorsal appendages (such as wings, halteres and other derivatives). The body of Drosophila like other insects is made up of 14 segments: 3 cephalic segments (with antennae and mouthparts), 3 thoracic segments (with a pair of legs on each segment and a single pair of wings and a pair of halteres on the second and third thoracic segment) and 8 abdominal segments. Position and patterning of the appendages are equally important.
1.4.2.1. Early Development of Wing

Dorsal appendages, wings and halteres, are present on the second and third thoracic segment of *Drosophila*. They are developed from epithelial sheets called imaginal discs, a tissue that is specified during embryogenesis (Bate and Martinez-Arias, 1991). Initially, imaginal discs are made of a small number of cells, which divides during post embryonic stages to increase in size, differentiate during pupation and develop into an adult structure with more than 50,000 cells (Garcia-Bellido and Merriam, 1971). Wing discs develop into a highly patterned adult wing containing stereotyped structures such as wing margin, veins and interveins (Fig 1.3A) (also details in Chapter 4 and 5).

Patterning of appendages is initiated during the late embryonic stages and early larval stages, which specifies the anterior-posterior and dorso-ventral compartments respectively. During embryogenesis, across the parasegment boundary, the disc founder cells are specified (Cohen, 1990; Couso et al., 1994) by Wg- and En. Imaginal disc primodia are set up in those cells present near the intersection between Wg and Dpp stripes that are exposed to both these signals (Fig 1.3B). A subset of cells in this cluster derived from En expressing cells confers posterior identity to the developing disc. Until late development of embryogenesis, most of the ectodermal cells remain pluripotent in nature and hence cell-cell interactions play a major role in assigning the final choice between alternative fates based on the position of cell in the population. Early during the larval development, the wing primordium separates out to develop into an independent entity, from the imaginal cells that develop into future legs.

During wing development, the combined action of selector genes namely *engrailed* (*en*) and *apterous* (*ap*) and morphogens such as Dpp and Wg (signaling molecules that act on cells to induce unique cellular responses in a concentration dependent manner) sets up organizers along anterior-posterior and dorso-ventral axis and compartmentalizes the wing disc into four boundaries (Fig 1.4A). Expression of the homeobox gene *engrailed* specifies the posterior compartmental identity in the developing first instar larva. En activates a short-range morphogen Hedgehog (Hh), which signals from posterior to anterior compartment, thereby inducing Decapentaplegic (Dpp) expression in anterior
Fig. 1.3. Patterning along the secondary axis-1. (A) Eyes, antennae, legs, wings and halteres are some of the appendages, which develop from imaginal discs in *Drosophila*. (B) During early embryogenesis imaginal disc primodia (in blue) are specified at the intersection of Wg (grey) and Dpp (green) stripes. A subset of the cells in this cluster is derived from *en* expressing cells which confer posterior identity to the developing discs.

Image from (A) From Fly move and (B) from Cohen, 1993.
cells (Basler and Struhl, 1994; Tabata and Kornberg, 1994). The long-range morphogen
Dpp signals the cell to have positional identity in respect to antero-posterior axis and
further controls the growth of the disc (Capdevila and Guerrero, 1994; Zecca et al., 1995;
Ingham and Fietz, 1995; Burke and Basler, 1996). Dpp exerts this organizing activity by
regulating downstream genes in a concentration dependent manner (Nellen et al., 1996;
Lecuit et al., 1996).

Similarly during the second instar larval stage, a second organizer is set up at the dorso-
ventral (D/V) boundary, by the localized expression of another selector gene, apterous
(ap) that codes for LIM-homeodomain protein in the dorsal cells of the second larval
instar (Diaz-Benjumea and Cohen, 1993; Blair, 1993). Apterous in turn, activates the
expression of Fringe and Serrate in dorsal cells (Irvine and Wieschaus, 1994; Kim et al.,
1995). Serrate, one of the ligands of the Notch signaling pathway, activates Notch (N) at
the compartment boundary that controls growth and patterning of the wing (Diaz-
Benjumea and Cohen, 1993; Williams et al., 1994). N activates the expression of the
nuclear protein Vestigial (Vg) and Wg in cells at the DV boundary (Diaz-Benjumea and
Cohen, 1995; Rulifson and Blair, 1995; Kim et al., 1995; Couso et al., 1995). Wg
functions as a long range morphogen to activate the expression of target genes like
Distalless (Dll) and Vg and Achaete-Scute (Ac/Sc) and mediates the growth and patterning

Thus, the organizers set along the A/P axis and D/V axis compartmentalizes the wing
disc into developmentally distinct 4 domains, thereby specifying cell fate to different sub-
populations. During the next phase of development, activation of downstream genes take
place to further refine and pattern the wing disc (explained in detail in Chapter 4).
Differentiation of cells takes place during the pupal stage and the imaginal discs evert to
develop into adult wing and body wall (Fig.1.4B). Morphologically, the adult wing of
Drosophila differs from haltere, its serial homolog on the third thoracic segment, by
number of features such as size, prominent structures such veins, interveins and margin
(Fig.1.4C). At the molecular level, suppression of certain genes by the Homeotic gene,
Ubx, makes a major difference to specify the haltere fate.
Fig. 1.4. Patterning along the Secondary axis-II. (A) The selector genes *en* and *ap* specify the posterior and ventral identity respectively, which in turn, activate long range morphogens like Wg and Dpp that patterns the disc. (B) The wing imaginal disc gives rise to the adult wing and dorsal body structures of T2. As this fate map shows different parts of the disc give rise to different parts of the fly. The wing pouch (green) gives rise to the adult wing blade and by late third instar larva is patterned into a dorsal (D), ventral (V), anterior (A) and posterior (P) compartment by an intricate set of patterning genes. The yellow region gives rise to the wing hinge region and the blue region forms the bodywall called mesonotum to which the wing is attached. (C) A *Drosophila* wing with five longitudinal veins (L1 to L5) that form at precise locations along the A/P axis are interspersed with interveins and enveloped by wing margin anteriorly.
1.5. Homeotic genes- Regulation and Function

Homeotic genes (HOM/HOX genes) are homeodomain-transcriptional factors that confer segmental identity along the primary body axis (reviewed by McGinnis and Krumlauf, 1992) and are implicated in regionalization of the body plan of all bilaterally symmetrical animals (de Rosa et al., 1999). All Homeotic genes share a characteristic motif called homeodomain, coding for 61 amino acids, that recognizes specific DNA sequence and is required for activating and repressing downstream target genes. The homeodomain folds into 3 helices, helix 2 and 3 to generate a helix-turn-helix conformation, characteristic of transcriptional factors that bind to the major groove of the DNA. While the N-terminal region of this motif that precedes the helix 1 contacts the nucleotides of minor groove of the target DNA, the third helix or the recognition helix, recognizes a four-base motif, TAAT, which is conserved in nearly all sites recognized by homeodomain (Otting et al., 1990; Percival-smith et al., 1990; reviewed in Mann and Chan, 1996). In addition to the specification of segmental identity as described earlier (in sec 1.4.1.e.) in this Chapter, HOX genes are also required for both main body development and also appendage development.

The HOX/HOM genes were first characterized in the fruitfly, where mutations that cause ectopic expression of Antennapedia (Antp) gene product result in antenna to leg transformations (Fig 1.5) suggesting that master regulatory genes act as selectors for regional identities.

The identity of first, second and third legs are specified by the homeotic genes Sex combs reduced (Scr), Antennapedia (Antp) and Ultrabithorax (Ubx) respectively. In the absence of homeotic gene function, all these thoracic segments develop the same 'ground' pattern, a mixture of thoracic and cephalic pattern without any morphological identity along the A/P axis (Struhl, 1982). Similarly in vertebrates the Hox genes are implicated in the development of 13 thoracic, 6 lumbar and 4 sacral vertebrae. For example, Hox10 mutant
mice show complete loss of lumbar vertebrae and obtain the rib processes that are typical of thoracic vertebrae (Wellik and Capecchi, 2003).

Unique feature of Homeotic genes is the maintenance of the colinearity principle. The Antennapedia complex (ANT-C) that includes anteriorly expressed five genes proboscipedia (pb), labial (lab), deformed (dfd), sex comb reduced (scr) and Antennapedia (Antp) specify the head and the anterior thorax while the bithorax complex (BX-C) that includes three genes, Ultrabithorax (Ubx), abdominal A (AbdA) and abdominal B (AbdB) assign the identity from third thoracic segment onwards (Fig 1.6A). The homeotic gene, Caudal (cad), is exceptional which do not fall into any of these two clusters but specify the anal development. The clustered organization of the homeotic genes exhibits a close relationship to their mode of expression. The expression domains along the primary axis of the developing embryos reflect the location of individual genes within the clusters, in other words, the arrangement of the genes on the chromosome is same as that of the order of expression during development. Hence the more proximally (to the centromere) located genes have more anterior expression domains while the distal ones show posterior expression domain. This ordered relationship of arrangement of genes and its relative expression has been termed spatial colinearity. Mouse and human genomes contain four copies of Hox complexes per haploid set, located on four different chromosomes (Boncinelli et al., 1998; McGinnis and Krumlauf, 1992; Scott, 1992). Absence of one-to-one correspondence between Drosophila Homeotic and vertebrate Hox complex suggests that the four Hox complexes of the vertebrates arose from a single cluster of invertebrates by gene and chromosome duplication during evolution. While vertebrates retain spatial colinearity there is also a temporal colinearity, wherein the most proximal genes exhibit the earliest onsets of expression, followed by the sequential activation of distal genes (reviewed by Duboule, 1998) (Fig 1.6B). A recent study has shown that Hox clusters can be fragmented, reduced or expanded in many animals implicating a role in bringing about morphological changes during evolution (Lemons and McGinnis, 2006).
Fig. 1.6. Colinearity Principle. (A) Spatial Colinearity in *Drosophila* showing the position of homeotic genes and their domain of expression both in embryo and adult. (B) Conservation of Hox gene expression from invertebrates to vertebrates. In addition to spatial colinearity, vertebrates show temporal colinearity too.

Source: Carroll, 1995.
1.5.1. Regulation of Hox genes

As discussed earlier (in section 1.4.1.e), transcription of Hox genes in *Drosophila* is initiated by the combined action of both gap and pair-rule genes. For example, the cis-regulatory elements present in the Bithorax complex regulate the spatio-temporal expression of *Ubx*, *abadA* and *AbdB*. In addition, autoregulatory circuit mechanisms operating and interaction among Hox genes further refine the boundaries of Hox gene expression. The Hox genes located posteriorly repress anteriorly localized Hox genes, for example, the posterior most Hox gene *AbdB* functions to repress anteriorly localized *AbdA*, *Ubx* and *Antp*, while *AbdA* functions to repress *Ubx* and *Antp* while *Ubx* represses *Antp* (Gonzalez-Reyes *et al.*, 1990). Recent studies have shown that miRNAs are also potential regulators of Hox genes. It has been proposed that in *Drosophila*, *Scr*, *Antennapedia*(*Antp*), *Ubx*, *abd-A* and *AbdB* transcripts are potential targets of miRNA regulation (Enright *et al.*, 2003). For example miR-196 regulates the protein encoding Hox transcript from mouse *Hoxb8* (Yekta *et al.*, 2004; Mansfield *et al.*, 2004) and mir-10 that is conserved in both *Drosophila* as well as other vertebral Hox clusters between *Dfd/Hox4* and *Scr/Hox5* is proposed to be a potential regulator of Hox genes (reviewed in Pearson *et al.*, 2005).

By virtue of their function to specify segmental identities along the A/P axis, Hox genes play a major role in morphological diversification during evolution. Often, variation in the body plan among the arthropods has been due to variation in the expression and regulation of Hox genes (reviewed in Hughes and Kaufman, 2002). The sequences flanking and inside the homeodomain also aid in the target specificity and are subjected to evolutionary changes (Ekker *et al.*, 1994, Lin and Mc Ginnis, 1992). Hox proteins bind to the enhancers of downstream target genes in a specific manner, either independently or in collaboration with other transcriptional factors and cofactors. Both in vitro and in vivo studies have shown that heterodimers of Hox proteins bind exclusively with Pre-B-Cell homeobox (PBC) class proteins (PBX in mammals, CEH-20 and CEH-40 in *C.elegans* and EXD in *Drosophila*) to a large number of target enhancers, where HTH/MEIS superfamily of proteins are found nearby (Chang *et al.*, 1995; Mann and Chan, 1996;
1.5.2. Function of Hox genes

Hox genes are capable of regulating the segment specificity either at the cell or organ level. For example, Hox proteins Ubx and Abd-A regulate the expression of dpp in the A/P axis of the visceral mesoderm of Drosophila. While Ubx activates the dpp expression in visceral mesoderm and Ubx and AbdA together directly repress Dll in the epidermis (Bienz et al., 1994). Abd-B activates a group of transcriptional factors to specify the spiracle development (Merabet et al., 2005). Function of Hox genes at the cellular level has been shown in neuroblast (NB) lineages of the central nervous system of Drosophila. Hox genes Abd-A and Abd-B specify the abdominal NB6-4a lineage by down-regulating levels of the CycE (Berger et al., 2004). Hox genes are known for regulation of various events including cell cycle, cell death and cell adhesion and movement (reviewed in Pearson et al., 2005). In addition to regulating specific targets, synergistic and redundant regulation of a common target imposed by more than one Hox genes is also observed, which reinforces Hox function.

Despite a broad knowledge on the genetic, molecular and biochemical nature of these Hox proteins, their wide range of targets genes and their function as developmental and regulatory switches are unknown. Our lab interest is to address questions pertaining to the homeotic gene Ubx and thence to generate a vivid picture of Ubx function in specifying T2 and T3 flight appendages, wings and halteres.

1.6. Ultrabithorax and its function

Ubx, originally described by Hollander in 1934 (Lindsley and Grell, 1968) is one of the first homeotic genes identified that specifies the third thoracic segment in fruit flies. Not just historically important, Ubx is one of the important and best-known Homeotic genes implicated in the evolution of the order Insecta. Ubx imposes a major change to the hind wings of the orders such as Coleoptera (beetles), Lepidoptera (butterflies) and Diptera
(Drosophila). Coleopterans possess a thick fore wing called elytra and a normal hind wing (Tomoyasu et al., 2005). While in Lepidopterans like butterflies and silkworms, hind wings are morphologically similar to their respective fore wings (reviewed in Carroll, 2005). In Drosophila, a pair of wings and a pair of halteres, a club shaped balancing organ that provides a swift flight (Pringle, 1948; Chan et al., 1998) are present on T2 and T3 segments respectively. Presence of Ubx imposes T3 (and thereby hind-wing or haltere) identity in all these insects. Interestingly although Ubx specifies the hind wing morphology in these orders, changes are found neither in sequences nor in their expression pattern (Galant and Carroll, 2002; Ronshaugen et al., 2002). Probably Ubx functions by specifying different developmental events in different groups of insects.

During embryogenesis of Drosophila, Ubx is expressed in parasegments (PS) 5-13 with highest expression of Ubx in PS6, thereby determining the identities of PS5 (that corresponds to T2p+ T3a) and PS6 (that corresponds to T3p+ A1a) (White and Wilcox, 1985). Many tissue specific embryonic enhancers of target genes such as connectin (cnn) (Gould and White, 1992); Distalless (Dll) (Vachon et al., 1992); scabrous (sca) (Graba et al., 1992); Antp (Appel et al., 1993); Transcript 48 (Strutt and White, 1994); centrosomin (Heuer et al., 1995); serpent (Mastick et al., 1995); Wnt4 (Graba et al., 1995); β-tubulin at 60D (Kremser et al., 1999); La-related protein (Chauvet et al., 2000) are regulated by Ubx.

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. Ubx is expressed in halteres throughout the development (White and Wilcox, 1985). Removal of Ubx from T3 segment leads to haltere-to-wing transformation (generating four winged fly), while ectopic expression of Ubx in T2 segment wing-to-haltere transformation (generating wingless fly) (Lewis, 1978) (Fig. 1.7). In the wing imaginal disc, Ubx is expressed only in the peripodial membrane (squamous epithelium) but not in the disc proper (columnar epithelium that forms the future wing) of the wing imaginal disc. Ubx is strongly expressed in T3 legs and haltere imaginal discs. Presence of Ubx function in the late larval stage and early pupal stage is sufficient to bring about a change in cell morphology
Fig 1.7 Ubx-mediated Haltere specification. (A) Homeotic gene *Ultrabithorax* (*Ubx*) suppresses wing development and specifies haltere identity in T3 segment of *Drosophila*. (B) Loss of Ubx in T3 segment gives rise to haltere to wing transformation. (C) Gain of Ubx in T2 segment gives rise to wing-haltere transformation.
and patterning to assign the haltere identity. At the end of pupal stage the nature and size of cuticular area between wing and haltere differ drastically. Wings are bigger in size with 8 times larger cuticular surface with 5 times more number of cells than halteres (Cohen, 1993; Held, 2002; Crickmore and Mann, 2006; Makhijani et al., 2006), possessing prominent structures like veins and margin.

1.7. Regulation of haltere identity by Ultrabithorax

Ubx functions as a developmental switch in the thoracic serial homologs of Drosophila to suppress wing fate and specify haltere fate (Cabrera et al., 1985; White and Akam, 1985; Makhijani et al., 2006). Major difference between wing and haltere development takes place during the later larval stage and pupal stages. Hence patterning genes involved in differentiation of cells during late larval stage and pupal stages probably are potential targets of Ubx (Roch and Akam, 2000).

The selector genes en and ap set up similar compartmentalization in these serial homologs and hence it is unlikely that Ubx regulates the expression pattern of these compartmental selector genes to specify haltere identity. However, many signaling molecules, morphogens at the compartmental boundaries and their targets are found to be downregulated by Ubx in the haltere disc. Deficiency and microarray screens to identify potential targets of Ubx have listed large number of regulatory molecules, transcriptional factors and other signaling molecules (Graba et al., 1997; Mohit et al., 2006; Hersh et al., 2007). It has also been found that numerous wing patterning genes such as wg, cut (ct), Serum Response Factor (Dsrf), spalt-related (sal) and vg are down-regulated in the developing haltere (Weatherbee et al., 1998; Shashidhara et al., 1999). Ectopic expression of pro-wing gene vg in the haltere pouch is sufficient to induce haltere-to-wing transformations, albeit partially (Prasad et al., 2003). In the fore wings of Drosophila and butterflies expression pattern of genes such as wg, Achaete-scute and Dsrf are similar while they are either absent or repressed by Ubx in the hind wings of only Drosophila. Therefore it is likely that changes in the Ubx binding sites of the cis-regulatory sequences of target genes generate differences in the hind wing morphology (Weatherbee et al., 1999; reviewed in Carroll, 1995). Unlike other Hox genes, Ubx
possesses certain unique features. During haltere specification, Ubx does not require the activity of cofactors like Exd and Hth (Azpiazu and Morata, 2000; Casares and Mann, 2000; Galant et al., 2002).

In haltere imaginal discs, to date, only two direct targets of Ubx are known. spalt-major (sal), which is implicated in Dpp signaling (Galant et al., 2002) and knot (kn), which is implicated in Hh signaling (Hersh and Carroll, 2005) are known. For these two targets, wing specific regulatory elements have been narrowed down to minimum sequence that is required for Ubx function (Galant et al., 2002; Hersh and Carroll, 2005). Thus, Ubx directly or indirectly regulates target genes at several levels of wing patterning hierarchy to shape and pattern the development of haltere (Weatherbee et al. 1998; Shashidhara et al., 1999; Galant et al., 2002; Mohit et al., 2003; Hersh et al., 2007). Ubx-mediated haltere specification thus sets a platform to reflect the Hox gene mediated specification of morphology and organ development along the body axis of an organism.

1.8. Earlier work in the laboratory

A clear inference drawn from previous extensive studies is that Ubx downregulates genes that are involved in growth and patterning of wing discs. Hence different approaches are to be utilized for identifying the potential targets of Ubx implicated in different developmental pathways to specify haltere development. Different approaches and strategies to identify such differentially expressed genes have been taken up in our laboratory.

1.8.a. Enhancer trap Approach

To identify novel targets of Ubx, Enhancer-Trap based genetic approach was used. In this study, EN403, the enhancer trap of the gene CG32062 that is differentially expressed between wing and haltere was identified. CG32062 was found to non-cell autonomously regulated by Ubx, indicating that it is an indirect target of Ubx (Bajpai et al., 2004).
1.8.b. Microarray Analysis Approach

Expression profiling of genes differentially expressed between wings and halteres were compared by this approach. Nearly 500 differentially expressed genes were identified in this screening. Validation of number of genes identified in this screening is going on in our laboratory. Preliminary validation of 18 genes suggested that downregulation of A/P ad D/V signaling pathways and their targets would be a probable mechanism by which Ubx assigns haltere identity (Mohit et al., 2006).

1.8.c. Enhancer Promoter (EP) overexpression screening Approach

A P-element with a GAL4 regulated promoter that is capable of activating the genes downstream to the insertion site is used in this method (Rorth, 1996). By mobilizing a starter P element, a new EP library was generated which was crossed to Ubx-GAL4 driver. Using this approach, novel targets of Ubx function such as Syntaxin, Derailed and Alhambra were identified. In the same study Egfr was also identified as a direct target of Ubx and it was shown that downregulation of EGFR signaling is critical for haltere specification (Pallavi et al., 2006).

1.8.d. Proteomics approach and Bioinformatics approach

Protein profiling of Ubx targets that are differentially expressed between wing and haltere was taken up in this approach. Few targets identified in this approach require validation. Simultaneously, Bioinformatics strategies that predict the target genes with cis-regulatory consensus sequences, called Ubx-binding motif, that bind to the Ubx homeodomain, was taken up and they were further validated for their genuiness by other biochemical and genetical approaches. In this study two genes, division abnormally delayed (dally) and thickveins (tkv) implicated in Dpp signaling were identified as direct targets of Ubx and validated for Ubx function on these genes. This study indicated that the downregulation of Dpp signaling along the A/P axis in haltere by Ubx is indispensable for Ubx mediated haltere specification (Makhijani et al., 2007).
1.9. Objectives of the present study

As described above, Ubx regulates target genes at several levels of wing patterning hierarchy to shape and pattern the development of haltere (Weatherbee et al. 1998; Shashidhara et al., 1999; Galant et al., 2002; Mohit et al., 2003; Hersh et al., 2007). Recent study based on microarray-based differential gene expression analysis between wing and halteres has indicated both repression of wing-specifying genes and activation of genes that confer haltere identity are required for haltere specification (Mohit et al., 2006; Hersh et al., 2007). Interestingly, although Vg is an indirect target of Ubx, its downregulation is important for haltere identity. Thus, there could be number of indirect targets of Ubx that play a major role in wing patterning while repressed during haltere development.

Earlier in our lab, a gene CG32062 that is differentially expressed between wing and haltere was identified in an enhancer trap screen (Bajpai et al., 2004). It is an indirect target of Ubx in the haltere. The present study is to characterize the function of CG32062 during wing development and thereby gain insights into the developmental mechanisms that are targeted by Ubx during haltere specification. Following are the objectives of the current study.

1. Raising antibodies against the protein encoded by CG32062, which helps in understanding of its expression patterns and function.
2. Generating additional mutations and transgenic flies for RNAi-mediated knock-down to study the function of CG32062 in appendage development.
3. Generating transgenic flies that enable gain-of-function studies on CG32062.
4. Studying possible interactions between CG32062 with other signaling pathways (Wg, EGFR/Ras, Notch or Hedgehog signaling pathway.