Ubx mediated haltere formation

: a model system used to identify a novel marker for appendage development in Drosophila
3.1 Introduction

3.1.1 Homeotic transformations: an unsolved problem

One of the more intriguing problems in pattern formation is how a combination of homeotic genes expressed in domains along the anterior-posterior axis specifies the identity of each segment (Fig 3.1 below).

Homeotic genes are highly homologous to the helix-loop-helix family of transcription factors. *Ultrabithorax* (*Ubx*), one of the first homeotic genes identified, specifies the thirds thoracic segment in fruit flies. Besides a pair of legs this segment also bears a pair of small balancing organs called the haltere. Since fruitflies are believed to have evolved from four winged ancestral forms, the fly halteres are thought of as modified wings. Supporting this idea the loss-of-function mutations of *Ubx* result in haltere to wing transformations (Lewis, 1978)(Fig3.2). Further the suggestion that *Ubx* may function as a developmental switch is strengthened by observations that the presence of Ubx in the developing wing disc is sufficient to override wing determination and impose haltere fate on it (Cabrera et al., 1985; White and Akam, 1985). Hence this developmental process is a good starting point for identifying events that are required not only for the formation of insect wing but are modulated by *Ubx* to give rise to a phenotypically distinct structure, the haltere.
Fig 3.2 Homeotic transformation in *Drosophila* a) Wild type flies with a pair of wings (black arrow) on the second thoracic segment and a pair of balancing organs, haltere (red arrow) on the third thoracic segment. The halteres are completely transformed to wings in homeotic mutants.
Ubx, originally described by Hollander in 1934 (Lindsley and Grell, 1968), is one of the very first homeotic mutations identified in Drosophila. Ubx was shown to belong to a locus containing different homeotic mutations that formed a pseudoallelic series. This locus was named the bithorax complex by Ed Lewis who pioneered the field of developmental genetics in Drosophila (Lewis, 1951, 1955, 1968, 1978). Ubx was the first homeotic gene to be cloned (Bender et al, 1983) and Ubx is one of the first few gene products that were the basis of the discovery of the homeobox, the signature motif of a large number of master regulatory transcription factors (McGinnis et al, 1984; Scott et al, 1984).

Ubx mutations are homozygous lethal at early larval stages. In the first instar larva the parasegments 5 and 6 are transformed into copies of PS4. Thus, the function of the Ubx product is to provide the proper identity of metameres in the posterior thorax and the anterior abdomen of the fruit fly. Ubx mutations are haplo-insufficient; heterozygous flies (Ubx+/) have slightly enlarged halteres on the third thoracic segment. The Ubx gene is one of the three homeotic genes of the bithorax complex (Sanchez-Herrero et al, 1985). The finding that parasegmental identity can be switched by a single mutation suggested that homeotic genes (selector genes) control developmental pathways by regulating subordinate target genes responsible for parasegment specific morphogenesis (realisator genes: Garcia Bellido). Several evidences strongly support this hypothesis:

i) Ubx contains a homeodomain motif, which has sequence similarity to prokaryotic and eukaryotic transcriptional regulators.

ii) Ubx product directly binds to DNA in vitro, for example, to the Antp and Dll promotor sequences.

iii) cloning of DNA sequences that bind to the Ubx products revealed the presence of genes that are regulated by Ubx (Gould et al, 1992).

The 70Kb long transcription unit of Ubx was discovered in 1981 in the laboratory of David Hogness. Alternate splicing and polyadenylation generates twelve different transcripts. Translation of these mRNAs yields a family of six Ubx proteins characterized by constant amino and carboxy terminal regions (the homeobox sequence is encoded by a common exon at the 3' end). The members of this family are distinguishable by a short variable region that links the constant regions and consists of different combinations of
three optional elements of 9, 17 and 17 residues. The spectrum of RNA products changes
with time and tissue. It has been demonstrated that Ubx protein carrying the second mini-
exon is not expressed in the nervous system. However all different isoforms of the Ubx
product are not essential since a mutation that eliminates the second micro exon (type4
isoform) has no effect on fly viability and development (Busturia et al, 1990). The
patterns of expression of the Ubx product have been extensively studied by in situ
hybridizations to RNA on frozen sections or antibody staining of whole embryos (Akam,
1983; Akam and Martinez-Arias, 1985). Ubx is expressed in parasegments affected by
Ubx mutations. The pattern appears in PS5 in few cells of the epidermis and central
nervous system (CNS). In PS6, nearly all cells of the epidermis and CNS express Ubx.
The parasegmental expression patterns of Ubx seem to be regulated by distinct enhancer
elements. There are mutations that affect specifically the identity of PS5 (the abx/bx
mutations) or of PS6 (the bxd/phx mutations; Lewis, 1978). The abx/bx mutations are
spread over 28kb of DNA within the large intron of Ubx transcription unit. While the
bxd/phx mutations are distributed over a 40kb region of DNA upstream of the promoter
(Bender et. al., 1983; Peifer and Bender, 1986). The abx/bx and bxd/phx mutations define
very large parasegment specific enhancers that direct the very intricate patterns of
expression of Ubx in PS5 and PS6 respectively (White and Wilcox,1985 a & b; Castelli-
Gair and Akam1995, Little et al., 1990; Irvine et al., 1991). The pattern of expression in
PS7 and in more posterior parasegments is less intense. This is due to the expression of
two other homeotic genes of the BX-C, abd-A and Abd-B genes, which down regulate
Ubx expression in these segments.

In spite of the wealth of information available on the structure and biochemical
function of Ubx, its mechanism of action remains elusive.

3.1.2 The Search for Downstream Genes
The role of the homeotic loci is best viewed as a series of developmental switches for
either/or decisions of cellular fate. The homeodomain, that endows the proteins with
DNA-binding ability, indicates that the switch activity of the loci is reflected in their
functioning as regulators of specific downstream target genes. Hence, identifying genes
that are required to translate the positional information delivered by Hox proteins into diversified morphogenetic programmes has been the focus of attention for several years.

Various strategies have been utilized in order to gain further insights into the function of the homeotic transcription factor proteins. While the targets of regulation are likely to be divergent in different tissues a holistic approach for target gene identification was adopted for *Ubx* early on. Botas et al. (1996) have shown over a hundred Ubx binding sites in salivary gland polytene chromosomes of flies. This list is by no means exhaustive, as there seem to be additional transient or tissue specific targets. A method based on the transcriptional activation of a selectable reporter in yeast cells also predicted 85-170 target genes of Ubx (Mastick et al, 1995).

Based on the formation of DNA-protein adducts in intact nuclei and immunoselection procedure, few genomic targets for Ubx proteins have been identified. These included *scabrous (sca)* and *connectin (con)* genes. *sca* encodes a secreted protein involved in cellular communication during neurogenesis. Dynamic pattern of *sca* transcript accumulation during embryogenesis and its ectopic expression in the first abdominal segment in *Ubx* mutants led to the proposal that it is directly repressed by *Ubx*. While *con* encodes a GPI linked cell surface protein, which acts as a homophilic cell adhesion molecule, and is believed to have a role in the formation of neuromuscular connections.

Although these "*in vivo molecular" approaches identify the direct effectors of homeoproteins, the methods do not have significant advantage to select context specific targets. In spite of the efficiency, this method is only confined to genes with Ubx regulated cis-acting elements. Since a large proportion of Ubx targets identified by candidate gene approach (see Table 1 in Graba et al.,1997) fall under the category of regulatory molecules of transcription factor or signaling types, the true effectors of homeotic genes (realizator molecules, that directly specify a cellular level characteristic and are central in the process of morphogenesis) may require alternate approaches for identification.

More recent attempts at understanding mechanism of homeotic gene function have been directed at assaying effect of mutation and over-expression of Ubx on tissue-specific candidate genes. In a seminal paper, Weatherbee et al. (1998) have done a
comprehensive comparison between the developing wing and haltere discs and have come to the conclusion that Ubx regulates genes at several levels of the wing-patterning hierarchy to shape the development of the *Drosophila* haltere.

In order to identify novel markers that are potential targets of Ubx activity in the developing haltere, I have used an enhancer trap based genetic approach, in this work. The aim was to determine how the activity of target genes is regulated and how the target genes are involved in the processes of cell growth, organization and differentiation required to modify a wing into a haltere. Before presenting the experimental results, I will briefly describe the hallmarks of haltere development in the next few paragraphs.

3.1.3 Developing haltere: modification of the wing

Halteres are the reduced and highly modified hind wings borne on the third thoracic segments of diptera (true flies). These club-shaped appendages bear a complex array of sensory organs in their basal part (Fig.3.3b). They play a crucial role in steering and maintaining balance during flight and motion (Pringle, 1948; Chan et al., 1998). Primordia for the marginal bristles and territories for the veins are specified in the wing during the larval period. These patterning events are suppressed in the haltere (Fig.3.3). Presence of functional Ubx during the late stages of larval development and possibly the first few hours in the puparium is sufficient to elicit a program of differentiation in the haltere that will shape cell morphology during the remaining 4 days of pupal development. By the end of pupal development, epithelial cells of the wing blade and the haltere capitellum differ dramatically. The most obvious difference being the size of the cuticle area secreted by each of the two kinds of cells. In the developing wing there are five times as many cells and each secretes an eight fold larger area of cuticle as the haltere. Each wing cell also bears one distinct bristle unlike 3 to 4 small bristles present on haltere cells.

3.1.4 Cell autonomous specification

Earlier reports on homeotic genes provide evidence for cell-autonomy of homeotic selector gene functions. X-ray induced, clonal analysis shows that Ubx- mitotic clones generated in the haltere D/V boundary produce localized margin-specific haltere-to-wing
Fig 3.3 Wing patterning events are repressed by Ubx during haltere development. The characteristic features of the wing blade like a specialized margin, demarcated veins, and intervein regions are not formed in the haltere. Instead this club shaped structure bears sensory bristles at its base. Ubx is known to directly repress wing cell growth and identity and intervein specification while its influence on bristle differentiation and vein formation may be indirect.
homeosis (Morata and Garcia-Bellido, 1976; for a review see Lawrence, 1992). However, this clonally induced wing margin does not alter cell fate or growth in the rest of the haltere cells. This is further supported by *Ubx* regulatory mutations (*pbx, abx* etc.) that result in the transformation of only part of the haltere.

An exception to this rule is reports on homeotic gene function in the induction process across germ layers (visceral mesoderm and endoderm; Thuringer and Bienz, 1993) and in some *Contrabithorax (Cbx)* alleles (Cabrera *et al*., 1985). Since a number of identified targets of homeotic genes encode regulatory molecules, a non-cell autonomous effect of *Ubx* mutations is predicted. Even if the effect is not manifested as an adult phenotype it is likely to be noticed as subtle molecular changes.

### 3.1.5 Molecular Basis of haltere specification

Compartments appear to be identically established in the two serial homologs, wings and halteres. The expression patterns of *en* and *ap* are essentially the same in the haltere disc as in the wing disc indicating that *Ubx* is not regulating haltere identity by altering the expression of these compartmental selector genes. However, morphogens at the compartment boundaries and/or their known targets have been shown to be down regulated by *Ubx* to varying degrees in the haltere disc. A detailed account of genes differentially expressed between the wing and haltere is shown in Fig 3.5.

Interestingly genes, known to be developmentally significant for the formation or patterning of major wing characters, have no effect when over-expressed in the haltere, suggesting that perturbing one component may not be sufficient to destabilize the system.

### 3.2 Results and discussion:

#### 3.2.1 Negative regulation of wing type D/V signal by *Ubx*

Several lines of evidence available in literature suggest that *Ubx* down regulates wing type D/V signals in the haltere.

i) Removal of *Ubx* from the haltere D/V boundary is sufficient to form wing like margin bristles, a hallmark of cell autonomous function of the wing D/V organizer.
Fig 3.5 Differential gene expression between the wing and the haltere. Wing disc is to the left and haltere disc to the right in each panel. Arrow in a and b points to the repression of Wg and Cut in the posterior haltere respectively. On the other hand Dll (in c) like Q-vg (data not shown) is completely repressed in the haltere pouch.
ii) The expression of Wg, a secreted morphogen from the D/V boundary, is restricted to the anterior compartment (Fig 3.5a)

iii) Targets of Wg, e.g. the quadrant enhancer of vestigial (Q- vg) and Distal-less (Dll) are not expressed even in the anterior compartment (Fig 3.5b,c)

iv) Over-expression of Ubx in the wing D/V boundary is sufficient to induce varying degrees of transformations in the entire wing.

X-ray induced loss-of-function clones of Ubx cause cell- autonomous transformation of haltere (described in Lawrence, 1993; schematically represented in Fig 3.6). However distinct wing fates are visible only when the clones covered part of the dorso-ventral boundary. Capitellum-only clones would assume trichome identity, albeit partial, but sort out from the neighbouring Ubx expressing haltere cells and remain inside the haltere. This aberrant cell sorting behaviour was probably due to incomplete transformation. To analyse if wing type D/V signal is required for the complete transformation of capitellum clones in the haltere, I generated a large number of Ubx- clones using the FLP-FRT technique during different developmental stages and scored halteres that contained numerous mitotic clones (Table 3.1). Long distance signaling from the D/V organizer was assayed by the ability of a Ubx- clones induced in the haltere D/V boundary, to affect complete homeotic transformation (i.e. rescue of the sorting behaviour) of capitellum clones away from the boundary. It was observed that a significant number of capitellum clones remained on the haltere surface and showed complete differentiation into wing trichomes only when the mosaic haltere also carried one or more Ubx clones on the margin. In fact, the position of the second Ubx clone on the margin was critical for the rescue of capitellum clones from sorting out. For instance, a single Ubx clone in the D/V margin was enough to rescue another Ubx clone in the capitellum from sorting out (Fig 3.7). In contrast, in a haltere carrying as many as 12 independent clones, all in the capitellum, none was rescued. Although D/V margin clones capable of rescuing capitellum clones as well as rescued capitellum clones were found in both anterior and posterior compartments, in the absence of clear landmarks such as veins in wings, I could not precisely estimate the distribution of rescued capitellum clones along the A/P axis.
**Fig 3.6** Schematic showing positions of $Ubx^-$ clones in the developing haltere discs and their behaviour. D/V boundary/margin is represented in red, $Ubx^-$ clones in black and completely transformed $Ubx^-$ clones are hashed. Actual clones are shown in Fig. 3.7.

**Fig 3.7** Long distance signalling from $Ubx^-$ clones in the haltere D/V margin as schematized in Fig 3.6. The experimental data is represented here. (a) a wild type haltere. (b) $Ubx^-$ clones in the haltere margin display wing-margin specific bristles. (c) $Ubx^-$ clones in the haltere capitellum sort out within the haltere. (c1) The boxed region in c is shown at higher magnification (800X). Note that $Ubx^-$ clones appear as both differentiated and undifferentiated clones. (d and e) Two independent clonal events to show rescue of $Ubx^-$ clones from sorting out within the capitellum. Note that in both cases (and as in other 25 cases; Table 3.1) the mosaic haltere carries a second $Ubx^-$ clones on the D/V margin.
Such rescue of capitellum clones was observed in nearly 25–30% of the halteres carrying $Ubx^-$ clones both on the D/V margin and in the capitellum ($n=102$).

### TABLE 3.1

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<th>$Ubx^-$ clones only in the capitellum</th>
<th>$Ubx^-$ clones both in the D/V margin and the capitellum</th>
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<td><strong>a</strong></td>
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<tr>
<td>0% 0%</td>
<td>25% 31%</td>
<td>93% 98%</td>
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<td>(54) (27)</td>
<td>(76) (26)</td>
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Percentage of rescued $Ubx^-$ clones in the capitellum of mosaic halteres displaying different categories of $Ubx^-$ clones induced at 48-60 AEL (a) and 36-48h AEL (b). $Ubx^-$ clones, which remain on the surface and show complete differentiation into wing trichomes are considered as "rescued" capitellum clones. Total number of mosaic halteres scored for each category of clones is shown in parenthesis. In addition, none of the mosaic halteres carrying $Ubx^-$ clones only in the margin ($n=138$) showed any effect on the capitellum growth.

* two (or more) independent clones one on the margin and one in the capitellum.
# single clones spanning both the margin and the capitellum or fused margin and capitellum clones.
Identification of new genetic markers for segment specific morphogenesis:

In the above experiments it is clear that Ubx does modify D/V signaling in the haltere disc. However, this modification is not reflected in the adult cuticle phenotype when Ubx is clonally removed from the D/V boundary. The phenotype could be subtle and/or may affect expression of specific genes. Since Ubx regulates haltere development by modifying several patterning genes, removal of Ubx only from the D/V boundary may not be enough to see its effect on adult halteres. Thus, there is a need to identify genes/markers that respond to removal of Ubx from the D/V boundary.

I utilized an enhancer-trap approach to identify genes whose expression pattern may reflect a regulation by both Ubx and D/V signals to answer one or more of the above questions. A collection of GAL4 lines available in the lab, which show a segmentally modulated expression of the reporter gene in a small subset of neurons in the CNS, was screened for “interesting” expression in the developing wing and haltere discs. The criteria for the screen was to identify lines expressing the reporter gene in either the D/V or non-D/V cells and is differentially expressed between wing and haltere discs. 403 GAL4 was identified in this screen and the enhancer that is trapped is hereafter referred to as EN-403.

EN-403 satisfied both of the above criteria. First it is differentially expressed between wing and haltere discs. In wing discs, it is expressed in both anterior and posterior compartments, whereas in haltere discs it is expressed only in the posterior compartment. Second, its expression is modulated along the D/V axis. The GAL4 is expressed exclusively in a subset of non-D/V cells, seen as a broad band on either side of the D/V boundary in both wing and haltere discs (blue staining of reporter gene in Fig 3.8a).

Interestingly, at the levels of both the expression pattern and differential expression between wing and haltere discs, EN-403 is complementary to Wg and Ct. The latter two are expressed in D/V cells, whereas EN-403 is restricted to non-D/V cells. In haltere discs, Wg and Ct are not expressed in the posterior compartment, whereas EN-403 is not expressed in the anterior compartment (Fig 3.8b). The functional significance of this interesting comparison in expression patterns is discussed below.
**Fig 3.8** Pattern of expression of EN-403 in late third instar wing (a) and haltere (b) imaginal discs. Anti-engrailed (brown) antibody staining was done on X-Gal stained discs (dark blue staining) of EN403 larvae expressing beta β-galactosidase. EN-403 expresses as a broad band on either side of the D/V in both the anterior and posterior compartment. While the expression is primarily restricted to the posterior half in the haltere disc.

**Fig 3.9** Pattern of expression of EN-403 in other larval discs. (a) Central nervous system of late third instar larvae expresses EN-403 in a subset of cells in a bilaterally symmetric pattern. There also appears to be a segmental regulation of the enhancer. (b) an eye antennal disc of the same age expresses EN-403 only in the differentiating neurons posterior to the morphogenetic furrow (arrow in b). (c) Leg disc showing a discontinuous pattern of expression in a single segment.
Mobilization of EN-403 insertion

One of the objectives of employing GAL4 enhancer trap approach was to use the selected GAL4 insertions to drive genes of interest in specific tissues during development. Since EN-403 expression was specific to non-D/V cells, it would be very useful in studying events, which occur specifically in non-D/V cells. Such events could be dependent or independent of D/V signaling, validation of which would be possible with the help of a combination of GAL4 lines expressed in only D/V boundary (such as vg-GAL4), only in non-D/V (such as EN-403) and in both types of cells (such as omb-GAL4).

vg-GAL4 driven expression of Ubx in D/V cells alone is enough to down regulate Q-vg activity in non-D/V cells, suggesting down-regulation of D/V signaling by Ubx (Shashidhara et al., 1999). To ascertain if Ubx functions in both D/V and non-D/V cells to down regulate Q-vg activity and other targets of D/V signaling, I attempted to express Ubx only in non-D/V cells using 403-GAL4 driver. However, ectopic expression of Ubx using 403-GAL4 caused embryonic lethality.

In addition to the dorsal discs, EN-403 is expressed in a subset of cells in all other developing discs, central nervous system and salivary glands (Fig3.9). Late embryos and first instar larvae also show segmental expression of this enhancer. Hence, misexpression of most genes using this GAL4 driver resulted in embryonic lethality. To overcome this problem EN-403 was mobilized in the hope of de-linking the embryonic and late larval enhancer activities and trapping only the larval disc specific enhancer.

The strategy used was to select new local insertions. Since the frequency of transposase induced local hops is extremely high up to 67% (Tower et al, 1993), such events were selected based on eye colour variations. Each of these lines was used to over-express Ubx. EN-403 directed Ubx expression causes embryonic lethality, while animals expressing Ubx driven by a number of new GAL4 insertions, N23 and N27 in particular, survived till late pupal stages. The expression of the reporter in larval discs of both of these GAL4 lines was identical to that of EN-403, however both N23 and N27 completely lacked any embryonic expression.

N23-GAL4 or N27-GAL4 (their expression patterns were identical) driven expression of Ubx caused early pupal lethality, thus, allowing us to examine the effect of Ubx expression in non-D/V cells, at least, in the third instar wing discs. N23-GAL4
expression partially overlaps with quadrant-vg (hereafter referred to as Q-vg) expression in the farthest non-D/V cells. Ubx expression using N23-GAL4 did not affect Q-vg expression suggesting that down regulation of Q-vg in haltere discs could entirely be due to down regulation of D/V signaling. However, the failure of Ubx to cell-autonomously down regulate Q-vg in this experiment could also be attributed to the fact that Q-vg expression may precede that of N23-GAL4. Thus, ectopic expression of Ubx at a later stage may not affect Q-vg, unless D/V signaling is also down regulated. Consistent with this over-expression of Ubx in both D/V and non-D/V cells using vg-GAL4 and N23-GAL4 shows severe reduction in Q-vg expression (data generated by others in our laboratory).

3.2.2 Negative Regulation of EN-403 by Ubx

To determine whether Ubx regulates EN-403 in the haltere disc, I analysed its expression in various Ubx mutant backgrounds. The assay was activation of EN-403 in the anterior compartment of haltere discs in different genetic backgrounds. The other known non-D/V marker, which shows differential expression pattern between wing and haltere discs, is Q-vg. This enhancer, whose expression is also restricted to non-D/V cells of wing discs, is not expressed in haltere discs. Interestingly, in Ubx\textsuperscript{1} heterozygous background, Q-vg activity is de-repressed, although not to the same extent as in Ubx\textsuperscript{+} homozygous background. Halteres of Ubx\textsuperscript{1} heterozygotes are slightly larger, characteristically with one or more bristle on the anterior surface. This phenotype is due to Ubx haplo-insufficiency. It is, therefore, likely that repression of Q-vg by Ubx is dose dependent. Since Q-vg responds to low levels of Wg signaling from the D/V organizer, it may be sensitive to small changes in D/V organizer activity.

The expression of EN-403 was unaffected in heterozygous Ubx background. This suggests that, unlike Q-vg, down regulation of EN-403 in haltere discs is not dependent on the dosage of Ubx. Ubx\textsuperscript{1} in combination with wing specific abx, pbx and bx\textsuperscript{3} mutations results in the complete transformation of haltere to wing. Even the transformed haltere disc is indistinguishable from the wing disc. EN-403 expression is de-repressed in the anterior compartment of the haltere and becomes identical to that in the wing (Fig3.10b). Gain-of-function, Contrabithorax alleles such as Ubx\textsuperscript{cbx-Hm}, on the other hand affect only
Fig 3.10 Negative Regulation of EN-403 by Ubx. (d) Expression pattern of EN-403 in wild type (a) and in Ubx heterozygous background. (e) En-403 expression in abx bx3 pbx/ Ubx1 background (b, fly of the same genotype with wing to haltere transformation). Clear de-repression of the enhancer is seen in the anterior compartment of the transformed haltere. (f) Expression of the same in Ubxbx-Hm/+ background (c, flies with wings transformed to haltieres due to aberrant expression of Ubx in the wing disc). In this converse experiment, strong repression of the enhancer is evident (arrow in f).
the wing, which is strongly transformed to haltere. A strong repression of the enhancer was seen in the Ubx<sup>cbx-Hm</sup> transformed wing discs (Fig3.10.c) suggesting that presence of UBX in the developing wing disc is sufficient to down regulate EN-403 expression albeit, only in the anterior compartment.

3.2.2.1 Temporal Expression of EN-403
The transformation of the D/V boundary, an interface between the Ap expressing Dorsal and non-expressing ventral cells into a functional organizer in the wing disc is marked by the activation of Notch, which in turn is known to activate wg and the boundary enhancer of vg. Wg, a molecular marker for the D/V organizer, is a morphogen that is known to diffuse and pattern the entire wing as described earlier. Q-vg is activated in non-D/V cells in response to Wg in the late third instar larval discs.

A gene activated as a result of the function of D/V signal is likely to express only after the establishment of a functional D/V boundary. Hence the temporal expression of EN-403, with reference to known wing markers was analysed. Comparative analysis of Wg, Q-vg and EN-403 expression showed that in the wing and haltere discs, EN-403 is activated late during development after the establishment of Wg domain and the initiation of Q-vg expression (Fig3.11). This suggests that EN-403 may represent the expression of late effectors of D/V signaling.

3.2.2.2 Clonal removal of Ubx from the D/V boundary is sufficient to activate EN-403 expression in the anterior haltere
To determine the developmental stage during which Ubx is required for repression of EN-403 in the haltere disc, I generated a variety of homozygous Ubx' clones. EN-403 is inserted on 3<sup>rd</sup> chromosome, wherein Ubx is also located. First, I recombined EN-403 and P[FRT]<i>Ubx</i>'<sup>l</sup>. EN-403 expression in <i>Ubx'</i> clones was monitored using UAS-GFP. Small clones generated late during development (96h AEL) failed to activate EN-403 in the anterior non-D/V cells of haltere discs (data not shown). However a few (n=8) larger clones made earlier (72h AEL) during development resulted in de-repression of the marker in the anterior haltere (Fig3.12). De-repression of EN-403 was always associated with a D/V boundary clone, although not all D/V boundary clones resulted in the
Fig 3.11 Temporal expression of EN403 with reference to Wg activation along the D/V boundary in developing dorsal discs. Wg expression visualised by anti WG antibody staining is shown in red and EN 403 expression using UAS-GFP in green. a) a set of early to mid third instar wing leg and haltere discs with clear Wg expression along the D/V boundary (white arrow in a and b). However EN 403 expression in the non-D/V cells is not seen (arrowhead points to the domain where EN 403 expression is expected). Compare this with EN403 expression (green) in late third instar wing disc (b). figc) shows a haltere disc of the same age as in b) with only EN403 expression.
**Fig 3.12 and 3.13** Clonal removal of *Ubx* from the D/V boundary is sufficient to activate EN-403 expression in the anterior haltere. Clones of *Ubx*^l^ were generated using the EN-403 P[FRT]82 *Ubx*^l^ and FRT 82 *arm-lacZ* (Fig 3.12) or FRT 82-Minute *arm-lacZ* (Fig 3.13). Loss-of-function clones were identified by the absence of anti β-galactosidase staining (red). In both figures the last column shows a merge of the individual staining. Green marks the expression of EN-403 and red marks *Ubx*^l^ heterozygous or wild type cells.

Fig 3.12 a-c. *Ubx*^l^ clones failed to activate EN403 cell autonomously (asterix in a and d). For example, removal of *Ubx* from non-D/V cells of the anterior compartment (shown in a) does not activate EN-403 (c). However a clone in the D/V boundary, inferred from its position (dotted line in e) resulted in EN-403 activation non-cell autonomously.

Fig. 3.13d-f shows occurrence of large *Ubx*^l^ clones covering both D/V and non-D/V cells. Such clones activate EN-403. However, activation of EN-403 is seen even in *Ubx*^+^ cells (blue arrows in f), reiterating non-cell autonomous activation. (Fig. 3.13 a-c) control Minute and *Ubx*^l^ clones in wing discs. No effect on EN-403 expression is observed.
activation of EN-403 in the anterior compartment. Anterior compartment cells now expressing EN-403 showed normal wild type Ubx expression (Fig3.12b). De-repression of the marker in the anterior compartment cells was never observed in the absence of a Ubx\(^{-}\) clone in the D/V boundary (Fig3.12a).

Since the frequency of large clones spanning the D/V boundary was low, I utilized the growth advantages conferred by the Minute technique. Minute (M) mutations slow down development and act dominantly and cell-autonomously. Hence, Ubx mutant clones that are wild type for the M locus outgrow their M\(^{+}\) neighbours. This independent experiment reconfirmed all of my earlier observations (Fig 3.13). When clones were induced at 72 ± 12 hrs all halteres primarily consisted of Ubx\(^{-}\) clones and showed expression of EN-403 even in Ubx positive cells, in the anterior compartment (Fig. 3.13b). The only requirement, however, was the presence of at least one Ubx\(^{-}\) clone in the D/V boundary.

Thus, it is evident that EN-403 regulation by Ubx is (i) non-cell autonomous and (ii) is mediated through its effect on D/V signaling.

3.2.2.3 Ectopic expression of Ubx in the D/V boundary is sufficient to repress EN-403 in non-D/V cells of wing discs

To further test the suggestion that de-repression of D/V signals in Ubx\(^{-}\) clones are required for EN-403 activation, I targeted the expression of Ubx in the wing disc D/V boundary using vg-GAL4. Vg is expressed in the D/V boundary in early 3\(^{rd}\) instar discs (Fig3.14a), while EN-403 (or N23-GAL4) is activated in late third instar discs (Fig3.14b). Hence, using vg-GAL4, Ubx can be expressed in the D/V boundary much before EN-403 is activated. This limited ectopic expression of Ubx was sufficient for the down regulation of EN-403 expression in the anterior wing disc, reproducing the haltere pattern of expression (Fig3.14d). The effect was most pronounced in the anterior most cells of the wing disc domain of expression, probably because vg-GAL4 does not express Ubx uniformly along the D/V boundary, with the expression in the center of the disc being very low (Fig 3.14b, see arrow).
**Fig 3.14.** Ectopic expression of Ubx in the D/V boundary is sufficient to repress EN-403 in non-D/V cells of wing discs. (a and b) vg-GAL4 and EN-403 GAL4 simultaneously driving expression of UAS-GFP in early and late third instar discs. vg-GAL4 specific expression is seen from early third instar stages in the D/V boundary, whereas EN-403 expression is seen only in late third instar discs (b). Expression of Ubx using these drivers results in repression of EN-403 in the anterior wing pouch (arrow in c). Incomplete repression along the A/P axis is probably due to low levels of expression of vg-GAL4 in that domain (arrow in b).

**Fig 3.15** Notch mediated regulation of EN-403. (a) Expression patterns of EN-403 and Wg in wild-type discs. Note that the two markers do not overlap in any part of the wing disc. (b) N* discs expressing EN-403 and UAS-GFP grown at permissive temperatures counterstained with antibodies against Wg. The expression pattern of EN-403 and Wg are normal. (c-d) N*; EN-403/UAS-GFP discs grown at non-permissive temperature show partial (c) to complete loss of EN-403 expression (left disc in d). However, partial loss of EN-403 is associated with very low levels of Wg (c) and complete loss of EN-403 is associated with moderate levels of Wg (d) expression in the D/V boundary suggesting that EN-403 regulation is not mediated through Wg.
3.2.3 Regulation of EN-403 by D/V organizer

Activation of EN-403 in the non-D/V cells of haltere discs carrying a clone of Ubx cells in the D/V boundary and in a converse experiment repression of the same in non-D/V cells of wing discs expressing Ubx in the D/V boundary, points to the fact that EN-403 expression is normally dependent on D/V signaling. N is required to establish a functional D/V boundary. To test if activation of EN-403 depends on N function, I analysed EN-403 expression in temperature sensitive N mutants. When larvae were at non-permissive temperatures, there was partial to complete absence of EN-403 expression both in wing (Fig3.15 b,c,d) and haltere discs (data not shown). The degree of repression of EN-403 in N's larvae depended on the length of time the larvae spent in non-permissive temperature, although the anterior expression domain of wing was more sensitive to repression in the absence of N (Fig 3.15b & c). The control larvae always had wild type expression pattern (Fig3.15a)

EN-403 does not require Wg signaling for activation

EN-403 is expressed in the posterior compartment of haltere discs. In this compartment, no Wg expression is observed in the D/V boundary. Ectopic expression of Ubx in the wing disc D/V boundary causes down regulation of EN-403 expression in the anterior compartment, although it does not affect Wg expression in the D/V boundary of that compartment. In fact, ectopic Ubx down regulates Wg expression in the posterior compartment of wing discs, wherein it does not affect EN-403 expression. Thus, EN-403 expression in non-D/V cells is likely to be independent of Wg expression in the D/V boundary.

To further test the requirement for Wg to activate EN-403 in non-D/V cells, I employed genetic backgrounds that remove Wg expression/function in the D/V boundary. In vg mutant discs, although initial activation of Wg in the early third instar disc by N is normal, its expression is not maintained (Fig 3.16a & b). Thus, late third instar vg' mutant discs completely lack Wg expression. However, vg' mutant discs continued to show robust EN-403 (and N23-GAL4) activation, suggesting that sustained Wg signaling is not required for continued expression of the same (Fig3.16c). In addition, down regulating the ability of D/V cells to transduce Wg by expressing
Fig 3.16 EN-403 does not require Wg signaling for activation. (a-c) patterns of Wg expression in vg\textsuperscript{1} mutant discs. Initial activation of Wg in the early third instar disc (a) is soon lost and late third instar vg\textsuperscript{1} mutant discs completely lack Wg expression. (d) robust expression of EN-403 (in green) in vg\textsuperscript{1} mutant discs. The middle one is a haltere disc.
dominant negative components of Wg signaling pathway (such as DN-TCF/pan) did not adversely affect EN-403 and N23-GAL4 expression patterns. However, there is a possibility that early Wg is required early during disc patterning to activate EN-403, which may continue to express either by auto-regulation or by some mechanism, which is independent of Wg function. In the previously described N\textsuperscript{as} experiments, I observed several wing discs, wherein EN-403 expression is completely repressed, while the D/V boundary continues to express low levels of Wg (Fig3.15d.)

Taken together, these experiments suggest the presence of a second signaling molecule in the D/V boundary in addition to Wg. Although it appears to be independent of Wg, there are many parallels. It is activated by N, it functions as a morphogen and activate gene expression at a distance. Finally, Ubx may repress the expression of this signal in a compartment specific manner during haltere development. Only difference is the two are repressed in different compartments. Henceforth I will refer to this additional D/V signal as twin of wingless (tow)

3.2.4 Strategy for identification of twin of wingless (tow)

3.2.4.1 Generation of EN403-lacZ reporter strain

Since EN-403 and N23 are both GAL4 enhancer-trap lines, in all mis-expression studies using vg-GAL4 ectopic expression of target genes in the EN-403 domain also occurs. Hence, prior to initiating experiments for identification of twin of wingless this GAL4 enhancer trap line was converted into a lacZ enhancer trap by a targeted P-conversion strategy (in collaboration with Nagaraj Sambrani). For the replacement of the GAL4 carrying P-element by lacZ carrying one (Fig. 3.17a), ap-lacZ (a lacZ enhancer trap of the ap locus carrying rosy\textsuperscript{+} marker) was used as the donor P-element. A balancer carrying the \Delta2-3 transposase source in a white background catalyzed the conversion event. Red eyed progeny representing the original GAL4 (w\textsuperscript{+}) were selected against. Progeny of all white-eyed flies (representing either the loss of EN-403 and/or insertion of lacZ) were screened for larval expression pattern mimicking that of EN-403 (Fig.3.17b). Only a single conversion event (L66) was identified that had a correct replacement of GAL4 by lacZ. Earlier results show that a smaller P element can be readily targeted to the locus of a larger one (Sepp et al, 1996). Hence, the large size (17.2-kb) of the P[lacZ, ry\textsuperscript{1}]
Fig 3.17 EN403-lacZ reporter strain, L66 and its pattern of expression. (a) L66 expression in the wing discs is similar to that of EN-403 in the wing and haltere discs (compare with Fig 3.8), however staining in the leg discs is much stronger than its GAL4 counterpart. (b) L66 is repressed in the anterior wing pouch on ectopic expression of Ubx only in the D/V boundary using vg-GAL4. For comparison vg-GAL4/UAS-GFP; EN-403/UAS Ubx discs are show again (also refer to Fig. 3.14). Note similar non-cell autonomous repression by Ubx in both the panels.
element, when compared to 11.3kb pGAL4 may have been the limitation in my experiment.

The newly generated EN403-lacZ, hereafter referred to as L66, showed an expression pattern similar to that of EN403-GAIA. Similar to EN-403 its expression in the anterior wing pouch was repressed on expression of Ubx along the D/V boundary using vg-GAL4 driver (Fig 3.17d).

3.2.4.2 Candidate gene approach

Several Wnts (such as DWnt2, DWnt4, DWnt10) are known to express in and around the D/V boundary of wing discs (Roel Nusse, personal communication), hence are likely candidates for the signaling molecule that activates L66. The Drosophila genome annotation project has identified as many as 7 Wnts in flies including Wg. Each of these Wnts was tested for their ability to activate L66 enhancer in non-D/V cells. None of them activated L66 in anterior haltere discs nor they induced ectopic L66 in wing discs. Thus, it is likely that L66 or EN-403 expression is not dependent on any member of the Wnt family of genes.

It has been shown that in addition to D/V signaling, Q-vg activation is also dependent on A/P signaling. Thus, Q-vg integrates both D/V (in the form of Wg) and A/P (in the form of Dpp) signals. Non-D/V specific expression is one of the common features of Q-vg and L66. I therefore, tested if L66 is also regulated by A/P signaling. Ectopic expression of Hh using vg-GAL4 non-cell autonomously activated L66 (Fig3.18b) in the anterior compartment of haltere discs, while ectopic expression of Ci using omb-GAL4 activated L66 expression. The expression of L66 almost filled the pouch, except in the D/V boundary suggesting its cell-autonomous activation by Ci (Fig3.18c). However, neither Dpp nor its activated receptor thick vein (tkv) activated L66 (the two were expressed using both vg-Gal4 and omb-GAL4). The mechanism of Hh-induced activation of L66 in haltere discs requires further investigation. Future work in this line includes examination of L66 expression patterns in the background of mutations in hh and its downstream effectors such as PKA, ptc, smo, fu, Ci etc. Since both L66 and hh are on 3rd chromosome, it requires recombination of the two. Temperature sensitive alleles of hh would be of particular use in such studies.
Fig 3.18 EN-403 is also regulated by A/P signaling. (a) expression of L66 and Wg in a wing and two haltere discs, expressing Hh along the D/V boundary (Genotype: vg-GAL4, UAS-Hh; L66). (b) Single channel staining of (a). Arrow points at the activation of L66 in the anterior haltere. Hh expression in the haltere D/V boundary however had no effect on Wg expression, which continued to be repressed in the posterior compartment (red arrows). (c) Ectopic Ci cell-autonomously activates L66. Discs are of genotype omb-GAL4;UAS-Ci/L66. The disc on the right side is an haltere disc showing L66 expression in the anterior compartment. Please compare with the wing disc, shown on the left side.
3.2.4.3 A Screen for novel wing and haltere patterning genes in Drosophila

A gain of function screen has been initiated to identify and characterize the genes that can regulate L66 expression. This screen utilizes the advantages of the GAL4-UAS system to target over-expression of random genes in either the D/V boundary of both wing and haltere discs (using vg-GAL4) or in the entire pouch (using omb-GAL4). A few of the predictions that are being currently tested are:

(a) If UBX directly represses the twin of wingless, its over expression via EP insertion in its locus is predicted to transform haltere like expression to wing kind and would be visualized as activation of lacZ in the anterior haltere.

(b) If UBX inhibits function of the twin of wingless by inducing repressors (transcriptional or at protein level) their over-expression in the wing D/V boundary is likely to cause the repression of lacZ in anterior wing.

(c) Over-expression of molecules that are cell autonomously involved in the pathway may cause lacZ activation in the D/V boundary.

3.3 Significance and conclusions:

While we know a great deal about the fly homeotic genes and regulators of wing morphogenesis, we have very little idea about other genes involved in the establishment of haltere identity. In this piece of work we have used a genetic approach to identify additional genes involved in this process.

3.3.1 Developing Drosophila wing: lessons from the haltere

The differentiation of the Drosophila haltere from the wing through the action of the Ubx gene is a classic example of Hox regulation of serial homology, and has served as the paradigm for understanding the nature of homeotic gene function. One unexpected and very informative finding of this work was the possible existence of a novel morphogen constituting the functional organizer in the developing wing.

Long-distance signaling from this organizer was analyzed by assaying the ability of a Ubx clone induced in the haltere D/V boundary to effect homeotic transformation of capitellum cells away from the boundary. The clonally restored wing D/V organizer in
mosaic halteres not only enhanced the homeotic transformation of $Ubx^-$ cells in the capitellum but also caused activation of a novel non-D/V specific marker (EN-403) in $Ubx^+$ cells. The suppression or activation of EN-403 was entirely independent of Wg activity in the D/V boundary, thus suggesting the presence of another signal molecule at the D/V boundary. Existence of such a molecule has not been predicted earlier, although wing development has been subjected to a large number of experiments, both at genetic and molecular levels. It is interesting to note here that Wg fails to rescue $N$ mutant wing discs, although its downstream target Vg is capable of the rescue.

I have been able to demonstrate the existence of such a molecule based on a completely different approach. My enhancer-trap screen was to identify markers that are differentially expressed between wing and haltere discs. The identified marker EN-403, which is expressed only in non-D/V cells, is not dependent on Wg function, although it responds to D/V signals. This I have shown both by activating D/V signals in haltere discs (by clonal removal of $Ubx$ from the D/V boundary) and by inactivating D/V signals in wing discs (by targeted expression of Ubx in the D/V boundary). Thus, I have successfully demonstrated that studies on differential development of wing and haltere not only help us to understand how Ubx modifies wing fate into haltere fate, would also provide insights into the mechanism of wing patterning.

A haltere is a modified wing with modified growth and development properties. If it had evolved by mere repression of growth, it would have resulted in a miniature wing rather than a haltere-like structure. Indeed, mutant phenotypes of $N$, wg and vg suggest that they all functional in haltere development, although D/V signaling is repressed by $Ubx$. This raises the question, how growth and patterning of the posterior compartment of haltere discs is achieved, wherein Wg expression is repressed by Ubx.

In this context the evidence that suggests that there might exist a second signaling molecule, other than Wg, in the D/V boundary and is functional in the posterior compartment of haltere discs, seems very attractive. In other words, while Wg confer D/V signaling to the anterior compartment of haltere discs, “twin of wingless” may transduce D/V signaling in the posterior compartment.

3.3.2 Evolutionary Possibilities: How did the fly haltere evolve?
One of the questions often raised in the evolutionary developmental biology (Evo-Devo) is as newer life forms evolved, did newer proteins get added to the proteome or did existing genes/proteins acquired new functions to their repertoire.

In the context of presence or absence of flight appendages, arthropods can be classified into two categories. One, such as Onychophora without wings. Second, insects such as butterflies and Drosophila with wings. Both the categories of arthropods express Ubx. Recently it has been shown that the ability of Ubx in insects to specify more diverse body pattern is due to the presence of a transcriptional repression domain in the C-terminal, which is absent in Ubx of other arthropods (Galant and Carroll, 2002).

Even in majority of the insect species, Ubx does not seem to have added complexity to the hind wings. Butterflies are the first to show distinction between fore and hind wings by the acquisition of subtle differences. The differences in the eyespot patterns in between the fore and hind wings have been shown to be a direct consequence of Ubx function in the hind wing (Weatherbee et al., 1999). Dipteran haltere, an extreme case of wing modification, is a direct consequence of Ubx-mediated modification of wing fate. Probably, during the evolution of body plan, Ubx acquired additional functions to increase the diversity in the anterior-posterior axis.

Unlike the differences between Onychophora and Drosophila, butterfly and Drosophila Ubx proteins are quite similar. This suggests that subtle molecular changes in Ubx protein in Drosophila might have resulted in gross morphological changes, such as wing-to-haltere modifications. This is possible if Ubx has targeted signal molecules that have long-distance activity, repression of which will have wider consequences. Results described in this chapter reinforce this idea in following ways,

1. Ubx down-regulates D/V signaling. This I have concluded based on the relative degree of transformations of Ubx clones generated in D/V, non-D/V or in both D/V and non-D/V cells. Since D/V signaling regulates the development of the entire wing blade, any influence of Ubx on D/V signaling will affect the entire wing blade.

2. I have identified a non-D/V-specific marker (EN-403), which is down regulated by Ubx in the anterior compartment of haltere discs. However, Ubx modifies the expression of this marker by modifying the D/V signaling.
3. Wg is a known morphogen expressed in the D/V boundary and influences growth and patterning in non-D/V cells. Earlier results suggest that Ubx down-regulates Wg signaling. In this study, I have demonstrated the possible existence of a second signal in the D/V boundary, in addition to Wg, whose activity is also modified by Ubx in haltere discs. It is therefore, likely that Ubx affects D/V signaling by regulating both the signaling molecules, which will have an enhanced effect on the wing blade development.

3.3.3 Segmental vs. compartmental mode of development

Although insect body plan is distinctly segmental, based on the expression patterns of homeotic genes in developing embryos, it has been proposed that development may not take place in the same way as we look at the adult organisms. It is now widely accepted that development takes place in compartments, although the two compartments, the anterior and the posterior, within a segment share several common features.

Although Ubx is required for the specification of the entire haltere, its expression in haltere discs seems to be regulated in a compartment-specific way rather than segment-specific manner. abx mutations in Ubx affect its expression only in the anterior compartment, whereas pbx mutations affect its expression only in the posterior compartment. These two classes of mutations induce phenotypes accordingly; i.e. transformation of only the anterior or posterior compartment, respectively.

It has been proposed earlier that wing development in Drosophila is not influenced by the function of any of the homeotic class of genes and it represents a ground state as far as the dorsal appendages are concerned (Carroll et al., 1994). Furthermore, fossil records suggest that modern-day insects evolved from ancestors, which had wing-like appendages in all trunk segments. Since homeotic genes such as Ubx modify this ground state, the prediction would be modification of target genes in compartment-specific manner. Indeed, amongst the genes that are differentially expressed between wing and haltere discs, Wg and Ct express only in the anterior compartment of haltere discs. Although these two genes are involved in patterning wing disc along the D/V axis, their expression seem to be affected by Ubx in a compartment-specific way along the A/P axis. Ubx represses Wg and Ct expression only in the posterior compartment.
compartment. Furthermore, over-expression of Ubx in the wing disc affects their expression only in the posterior compartment.

Here I have shown that regulation of EN-403 expression by Ubx is also compartment-specific. In haltere discs it is expressed only in the posterior compartment, whereas in wing discs it is expressed in both anterior and posterior compartments. Over-expression of Ubx in the wing disc affects EN-403 expression only in the anterior compartment. Thus, differential regulation of EN-403 is an additional evidence for compartment-specific regulation of T3 segment by Ubx.