A micro-evolutionary change in target binding sites as key determinant of Ultrabithorax function in *Drosophila*.

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Abstract

All Hox proteins are known to recognize, in vitro, similar DNA-binding sites containing a TAAT core sequence. This poor DNA-binding specificity is in sharp contrast with their specific functions in vivo. Here we report a new binding motif with TAAAT core sequence to which the Hox protein Ultrabithorax (Ubx) binds with higher affinity and specificity. Using transgenic and luciferase assays, we show that this new motif is critical for Ubx-mediated regulation of a target gene in *Drosophila melanogaster*. Interestingly, this new motif with TAAAT core sequences is not associated with the targets of Ubx in the honeybee, *Apis mellifera*, wherein hindwings are nearly identical to the forewings. We show that introduction of TAAAT motif in the place of TAAT motif is sufficient to bring an enhancer of a wing-promoting gene of *A. mellifera* under the regulation of Ubx. Our results, thus, suggest that binding motifs with a TAAAT core sequence may help identify functionally relevant direct targets of Ubx in *D. melanogaster* and the emergence of these binding sites may be crucial for Hox-mediated morphological changes during insect evolution.

Introduction

The diversification of morphology and emergent function of appendages have been important features of animal evolution. This is best exemplified in the diversity that is seen in closely related species of Arthropoda. In Bilaterans, *Hox* genes regulate the development of serially homologous structures along the anterior posterior axis (Lewis 1978; reviewed in

McGinnis and Krumlauf 1992). While genetic studies unequivocal on the role of Hox genes in the specification of various body parts along the antero-posterior axis, the mechanisms by which Hox proteins specifically identify and regulate the expression of their target genes remain elusive. As expression, structure, and function of Hox proteins are comparable among closely related species, it is postulated that variations in the regulation of targets of Hox proteins are critical for morphological diversification.

Multiple monomeric and low affinity binding sites are reported to confer specificity to Hox proteins (Galant et al. 2002; Crocker et al. 2015), which may also correlate to higher levels of Hox protein needed to achieve robustness in their function (Paul et al, 2021). Cofactors of Hox proteins such as TALE class of cofactors, particularly PBC and Meis family proteins, are also shown to be involved in the regulation of Hox target gene expression (reviewed in Merabet and Galliot 2015; Mann and Affolter 1998). However, various distal appendages in arthropods and vertebrates do not require the input of either the PBC or Meis family of cofactors which might be suggestive of a cofactor independent mechanism of target regulation.

In *Drosophila melanogaster*, the Hox protein Ultrabithorax (Ubx) specifies the development of halteres in the third thoracic (T3) segment (Lewis, 1978 and many other reports; reviewed in Khan et al. 2020). Loss of function mutations of Ubx gives rise to four winged flies with complete duplication of the T2 segment in place of T3 (Lewis 1978; Bender et al. 2007), while overexpression of Ubx in larval wing discs lead to wing to haltere transformations (Cabrera et al. 1985; White and Akam 1985; White and Wilcox 1985; Castelli-Gair et al. 1990). Ubx is known to bind, in vitro, to motifs with consensus binding site (TTTAATTA) having a TAAT core sequence (hereafter referred to as TAAT motif) (Egger et al. 1991; Noyes et al. 2008; Berger et al. 2008). ChIP-Chip studies suggest that Ubx binds to a large number of sites in the genome (Slattery et al. 2011; Choo et al. 2011; Agrawal et al. 2011). While most of those Ubx-binding sites have TAAT motif, they are not specifically enriched in pulled down sequences compared to their prevalence in the whole genome (Agrawal et al. 2011). Furthermore, many of those direct targets of Ubx are not differentially expressed between developing wings and halteres (Agrawal et al. 2011) . One possibility is due to its low-affinity binding to the chromatin, ChIP may pull down a very large number of

enhancers and not all of them are real targets of Ubx. Thus, it is not well understood what is the mechanism by which Ubx specifically recognizes and regulates its targets in the context of haltere specification.

Ubx is expressed in hindwing primordia of different insect species (Carroll 1995; Prasad, Tarikere et al. 2016). For example, in the Coleopteran *Tribolium castaneum*, Ubx is expressed and required for the specification of hindwings in the T3 (Tomoyasu et al. 2005), while in Lepidopterans like *Precis coenia*, it specifies differences in eyespot patterns between the hindwings and forewings (Weatherbee et al. 1999). In Hymenopterans like *Apis mellifera* which bear a marginally smaller hindwing as compared to the forewing, Ubx is expressed in both forewing and hindwing primordia. The expression, however, is stronger in the hindwing primordia (Prasad, Tarikere et al. 2016). In *Bombyx mori*, although hindwing is morphologically similar to the forewing, Ubx is expressed only in developing hindwings. Over-expression of Ubx derived from *A. mellifera* or *B. mori* or *T. castaneum* can suppress wing development and specify haltere fate in transgenic *D. melanogaster*, suggesting that changes in Ubx at the protein levels may have contributed only marginally to the evolution of its function. A study comparing genome-wide targets of Ubx in developing halteres of *D. melanogaster* and developing hindwings of *A. mellifera* and *B. mori* reveal that a large number of genes have remained common targets of Ubx between the two species. Among those common targets, a few wing patterning genes are differentially expressed in *D. melanogaster*, but not in *A. mellifera* or *B. mori* (Prasad, Tarikere et al. 2016). Evolutionary changes in cis-regulatory elements are thought to be associated with this phenomenon.

In this study, we performed ChIP-sequencing for the Ubx protein in third instar haltere imaginal discs from *D. melanogaster*. Our analysis led to the identification of a motif (MATAAATCAY) containing a TAAAT core sequence (hereafter referred to as TAAAT motif) that was enriched in ChIP pulled-down sequences. The prevalence of this motif, however, was found to be similar between putative enhancers of direct targets of Ubx that are upregulated or downregulated in the haltere. In vitro analyses suggest that Ubx binds strongly to TAAAT motif than the previously reported TAAT motif. We experimentally analyzed enhancer of one target that is upregulated (*CG13222*) by Ubx during haltere specification and one that is down

regulated (*vestigial*). Studies using the *CG13222* enhancer in *D. melanogaster* halteres suggest that the TAAAT motif is functionally relevant and is critical for its upregulation by Ubx.

In *A. mellifera*, the enhancer of the *vestigial* (*vg*) gene has a TAAT motif, and no TAAAT motifs. In transgenic *D. melanogaster*, this orthologous enhancer of *vg* of *A. mellifera* drives reporter gene expression in both wing and halteres and is not sensitive to over-expression or loss of Ubx. Changing the TAAT motif to TAAAT motif was sufficient to bring this, otherwise unresponsive, enhancer under the regulation of Ubx. Comparative genome-level analyses suggest that, unlike in *D. melanogaster*, TAAAT motifs are not enriched in ChIP-pulled down sequences by Ubx from developing hindwings of *A. mellifera*. Taken together, our study suggests that evolutionary changes in the regulatory sequences in the targets of Ubx in dipteran lineages, specifically from TAAT to TAAAT as core sequence in Ubx-binding sites, is correlated to the evolution of Ubx-mediated specification of haltere.

Results

Motif with a TAAAT core sequence is enriched in direct targets of Ubx in developing halteres in *D. melanogaster*

Several previous studies, many from our own group, have reported identification of direct targets of Ubx in developing halteres. Those studies employed single gene approaches or ChIP-chip based methods. In order to identify Ubx-binding sites with higher accuracy and resolution in the genome, we employed ChIP-sequencing, using antibodies that are specific to Ubx to pull down the chromatin from the third instar haltere discs. Our analysis revealed 2171 high confidence peaks for Ubx annotating to 1236 direct targets, of which 418 genes were common to ChIP-microarray data from Choo. et al, 2011 and 148 genes were common to Agrawal et al, 2011 (Fig. S1 A). We observed greater occupancy of Ubx at intronic and intergenic regions (Fig. S1 B). Several previously reported direct targets of Ubx such as *CG13222* and *spalt* were also part of the ChIP-seq data (Fig. S1 C, S1 D).

We carried out extensive bioinformatic analysis to identify common sequence motif/s in pulled down sequences, which is/are expected to be recognition sites for Ubx to bind to its targets. Using a de-novo motif finding algorithm, we observed that a motif (MATAAATCAY) with TAAAT core sequence is enriched in Ubx pulled down sequences. This motif was found

to be 1.7fold overrepresented in Ubx pulled down sequences as compared to its prevalence in the whole genome (Fig. 1B). The canonical TAAT motif is not enriched in Ubx-pulled down sequences, rather it was found under-represented compared to its prevalence in the whole genome (Fig. 1B). Similar observations are reported earlier when ChIP-chip method was employed to identify direct targets of Ubx (Agrawal et al., 2011).

Earlier studies have reported that TAAAT motif is bound by the Ubx-Extradenticle (Exd) complex during embryonic development. However, Exd is not required for the development of the haltere capitellum (Casares and Mann 2000). In this context, we first sought to determine functional significance of Ubx-binding to the TAAAT motif. We carried out RNA-seq analyses to identify genes that are differentially regulated between the third instar wing and haltere imaginal discs (generation of RNA-seq and analyses- unpublished data). Among 611 genes that are differentially expressed between wing and haltere discs, 71 genes are common to ChIP-Seq data suggesting them to be potential direct targets of Ubx. We categorised direct targets of Ubx expressed in wing and/or haltere discs into three categories: genes that are upregulated in haltere discs (44), downregulated in haltere discs (27) and genes that are expressed at equal levels between wing and haltere discs (99) (Fig. 1C). We found that the frequency of the TAAAT motif in putative Ubx response elements in these three categories of targets are similar (Fig. 1D). While TAAAT motif may be preferred site for Ubx to bind on the enhancers of its targets, it was not obvious from these analyses its significance in the context of Ubx-mediated regulation of its targets.

The TAAAT motif in the CG13222 enhancer confers high-affinity and specificity in Ubx binding *in-vitro*

We next evaluated relative affinities and specificities of Ubx binding to the TAAAT motif to regulate its targets as against binding to the TAAT motif. We chose two well-characterized targets of Ubx, Spalt (*sal*) and CG13222. While Ubx downregulates *sal* (Galant et al. 2002), it upregulates CG13222 (Mohit et al. 2006; Hersh et al. 2007). The enhancer of *sal* contains several sites with the TAAT motif (Fig. 2A) and these sites were shown to be recognized by Ubx for effective repression in the haltere imaginal disc (Galant et al. 2002). The “edge” enhancer of CG13222 has two TAAT motifs at 17 bp apart. Hersh et al. (2007) have named the two motifs as site 1 and site 2. In their transgenic assays, site 1 appears to be critical for Ubx-

mediated regulation of *CG13222*, while site 2 is dispensable. Interestingly, the site1 in the *edge* enhancer which is critical for its regulation by Ubx has a TAAAT motif in proximity to the TAAT motif (Fig. 2A). Their analysis of site1 involved mutating both TAAT and TAAAT motifs, which leads to loss of enhancer driven reporter gene expression (Hersh et al. 2007). We mutated site1 in such a way that it affected only the TAAAT (M1_A) or the TAAT motif (M1_B) (Fig. 2A) and examined the relative affinities of binding of Ubx to these sites.

First we directly estimated binding affinities by employing Electrophoretic mobility shift assays (EMSA). We used a 29bp long probe for *sal* and a 21bp long probe covering site 1 of *edge* enhancer of *CG13222* for EMSA. Two different Hox proteins, Ubx and Sex combs reduced (Scr) were tested on these probes for assessing DNA-binding specificity *in vitro*. Results showed that both the Hox proteins bind on the probe derived from the *sal* enhancer, although with less affinity for Scr (probe Spalt 5/6 in Fig. 2B). In contrast, only Ubx could bind on the probe derived from the *CG13222 edge* enhancer suggesting a higher level of DNA-binding selectivity when compared to the *sal* enhancer (probe WT (site1) in Fig. 2B). We find that the mutation in TAAAT motif in site1 of *CG13222* led to a complete loss of Ubx binding, while the mutation in TAAT motif had no significant effects (probes M1_A and M1_B, respectively in Fig. 2B). These experiments, thus, demonstrate that the TAAAT motif may confer higher affinity and specificity for Ubx to bind on the enhancer of *CG13222* (Fig. 2B).

Next we examined the relative importance of the two motifs in gene regulation using Luciferase reporter assays in S2 cells. For these functional assays, we used a 512bp long region spanning the *edge* enhancer of *CG13222* as reported by Hersh et al. (2007), which has both site 1 (consisting of one TAAT motif and one TAAAT motif) and site 2 consisting of one TAAT motif). Consistent with *CG13222* being a target of Ubx upregulated during haltere development, a luciferase expression construct driven by the enhancer of the gene was upregulated in S2 cells only in the presence of Ubx (construct WT in Fig. 2C and Fig. S2 A). We observed a significant decrease in Ubx-dependent expression compared to the wildtype *CG13222* enhancer when TAAAT alone is mutated (construct M1_A in Fig. 2C), thus suggesting that TAAAT motif is essential for Ubx-dependent expression of *CG13222*. We also attempted to examine the effect of mutating the TAAT motif to a TAAAT motif (in site2), which results in two high-affinity binding motifs in the *edge* enhancer of *CG13222*. We observed marginal

reduction in the luciferase activity (Fig. S2 B), perhaps, due to negative effect of two high-affinity binding sites for Ubx in close proximity (however see below results of transgenic assays).

TAAAT motif is essential for Ubx-mediated activation of CG13222 *in vivo*

To assess the role of the TAAAT motif in Ubx-mediated activation of *CG13222* enhancer in the haltere imaginal discs, we generated transgenic strains of *D. melanogaster* carrying the wild type or mutant enhancers cloned upstream of a GFP reporter. The wildtype *CG13222* enhancer showed GFP expression at the posterior edge of the haltere imaginal discs consistent with earlier reports (Fig. 2D; Mohit et al. 2006; Hersh et al. 2007). We generated transgenic lines for multiple mutant versions of the *CG13222* enhancer. The reporter gene expression is significantly reduced when driven by the M1 mutant enhancer, which mutates both TAAT and TAAAT motifs at site 1 of the enhancer (Fig. 2E). We observed similar loss of reporter expression when the GFP was driven with a version of mutant enhancer (M1_A mutant) which mutates only the TAAAT motif (Fig. 2F, 2H). This suggests that Ubx mediated activation of the *CG13222* enhancer is dependent on the TAAAT motif.

As described earlier, the *edge* enhancer of *CG13222* has a second TAAT motif (site 2), which is redundant for Ubx-mediated upregulation of *CG13222* in the haltere discs (Hersh et al. 2007). We generated a transgenic line (M2_A) wherein this TAAT core sequence is mutated to TAAAT core sequence resulting in two TAAAT motifs in the modified enhancer as against one in the wildtype. This transgene showed much stronger expression of the GFP reporter compared to the wildtype *CG13222* enhancer. Enhancer driven expression was also observed in ectopic regions in the posterior compartment (red arrows in Fig. 2G), further suggesting that the presence of a TAAAT motif brings the, otherwise redundant, site2 under Ubx regulation.

Introduction of a TAAAT motif is sufficient to bring an otherwise unresponsive enhancer of *vestigial* gene of *A. mellifera* under Ubx control in a transgenic *Drosophila* assay

Examination of *CG13222* enhancer suggested an essential role for TAAAT motifs in Ubx mediated upregulation of target genes. We next analysed cis-regulatory regions of *vg* (*vestigial*), a pro-wing gene, which is downregulated by Ubx in developing halteres,

(Weatherbee et al., 1998; Shashidhara et al., 1999) for the presence of TAAAT sites. Of the two well-characterized enhancers of *vg*, the quadrant enhancer (hereafter referred to as *quad-vg* enhancer) is differentially expressed between wing and haltere discs. Scanning for sequence motifs revealed that this enhancer has a 25bp cassette containing both TAAAT and TAAT motifs in close proximity (Fig. 3A). Sequence alignment of the region containing the Ubx binding motifs show high conservation of both TAAT and TAAAT among most *Drosophila* species (Fig. S3 A).

We examined the relative importance of TAAAT and TAAT motifs for Ubx-dependent downregulation of *quad-vg* enhancer in a transgenic assay. As reported earlier, we observed that the wild type *quad-vg* enhancer drives expression of the GFP reporter in the wing imaginal discs, albeit at low levels (compared to earlier reported *quad-vg* -lacZ reporter), but not in the haltere imaginal discs (Fig 3B). To test a possible role for the TAAAT motif in mediating differential expression of this enhancer, we generated transgenic flies carrying mutations (Fig. S3 B) in the Ubx-binding sites. However, we observed complete loss of reporter GFP expression in both wing and haltere imaginal discs for most mutant constructs of *quad-vg*, suggesting that mutations in Ubx-binding motifs of the *quad-vg* enhancer may completely inactivate the enhancer element (Fig. 3B, Fig. S3 C).

We had previously reported that the *vg* gene in *A. mellifera* (hereafter referred to as *Apis-vg*) is expressed in both fore- and hindwings (Prasad, Tarikere et al. 2016). In a transgenic *Drosophila* assay, an enhancer of *Apis-vg* equivalent to *quad-vg* showed similar patterns and levels of expression between wing and haltere discs (Prasad, Tarikere et al. 2016). However, we could not locate a specific region in the two enhancers that is relevant to understand the evolution of differential expression of *vg* in wing and haltere in *D. melanogaster*. We specifically searched for TAAAT motif/s in the enhancer of *Apis-vg*. While enhancer of *Apis-vg* contains a TAAT motif, we did not find any TAAAT motifs (Fig. 3C). We tested the significance of this in a transgenic *Drosophila* assay. We generated new transgenic flies with wild type and mutant enhancers of *Apis-vg*. As reported earlier, we observed similar and strong expression of GFP in both wing and haltere imaginal discs (Fig. 3D). *vg* gene in *D. melanogaster* is expressed in both the presumptive hinge and pouch regions. However, the *quad-vg* enhancer of *D. melanogaster* drives its expression only in the non dorso-ventral

boundary cells of the pouch. *Apis-vg* driven GFP expression, while was much lower in the dorso-ventral boundary cells, is at equivalent levels in both the presumptive hinge and the pouch regions. The expression pattern of GFP driven by the enhancer of *Apis-vg* in *Drosophila* wing discs is an indication of high degree of conservation of this enhancer between *A. mellifera* and *D. melanogaster* (which have diverged for more than 350 million years). Wing patterning genes upstream of *vg* are able to regulate *Apis-vg* enhancer in a pattern similar to its counterpart in *D. melanogaster*. Nonetheless, this enhancer is not sensitive to Ubx-mediated regulation of gene expression in haltere discs.

We generated a number of transgenic flies carrying various mutations in the *Apis-vg* enhancer with the aim to understand the significance of the absence of TAAAT motif vis-à-vis its expression in haltere discs in transgenic *Drosophila*. First we generated a chimeric enhancer (*Apis-vg_M2*), in which a 25bp Ubx-binding cassette from the *Drosophila quad-vg* enhancer replaced its counterpart in the *Apis-vg* enhancer (Fig. S4 A (iii)). *Apis-vg_M2* showed differential expression of GFP reported between wing and haltere imaginal discs (Fig. S4 C). Its expression was much lower in the haltere pouch compared to the GFP reporter driven by the wildtype *Apis-vg* enhancer (Fig. S4 C). In another chimeric enhancer, only a part of the cassette with the TAAAT motif was introduced from *quad-vg* of *D. melanogaster* (*Apis-vg_M3*). This too resulted in differential expression of the GFP reporter in wing and haltere discs (Fig. S4 C). Interestingly, the GFP expression was lower only in the pouch region and not in the hinge region suggesting that these manipulations of the sequence of *Apis-vg* enhancer brings its expression patterns closer to that of *quad-vg* of *Drosophila*. More importantly, changing only the TAAT motif of the *Apis-vg* enhancer to a TAAAT motif (*Apis-vg_M1*) was sufficient for the repression of the GFP reporter in the haltere pouch without affecting its expression in the wing pouch (Fig. 3D, 3E, 3F, Fig. S4 B). This suggests that micro-evolutionary changes in the enhancer sequence may have brought the *vg* gene under the regulation of Ubx in dipteran lineages. This gene being a pro-wing gene, its repression is critical for Ubx-mediated specification of haltere.

Enrichment of TAAAT motif in the enhancers of targets of Ubx is specific to *D. melanogaster*:

Our results suggest that differences in Ubx binding motif (TAAAT vs TAAT) in enhancers of orthologous targets may have brought key wing patterning gene *vg* under the differential

regulation by Ubx in dipteran lineage. To understand whether the difference in Ubx binding motif might be a recurrent theme that impinges on differential hindwing morphology, we compared genome wide binding of the Ubx protein between *D. melanogaster* and *A. mellifera*.

Previous studies indicate that despite the differences in the hindwing morphology, the Ubx protein is expressed in the third thoracic segment of both insects (as well as other insect lineages) (Fig. 4A) (Carroll 1995; Prasad, Tarikere et al. 2016). Additionally, Ubx is highly conserved across *A. mellifera*, *B. mori*, *T. castaneum*, *Junonia coenia* and *D. melanogaster* at the level of its DNA binding homeodomain and the protein interaction domains, YPWM and Ubda (Fig. S5 A). We have previously shown that overexpression of Ubx from different insect species in the wing imaginal disc of transgenic *Drosophila* resulted in wing to haltere transformation in a pattern similar to *Drosophila* Ubx (Prasad, Tarikere et al. 2016). This suggests that differences in insect hindwing morphology is a consequence of events occurring downstream or parallel of Ubx. Comparison of targets of Ubx between *D. melanogaster* and *A. mellifera* suggest that many functionally important genes are common to the two lineages (Fig. S5 B). This reconfirms that morphological differences may lie at the levels of regulation of those targets.

We analysed previously reported Ubx ChIP-seq data of *A. mellifera* (Prasad, Tarikere et al. 2016) for the presence of TAAT and TAAAT motifs. We did not observe any enrichment of either TAAT or TAAAT motifs in the *A. mellifera* Ubx ChIP-seq dataset (Fig. 4B, 4C). However, we observed enrichment for binding sites for other transcription factors GAGA, CTCF and Brk in both *D. melanogaster* and *A. mellifera* datasets (Fig. 4B). Interestingly, the frequency of the TAAAT motif in the entire genome is 1.8 fold higher in *A. mellifera* as compared to *D. melanogaster* (Fig. 4C). This suggests that the TAAAT motif is selectively enriched in the enhancers of targets of Ubx only in *D. melanogaster*.

In addition, we also observed differences in the clustering of peaks vis-à-vis transcription start site (TSS) in the two species (Fig. S5 C). While in *D. melanogaster*, Ubx binding sites are clustered away from the TSS, the reverse is observed in *A. mellifera*.

However, further studies are needed to understand functional relevance, if any, of this topology of binding sites.

Taken together, In the context of the fact that Ubx brings differential development of the dorsal appendages of T2 and T3 in *D. melanogaster*, but not in *A. mellifera*, our results help uncover a mechanism in which micro-evolutionary changes in enhancer sequences could bring in key wing patterning genes under the regulation of Ubx.

Discussion

Genetics and developmental biology methods have established, unequivocally, the role of Hox genes as master control genes in regulating segment-specific developmental pathways. However, precise mechanism by which each of the Hox proteins mediate development of a specific morphological feature is still largely debated. Main reasons this has eluded systematic study include their highly conserved DNA-binding domains in both Hox and non-Hox homeobox-containing transcription factors and their overlapping expression patterns in a given set of cells.

In *D. melanogaster*, Ubx activates and represses a number of genes at various levels of hierarchical wing patterning pathways, some by direct regulation and largely by indirect regulation. Identifying mechanisms by which Ubx directly regulates a few key wing patterning genes may throw a light on the mechanism by which it specifies haltere development. For example, in the context of unequal size of the anterior and posterior compartments in haltere discs (as against equal size in wing discs), multiple studies have elucidated an elegant mechanism (Mohit et al. 2006; de Navas et al. 2006; Crickmore and Mann 2006; Makhijani et al. 2007; Crickmore and Mann 2007). Ubx activates the expression of Thickvein (Tkv) at the A/P boundary and suppresses the expression of Dally in the posterior compartment making the gradient of growth molecule Decapentaplegic (Dpp) more pronounced in the anterior compartment than the posterior compartment.

Efforts over the past 3 decades from multiple laboratories, including ours, are centred around the hypotheses that Ubx-mediated specification of haltere development involves (i) a possible unique Ubx-binding motif that identifies a gene as a target of Ubx; (ii) modulation of

expression of a set of direct targets would lead to distinct changes in number, size, shape, and organization of epithelial cells resulting haltere structure as against wing structure and (iii) step-wise regulation of wing developmental pathways to modify its fate to haltere.

Earlier efforts involving whole genome approaches, such as microarray and ChIP-chip analyses, to identify all potential targets of Ubx have not yielded satisfactory results. With the advent of higher resolution, specificity and sensitivity in genome sequencing techniques, we envisaged ChIP-Seq and RNA-seq to identify direct targets of Ubx that are functionally relevant to haltere specification. Detailed lists of all genes identified using these methods and the implications of the same to understand the mechanism of function of Ubx would be published elsewhere. While analysing the enhancer regions of direct targets of Ubx identified by ChIP-Seq, we observed presence of a putative Ubx-binding motif with TAAAT core sequence, which is different from the one (TAAT motif) being studied for more than 2 decades now. The fact that Ubx binding to this motif is stronger suggests a possible mechanism for target identification. While low-affinity binding brings Ubx on the enhancers of a very large number of genes, only those enhancers which have stronger affinity for Ubx would be impacted at the functional level. Earlier reports suggest that specificity in gene regulation by Hox proteins may come from stronger binding to their targets, which in turn could be ascribed to higher levels of a given Hox protein in a specific segment (Paul et al. 2021) and/or to the cofactors binding to nearby locations on the chromatin (Agrawal et al. 2011). Our observation on the presence of high-affinity binding sites on the enhancers of targets of Ubx adds another dimension to the mechanisms by which robustness is achieved during Ubx-mediated haltere development.

We studied the consequence of binding of Ubx to TAAAT motif in the context of regulation of two different genes. *CG13222*, a gene upregulated by Ubx in the haltere and *vg*, a gene downregulated by Ubx. Our observations reported here suggest that Ubx binds strongly to TAAAT motif than to TAAT motif and its binding to former is critical for the regulation of *CG13222* expression. We also report that TAAT motif present in the enhancer of *CG13222*, which is also ubiquitously present in the genome, is dispensable for Ubx-mediated regulation.

However, we could not directly study Ubx-mediated regulation of *vg* in *Drosophila*, perhaps due to Ubx binding to a region which is critical for its expression in developing wings. As Ubx represses wing development to promote haltere development, this suggests a possible mechanism in which Ubx interferes with gene regulatory network to activate *vg* during wing development. Binding of Ubx with high affinity to this part of the enhancer of *vg* may interfere with the binding of other transcription factors. This, however, needs to be empirically validated.

An earlier report of ours (Prasad, Tarikere et al. 2016) and this report suggest that an enhancer of *vg* gene of *A. mellifera* can drive GFP expression in wing imaginal discs in a transgenic assay in a pattern similar to that of the quadrant enhancer of *Drosophila vg*. The enhancer of *Apis-vg*, however, is not sensitive to Ubx and drives reporter gene expression at high levels in the haltere. The enhancer of *Apis-vg* allowed us to dissect the importance of TAAAT motif as we could achieve its repression in haltere discs without affecting its expression in wing discs. We show that changing the TAAT motif to TAAAT motif, dramatically, brought the enhancer under the negative regulation of Ubx. As *vg* is a pro-wing selector gene and can assign wing fate to any group of dorsal epithelial cells (Kim et al. 1996; Klein and Arias 1998; Williams et al. 1991; Neumann and Cohen 1996), it is a critical target of Ubx to specify haltere development. In this context, a microevolutionary change (TAAT to TAAAT motif) in the enhancer of an ancestral *vg* may be a critical step in the evolution of dipterans.

We have observed that, in general, TAAAT motif is more prevalent among the direct targets of Ubx in *D. melanogaster* than in *A. mellifera*. The *Drosophila* Ubx protein has longer linker region (than the Ubx of *A. mellifera*) between the YPWM motif (which mediates protein-protein interactions) and the homeodomain (which mediates binding to DNA) in the isoform Ia that mediates haltere specification. Once Ubx binds strongly to the chromatin with the help of TAAAT motif, it may interfere with other proteins in the neighbourhood modifying the gene expression pattern from wing type to that of haltere. Thus, evolutionary changes in the protein structure as well in sequences in the enhancers of its target genes may both be involved in the evolution of function of Ubx as a modifier of wing development, re-specifying its fate to haltere. It is, however, very unlikely that a large number of convergent mutations would be involved in this process, wherein multiple TAAT motifs are evolved to TAAAT motifs.

A few critical genes, such as *vg*, upstream of wing patterning pathways may have evolved to become targets of Ubx through this route. Once regulatory networks of those genes are modulated by Ubx, chromatin landscape of many other targets (Ubx may bind to these targets via TAAT motifs) would change making them amenable for Ubx-mediated regulation and thereby specifying the haltere fate.

Materials and Methods

1. ChIP sequencing: The protocol was essentially as reported by (Agrawal and Shashidhara 2014). Third instar wandering larvae were cut, inverted and fixed with 1.5% PFA (ThermoFisher scientific) for 20 minutes at room temperature and subsequently quenched (125mM Glycine solution, 1XPBS). Samples were washed twice with 1XPBS (Sigma) for 10minutes each and dissected for wing and haltere discs (500 number of wing discs and 1000 number of haltere discs were used per replicate). Samples were lysed with Cell Lysis Buffer (10mM Tris-Cl pH 8, 10mM NaCl, 0.5% NP40, 1xPIC (Roche)) followed by mechanical shearing for 10minutes on ice. After centrifugation at 2000rpm for 10mins at 4 °C, the was pellet resuspended in Sonication buffer (50mM Tris-Cl pH 8, 1% SDS, 1% NP40, 10mM EDTA, 1xPIC) followed by incubation on ice for 30 minutes. Sonication was carried out using the Covaris S2 sonicator using settings (DC 20%, Intensity 5, Cycles of Burst 200, Time= 20 minutes). Samples were centrifuged at 14000rpm for 15mins at 4 °C and precleared using with 4ml of Magnetic A Beads (Diagenode) at 4 °C for 1 hour. Preclearing beads were removed and 2ml of Polyclonal anti-Ubx antibodies (Agrawal et al. 2011) were added with overnight incubation at 4 °C. Magnetic A beads were added and incubated for 4 hours at 4 °C. The supernatant was removed and beads washed twice with low salt buffer (20mM Tris-Cl pH 8, 150mM NaCl, 0.1 % SDS, 2mM EDTA, 1% TritonX-100), twice with High Salt buffer (20mM Tris-Cl pH 8, 500mM NaCl, 0.1 % SDS, 2mM EDTA, 1% TritonX-100), twice with LiCl buffer (10mM Tris-Cl pH 8, 250mM LiCl, 1% NP40, 1% Na-Deoxycholate, 500mM EDTA,), and once with TE buffer (10mM Tris-Cl pH 8, 500mM EDTA). Samples were eluted in elution buffer (50mM Tris-Cl, 1mM EDTA, 1% SDS, 50mM NaHCO₃). Samples were decrosslinked followed by treatment with RNase treatment at 37 degrees for 1hour and Proteinase K treatment at 42 °C for 2 hours. DNA was purified using PCI purification and quantified using the Qubit HS DNA quantification system.

For library preparation, equal amount of DNA (~2 ng) was used as an input for NEB Ultra II DNA library prep kits (NEB #E7645). Number of cycles for amplification of adapter ligated libraries were estimated by the qPCR before final amplification to avoid any bias arising due to PCR amplification and indexing (NEB #E7350). Final amplified libraries were purified twice, first with 1X followed by 0.8x volume of beads per sample using HiPrep PCR clean up system (Magbio #AC-60050). Library concentration was determined using Qubit HS DNA kit (Invitrogen #E7350) and average fragment size was estimated using DNA HS assay on bioanalyzer 2100 (Agilent #5067-4626) before pooling libraries in equimolar ratio. Sequencing reads (100bp PE) were obtained on the Hiseq 2500 V4 platform at Macrogen Inc, Korea.

2. ChIP seq analysis: For *D. melanogaster*, raw reads were trimmed using Trimmomatic software and aligned to the dm6 genome (version BDGP6.28). Peak calling was performed using MACS2 (maxdup=1, FDR 0.05) (Zhang et al. 2008) and high confidence peaks (occurring in at least two biological replicates) were identified (<https://ro-che.info/articles/2018-07-11-chip-seq-consensus>).

For *Apis mellifera*, raw reads were downloaded from NCBI (GSE71847). Reads were aligned to the Amel_4.5 genome (version 4.5.47) and peak calling done using MACS2 (maxdup=1, FDR 0.05). High confidence peaks occurring in both replicates were identified.

Peaks were annotated to their nearest TSS using the Homer software (annotatePeaks.pl) (Heinz et al. 2010). Gene ontology of targets was performed using GO (Ashburner et al. 2000; Carbon et al. 2021) and overrepresented GO terms plotted. The ChIPseeker program (Yu et al. 2015) was used to annotate peaks to genomic regions. Genome tracks were made using Deeptools (normalizeUsing RPGC, binsize 1, minMappingQuality 10, smoothLength 150) (Ramírez et al. 2016) and visualized using the IGV software (Robinson et al. 2011). Heatmaps were generated using Deeptools (referencePoint TSS).

3. Motif analyses and protein sequence comparison: De-novo motif analysis was carried out using Homer (findMotifsGenome.pl). The PWM for the TAAAT motif obtained from Homer was converted to Transfac format using RSAT (Nguyen et al. 2018) and finally converted to MEME format using transfac2meme command. A 100bp pad on both sides was applied to

ChIP peaks for calculation of frequency of motifs using the FIMO software from MEME suite. Identification of binding motifs in enhancers of *CG13222*, *quad-Vg* and *ApisVg* was performed using the MAST software from MEME suite (Bailey et al. 2009).

Protein sequences of Ubx from different insect species were downloaded from Uniprot and multiple sequence alignment performed using Clustal Omega (Madeira et al. 2019). Alignments were visualised and processed using the Jalview software (Waterhouse et al. 2009).

4. Comparative analyses of putative Ubx response elements: Genes that are differentially expressed between the wing and haltere were identified using RNA sequencing of third instar wing and haltere imaginal discs (unpublished data). A fold difference (FD) cut-off of 1.5 fold was applied to identify upregulated or downregulated genes in the haltere. Genes having a fold difference ranging between 0.95-1.05 between the wing and haltere imaginal discs were considered as not-differentially expressed. Ubx targets obtained from ChIP-seq was compared to RNA-seq data and frequency of the TAAAT motif calculated using an analysis module (unpublished data).

5. Plasmids constructs, cloning and transgenic fly generation: The full list of primers as well as sequences of enhancers of the *CG13222*, *quad-Vg* and *ApisVg* is provided as supplementary data (File S2). For Luciferase assays, site-directed mutagenesis was used to clone enhancer constructs of the *CG13222* gene between Kpn1 and Nhe1 restriction sites, upstream of a modified pGL3 vector containing a 5X Dorsal binding site. Metallothionein inducible pRMHa3 vectors containing Ubx were previously generated in the lab and empty pRMHa3 vector was generated by excising the cloned Ubx sequence.

For generating transgenics, all enhancer constructs were cloned between the Nhe1 and Kpn1 restriction sites of the pH-stinger-attb (a kind gift from Manfred Frasch) and injected into the attp40 site on the second chromosome using a mini white screen at NCBS fly facility, Bangalore. All constructs were sequence verified before use in functional assays.

6. Luciferase assays: S2 cells were checked for contamination before performing Luciferase assays. Cells were plated onto 24 well plates at a density of 3×10^5 cells per well 6 hours prior to transfection. For every construct, either wild type or mutant, two sets of experiments were designed; one well was co-transfected with the enhancer construct in pGL3 vector and the empty pRMHa3 vector whereas the other well was co-transfected with the enhancer construct and pRMHa3 vector containing Ubx. Renilla luciferase was used as an internal control and co-transfected in all experiments. Transfection was carried out using the Effectene transfection reagent and all experiments carried out in 3 technical replicates and at least 3 biological replicates. 48 hours post transfection, sterile CuSO₄ solution was used to induce expression of Ubx at a final concentration of 500µm and incubated for 24 hours. Cells were harvested, pelleted down (1000rpm for 4mins) and 100ul of 1X Passive Lysis buffer added and vortexed to dissolve the cell pellet. Cells were incubated for 15mins at room temperature and further spun at 10,000rpm for 90secs to collect the supernatant. The luminescence was measured with the Dual-glo Luciferase assay kit (Promega) on Enight Plate reader (Perkin Elmer). All readings were normalized to Renilla luminescence and datasets compared using the Prism software.

7. EMSA: VC-Ubx and VC-Scr were cloned in the PcDNA3 vector and produced with the TNT-T7-coupled in vitro transcription/translation system (Promega) for EMSAs, as previously described (Hudry et al. 2012). Shortly, between 3 and 6 microliters of programmed lysate was used for each protein (100 ng/ml of proteins were produced on average). The VC-Ubx and VC-Scr fusion proteins were produced separately (0.5mg of each plasmid was used for the in vitro transcription/translation reaction). Supershift against the VC fragment was performed by adding the anti-GFP antibody after 15 minutes in the binding reaction. Each band shift experiment was repeated at least two times.

8. Immunostaining and Microscopy: Wandering third instar larvae were cut and inverted in cold PBS followed by fixation with 4% PFA for 20mins with gentle rocking. Samples were washed thrice with 0.1% PBTX (0.1% Triton in PBS) for 10 minutes each, followed by one hour of blocking at room temperature (blocking solution: 0.5% BSA in 0.1%PBTX). Samples were incubated with primary antibodies (dilutions made in blocking solution) at 4 °C overnight followed by washing with 0.1% PBTX for 10mins (x3) at room temperature. Samples were

incubated with secondary antibodies for one hour at room temperature, washed with 0.1% PBTX for 10mins (x3) and finally with PBS before mounting. Wing and haltere imaginal discs were dissected out, mounted using Prolong Gold Antifade (Invitrogen) and stored at 4 degrees. Antibodies used in the study are Rb-GFP (1:1000) (Invitrogen), Rb-Ubx (1:1000) (Agrawal et al., 2011), m-Ubx (1:30) (DSHB), goat-Hth (1:100) (Santa Cruz Biotechnology), Alexa Fluor 568 (1:1000) (Invitrogen) and Alexa Fluor 633 (1:1000) (Invitrogen). All images were taken on Leica Sp8 system at Microscopy Facility, IISER Pune. Laser settings (Laser Power (LP) and Gain (G)) used for measuring GFP fluorescence have been indicated for each image panel. All images were processed using the ImageJ software and compiled using Microsoft PowerPoint and Adobe Illustrator.

Data availability: Raw fastq files of ChIP sequencing for the Ubx protein in *Apis mellifera* hindwings can be accessed from NCBI (GSE71847). Raw data for ChIP sequencing for Ubx in *Drosophila melanogaster* has been submitted to GEO. RNA sequencing data for the wing and haltere imaginal discs will be published elsewhere.

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Author contributions:

SK carried out all fly experiments, image analyses, and wrote the MS.

SK and SP did ChIP-Seq and RNA-Seq and analyses.

GG, FB and RP did EMSA experiments.

LSS and SM conceived the project and wrote the MS.

We declare “no-conflict-of-interest”.

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Figure and Figure legends

Figure 1: A motif containing a TAAAT core sequence is enriched in the enhancers of putative direct targets of Ubx in *Drosophila* halteres

Legend Fig. 1:

A) A motif containing TAAAT core sequence (hereafter referred to as TAAAT motif) is enriched by Ubx on denovo motif analysis of Ubx pulled down sequences from *Drosophila* halteres.

B) The frequency of the TAAAT motif in Ubx pulled down sequences is 1.7 fold more than the frequency of the motif in the entire genome. The motif containing TAAT core sequence (hereafter referred to as TAAT motif) was not enriched in Ubx pulled down sequences.

C) Comparison of Ubx direct targets to differentially expressed genes between the wing and haltere identified 27 targets that are downregulated and 44 targets that are upregulated by Ubx in the haltere.

D) Table showing the relative enrichment of the TAAAT and TAAT motifs in *Drosophila* ChIP-seq and Ubx Response elements of genes that are upregulated, downregulated or equally expressed between the wing and haltere imaginal discs in *Drosophila*

Figure 2: The TAAAT motif in the *CG13222* enhancer is critical for its regulation by Ubx

Legend Fig. 2:

A) Sequence of part of *sal* enhancer (Spalt 5/6), *CG13222* enhancer (WT (site1)) and mutations of the *CG13222* enhancer (M1_A, M1_B) used as probes for EMSA. The wild type *CG13222* enhancer has two Ubx binding sites (shown in green) termed here as WT (site1) and WT (site2). Mutations were generated to specifically modify either the TAAT (M1_B, M2_A) or the TAAAT (M1_A) motifs while keeping the other one intact in site1 of the *CG13222* enhancer. For Luciferase and Transgenic assays, the entire 512bp spanning the *CG13222* edge enhancer was used.

B) While both Ubx and Scr bind to TAAT motif of *sal*, only Ubx binds to TAAAT motif of *CG13222* (WT site1). Binding of Ubx to WT Site 1 is severely reduced when the TAAAT motif is mutated to TAAT motif (M1_A), whereas no such effect is seen on mutating the TAAT motif (M1_B).

C) The *CG13222* enhancer drives Luciferase activity in a S2 cell culture system in presence of Ubx. Significant loss of reporter activity is observed in the mutant enhancer (M1_A) where the TAAAT motif is affected. For statistical analysis, t-test was performed (two-tailed).

D-G). The *CG13222* enhancer drives reporter expression in the posterior edge of the third instar haltere imaginal discs (D). The M1 mutant where both the TAAT and TAAAT motifs are affected show a marked reduction in reporter expression (E). The M1_A mutant carrying mutations in the TAAAT site of the enhancer has a similar phenotype as the M1 mutant (F) and shows a significant reduction in GFP expression as seen from calculation of average fluorescence intensity (H). Mutation of TAAT to TAAAT in site2 results in higher level of reporter expression along with ectopic expression in some domains (shown by red arrows) (G). For statistical analysis, t-test was performed (two-tailed).

Figure 3: The TAAAT site is sufficient to bring the orthologous enhancer of the *vestigial* gene from *Apis* under Ubx control in a transgenic assay

Legend Fig. 3:

A) Sequence of part of the 805bp enhancer of the *vestigial* gene in *Drosophila* (*quad-vg*) containing both TAAT and TAAAT motifs and its mutant enhancer (*quad-vg_M*).

B) The *quad-vg* drives expression of the reporter gene GFP in the pouch of wing imaginal discs but is repressed by Ubx in the haltere. Mutations in the TAAAT motif lead to complete loss of reporter gene expression from both wing and haltere imaginal discs. The laser power (LP) and gain (G) used for imaging GFP fluorescence is indicated.

C) Sequence of part of the 575bp enhancer of the *vestigial* gene in *Apis* (*Apis-vg*), showing the presence of a TAAT motif the mutant enhancer (*Apis-vg_M1*), wherein the TAAT motif is changed to a TAAAT motif.

D) The *Apis-vg* enhancer drives expression of GFP in the pouch region in wing imaginal discs as well as haltere imaginal discs. The mutant enhancer (*Apis-vg_M1*) with introduced TAAAT motif shows reduced levels of GFP expression in the haltere pouch, while its expression in wing discs is not altered compared to the wild type enhancer.

E) Magnified images of the haltere pouch of *Drosophila* transgenics expressing GFP under *Apis-vg* and *Apis-vg_M1* enhancers. Note much reduced GFP levels specifically in the haltere pouch of *Apis-vg_M1* transgene. Orthogonal views of the haltere imaginal pouch indicate a clear difference in GFP expression driven by the WT and the mutant enhancers. Hth staining, which is hinge-specific is used to demarcate the pouch region.

F) Quantification of average fluorescence intensity in the haltere pouch for the *Apis-vg* and *Apis-vg_M1* transgenics. For statistical analysis, t-test was performed (two-tailed).

Figure4: The TAAAT motif is not enriched in the enhancers of targets of Ubx in *Apis*

Legend Fig. 4:

A) While Ubx is required for haltere development in Dipterans such as *Drosophila melanogaster*, differences in hindwing morphology in the third thoracic segment of diverse insects is not correlated to the mere presence of Ubx.

B) De-novo motif analysis of Ubx ChIP-seq in *Apis* hindwings and *Drosophila* halteres reveal enrichment of the TAAAT motif specifically in *Drosophila* but not in *Apis*. Binding sites for transcription factors like Trl, CTCF and Brk are, however, enriched in both datasets.

C) FIMO analysis of the TAAAT motif reveals a 1.7fold enrichment of sites in *Drosophila* ChIP-seq sequences as compared to the entire genome. No such enrichment was found for the *Apis* ChIP-seq datasets. The TAAT motif was not enriched in either of the datasets.