Modulation of AP and DV signaling pathways by the homeotic gene Ultrabithorax during haltere development in Drosophila

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Abstract

Suppression of wing fate and specification of haltere fate in Drosophila by the homeotic gene Ultrabithorax is a classical example of Hox regulation of serial homology (Lewis, E.B. 1978. Nature 276, 565–570) and has served as a paradigm for understanding homeotic gene function. We have used DNA microarray analyses to identify potential targets of Ultrabithorax function during haltere specification. Expression patterns of 18 validated target genes and functional analyses of a subset of these genes suggest that down-regulation of both anterior–posterior and dorso-ventral signaling is critical for haltere fate specification. This is further confirmed by the observation that combined over-expression of Decapentaplegic and Vestigial is sufficient to override the effect of Ubx and cause dramatic haltere-to-wing transformations. Our results also demonstrate that analysis of the differential development of wing and haltere is a good assay system to identify novel regulators of key signaling pathways.

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Introduction

The HOX genes in vertebrates and the homeotic/HOM genes in the fruitfly Drosophila melanogaster are a highly conserved family of regulatory genes controlling cell identities along the anterior–posterior body axis of the developing embryo. These genes encode proteins containing DNA-binding domains and function by regulating downstream target genes. It is generally thought that homeotic genes directly regulate differentiation-specific genes (or “realizator” genes; Garcia-Bellido, 1975), which execute homeotic information at the cellular level rather than regulating another set of master regulatory genes. The structure and function of homeotic genes are highly conserved across a wide range of species including humans. Although much information is available on the molecular and biochemical nature of homeotic genes, comparatively, little is known about the mechanism/s that are used to generate segmental diversity. Considering their biochemical function as transcriptional regulators, the identification of the targets of homeotic genes is critical to an understanding of the genetic control of segmental diversity. Although a few targets have been identified, a global view of the targets of homeotic gene control and how they may specify cell fate is still lacking.

In Drosophila, wings and halteres are the dorsal appendages of the second and third thoracic segments respectively. In the third thoracic segment, the homeotic selector gene Ultrabithorax (Ubx) suppresses wing development to mediate haltere development (Lewis, 1978). Loss of Ubx function in developing haltere discs induces haltere-to-wing transformations, whereas ectopic expression of Ubx in developing wing discs leads to wing-to-haltere transformations (Lewis, 1978; Cabrera et al., 1985; White and Akam, 1985). To specify haltere fate, Ubx functions at multiple levels in the hierarchy of wing development and represses several wing-patterning genes (Weatherbee et al., 1998; Shashidhara et al., 1999; Galant et al., 2002; Mohit et al., 2003). For example, expression of the secreted signaling molecule Wingless (Wg) is repressed in the posterior compartment of haltere discs (Weatherbee et al., 1998; Shashidhara et al., 1999; Mohit et al., 2003), while Wnt-
signaling is down-regulated in both anterior and posterior compartments due to enhanced degradation of its effector Armadillo (Arm). Consequently, Vestigial (Vg), a target of Wg signaling, is repressed in non-D/V cells (Mohit et al., 2003). In addition, Ubx inhibits events downstream to Arm in non-D/V cells to reinforce its repression of Vg (Mohit et al., 2003). However, it is not known if Wg and/or any other components of this pathway are direct targets of Ubx.

One way to approach the mechanism of Ubx function is to reconstruct a wing appendage in the third thoracic segment without altering the patterns/levels of Ubx expression. This necessitates identification of genes that are differentially expressed between wing and haltere discs and reverse-engineer the expression of one or more of those genes during haltere development. With this aim, we have employed DNA microarray analyses to identify potential targets of Ubx function. Here, we describe functional analyses of few selected genes, whose differential expression between wing and haltere discs is validated by means other than the microarray analyses. Our results suggest that Ubx represses the expression of several components of both D/V and A/P signaling. Earlier, we reported that over-expression of Vg, an effector of D/V signaling, causes significant haltere-to-wing homeotic transformations (Mohit et al., 2003). Here, we show that over-expression of Decapentaplegic (Dpp), a secreted factor that is a key component of A/P signaling, also results in similar phenotypes. Furthermore, combined over-expression of Dpp and Vg results in dramatic haltere-to-wing transformations, similar to phenotypes normally seen in strong allelic combinations of Ubx. These results suggest that negative regulation of both A/P and D/V signaling by Ubx is critical for haltere fate specification. In addition, we have identified 8 new genes, which show restricted expression patterns along the A/P or D/V axis of the wing disc. We have performed loss- and gain-of-function studies on two such genes, Cyp310a1 and CG17278. Our observations suggest that they may function to restrict Wingless expression to the D/V boundary. Taken together, our results identify a set of targets of Ubx that play a significant role in mediating the regulation of haltere fate by this homeotic gene.

Materials and methods

All microarray-based experiments conform to the MIAME guidelines developed by the Microarray Gene Expression Data Society (http://www.mged.org/miame). A description of the methodology used for microarray analyses is given in Appendix A. Both the array components and raw data of all microarray experiments have been submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo). GEO accession numbers for array elements are GPL1239 and GPL1240. GEO accession numbers for the raw data reported here are GSM23560 to GSM23275. This report is limited to only those candidate genes, whose differential expression between wing and haltere discs is validated by means other than the microarray analyses.

RNA in situ hybridization

RNA in situ hybridization was performed on late 3rd instar larval discs using the standard protocol (Sturtivant et al., 1993). The following cDNA clones were used to generate antisense probes for RNA in situ hybridization: GH13232 (for GleeC), LD44491 (for Cyp310a1), SD04019 (for CG17278) and GH13232 (for CG5119). Prior to their use in RNA in situ hybridization, the identity of all clones was reconfirmed by sequencing both 3’ and 5’ ends.

Immunohistochemistry

Immunohistochemical staining was performed essentially as described by Patel et al. (1989). The primary antibodies used were polyclonal anti-β-galactosidase (in house, CCMB), anti-cnc (McGinnis et al., 1998) and anti-Strausbiumis (Rawls and Wolff, 2003), monoclonal anti-Delta (Qi et al., 1999) and anti-Wg (Brook and Cohen, 1996). Anti-Delta and anti-Wg were obtained from DSHB, Iowa, USA. All primary antibodies were used at a dilution of 1:100 and secondary antibodies at 1:250. Fluorescence images were obtained either on Zeiss Apotome™ microscope or on Zeiss LSM/Meta Confocal microscope.

Real-time PCR

Taqman probes and PCR primers were designed using the Primer Express software provided by Applied Biosystems, USA. Real-time RT-PCR analysis was performed on the ABI Prism® 7500 Sequence Detection system of Applied Biosystems, USA. Levels of β-tubulin 56D and Rp32 transcripts were used as controls to normalize the real-time RT-PCR data. Details of the primer sequences/Taqman probes used are given in Appendix A. Dissociation curves were used to verify all amplicons, and all reactions were performed in triplicate. Values reported represent normalized cycle thresholds (2^{−ΔΔCt}).

Fly stocks

Canton-S was used as the wild type strain. Other fly stocks used were omb-GAL4 (M. Calleja, personal communication to FlyBase, 16 October 1996), vg-GAL4 (Simmonds et al., 1995), UAS-DI (Jonsson and Knust, 1996), UAS-Dpp (Frasch, 1995), UAS-THL-m8 (Giebel and Campos-Ortega, 1997), UAS-Ubx (Castelli-Gair et al., 1994), Chab (described in FlyBase), P{PTT-GB} CG10996 (Morin et al., 2001), Dda18A-lacZ (Tsuziezumi et al., 1997), P{PZ/Gspk}36016 (Schneider and Spradling, 1997) and P{m8-lacZ} (Lecourtois and Schweiguth, 1995). crumbs-lacZ and P{tkv-lacZ} are from the Bloomington Stock Center (Spradling et al., 1999). All genetic experiments were done at 25°C, except co-expression of Vg and Dpp, which was at 18°C.

Generation of transgenic flies

UAS-RNAi transgenic flies were generated for Cyp310a1 and CG17278. Primers were designed to amplify 3’ ends corresponding to base pairs 1465–1584 of cDNA clone LD44491 of Cyp310a1 and corresponding to base pairs 1201–1569 of the cDNA clone SD04019 of CG17278. These regions show negligible homology to other sequences in the Drosophila genome. The amplified fragments were sub-cloned into pUAST-sym vector (Brand and Perrimon, 1993). After sequence verification, these four constructs were injected into embryos of a fly strain with genetic source of transposase (Cooley et al., 1988). RNA in situ hybridization confirmed loss and gain of the corresponding transcripts in the transgenic flies (data not shown).

Results

Differential gene expression analyses of wing and haltere imaginal discs

To identify targets of Ubx action, we used RNA isolated from three pairs of tissue samples for microarray analyses. (i) To generate a global picture of differences between the two thoracic appendages, RNA isolated from wild type wing and haltere imaginal discs was used in the first set of microarray experiments. (ii) To focus on the Ubx-mediated development of
the appendage proper rather than the notum, we used Cbx<sup>Hm</sup> allele, in which the wing is completely transformed to the haltere, without affecting T2 notum (Supplementary Figs. 1D, E; Lewis, 1978; Cabrera et al., 1985; White and Akam, 1985). (iii) To identify genes related to D/V signaling, we used flies in which Ubx is over-expressed in the wing disc using a vg-GAL4 driver to down-regulate D/V signaling and non-cell autonomously affect wing development (Shashidhara et al., 1999). In this genotype too, the transformation is limited to the pouch without any effect on the notum (Supplementary Figs. 1F, G). Hereafter, the sample pair (i) wild type wing disc vs. wild type haltere disc is referred to as WH, (ii) wild type wing disc vs. Cbx<sup>Hm</sup> wing disc as WC and (iii) wild type wing disc vs. vg-GAL4/UAS-Ubx wing disc as WV (see Appendix A for more details).

To identify genes that show differential expression between a given sample pair, a threshold setting of 1.6-fold and above was applied to the data generated from two hybridizations per sample pair. Out of 8102 genes spotted on the array, 44 genes are differentially expressed in the WH pair. The number was relatively higher (136) for the WC pair and significantly higher (405) for the WV pair. The entire dataset is available on Gene Expression Omnibus (see Materials and methods for details).

### Validation of differential gene expression patterns

In order to validate our microarray analysis, we took two approaches. First, we searched for genes already reported to be specifically expressed in either wing or haltere discs. These include ac, ct, Dll, dsrf, E(spl) m6, kn, Ocho, salm, sca, Ser, twi, vg and wg (Fernandes et al., 1994; Gorfinkiel et al., 1997; Weatherbee et al., 1998; Shashidhara et al., 1999; Lai et al., 2000; Galant et al., 2002; Yan et al., 2004; Hersh and Carroll, 2005). Among them, ac, ct, Ser and vg are not represented in our arrays, whereas spots representing Dll, dsrf and salm were below the detection limit in all the three experiments suggesting either the amount of DNA spotted was not sufficient or very low mRNA abundance. Of the remaining, E(spl) m6, kn, Ocho, twi and wg were identified as positives in our microarray screens, while only sca was not detected.

Second, we validated differential expression of several new genes identified in our analysis using methods other than microarray (RNA in situ hybridization, immunohistochemistry, enhancer- and protein-trap insertions and real-time RT-PCR; Table 1). Genes were selected for validation based on the availability of antibodies and enhancer- and protein-trap lines. RNA in situ hybridization was carried out to determine the expression patterns of those genes for which such reagents are

### Table 1

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<th>Gene symbol</th>
<th>Gene ontology</th>
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<th>NLR WH</th>
<th>NLR WC</th>
<th>NLR WV</th>
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Attempts have been made to validate 27 genes, of which 18 were true positives. They were validated using RNA in situ, immunohistochemistry, protein or enhancer-trap lines that reflect endogenous gene expression patterns and real-time RT-PCR. Six genes have been reported elsewhere as differentially expressed between wing and haltere discs, thus making total number of validated genes from microarray analyses to 24. NLR values shown are average of forward and reverse experiments. WH = microarray analyses of wild type wing vs. haltere discs, WC = wild type wing discs vs. Cbx wing discs and WV = wild type wing disc vs. vg-GAL4/UAS-Ubx wing discs.
not available. Out of 17 genes examined by this method, 4 genes (CG10391, CG17278, CG5119 and CG6575) were differentially expressed between wing and haltere discs (Figs. 1A–D). Three genes did not show any signal in wing or haltere discs either by RNA in situ hybridization or real-time RT-PCR analyses, suggesting that they were either false positives or of very low transcript abundance. The remaining 10 genes showed ubiquitous expression in all cells of the discs, suggesting that differences between wing and haltere discs might be quantitative and are not detectable by RNA in situ hybridization. On quantitative real-time RT-PCR analyses, 5 of those 10 genes showed significant differences in their levels of expression between wing and haltere discs (Table 2). Interestingly, all 5 genes (CG13335, CG13670, CG3479, CG13222 and CG5171) showed higher levels of transcripts in haltere discs. However, they are of unknown function and require further characterization to understand the response to Ubx in haltere discs.

We have validated 4 genes (CG3619, CG17894, CG8075 and CG10990) using antisera against the proteins or the protein-trap line, and all the 4 genes are differentially expressed between wing and haltere discs (Figs. 1E–H). Finally, we have validated 5 genes using enhancer-trap lines (CG6383, CG8365, CG17998, CG5201 and CG14026). Although these lines may not reflect the complete expression patterns of the trapped genes, this aspect of Dl expression is conspicuously absent. (F) CG17894 is expressed in non-D/V cells of wing discs in a pattern similar to CG10391 and CG17278. (G) CG8075 is expressed only in cells adjacent to the D/V boundary, a pattern shared by CG5119 and CG6575. (H) Protein-trap GFP marker of CG10990 shows its expression along the D/V boundary of wing discs. CG17894, CG8075 and CG10990 are not expressed in haltere discs. In this figure and in all subsequent disc images, anterior is oriented to the left.
Validation of microarray positives for their differential expression between wing and haltere discs by using real-time RT-PCR

<table>
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<tr>
<th>Sl. no.</th>
<th>Gene ID</th>
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<th>Fold difference in transcript levels in haltere discs compared to Wing discs ± SD</th>
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<td>Haltere</td>
<td>15.232 ± 0.092</td>
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<td>Gti5171</td>
<td>Haltere</td>
<td>5.023 ± 0.312</td>
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The table shows fold difference in the levels of a given transcript in haltere discs relative to wing discs. β-tubulin 56D (CG9277) and RpL32 (CG7939) were used as standards against which all other samples are normalized. vg, kn, wg (latter two are also identified in the microarray analysis) and Ubx are used as positive controls. Transcript levels of kn, vg and wg are lower in haltere discs, whereas those of Ubx are higher compared to wing discs. All the validated genes (CG13335, CG13670, CG3479, CG13222 and CG5171) show higher levels of transcripts in haltere discs than in wing discs. SD is the standard deviation of normalized threshold cycle for a given sample.

D/V signaling

We have identified 4 known components of N signaling as differentially expressed between wing and haltere discs. Activation of N in the D/V boundary occurs via interactions between dorsal and ventral cells of the wing pouch to set up the organizer (Diaz-Benjumea and Cohen, 1993, 1995; Williams et al., 1994; Irvine and Wieschaus, 1994; Kim et al., 1995; de Celis et al., 1996). N, in turn, activates Wg, Ct and Vg in the D/V boundary (Couso et al., 1995; Kim et al., 1995; Rulifson and Blair, 1995; Kim et al., 1996; Neumann and Cohen, 1996). Previous reports have shown that several downstream targets of N pathway, such as wg, ct and vg, are down-regulated in haltere discs (Weatherbee et al., 1998; Shashidhara et al., 1999). We have observed down-regulation of Delta (Dl) and HLHm8 (a member of Enhancers of Split (E(spl)) complex genes) expression in haltere discs (Figs. 1E, 2B). Previous reports indicated lower levels of E(spl)m6 (another member of E(spl) complex genes) and higher levels of Ocho (a negative regulator of N pathway) in haltere discs than in wing discs (Lai et al., 2000). Thus, more than one mechanism may operate in haltere discs to down-regulate N signaling.

Wg and Ct are not expressed in D/V cells of the posterior compartment of haltere discs, while Vg is expressed in D/V cells of both anterior and posterior compartments (Weatherbee et al., 1998; Shashidhara et al., 1999; Mohit et al., 2003). Over-expression of an activated form of Notch fails to activate Wg expression in the posterior compartment (Mohit et al., 2003), suggesting that repression of Wg is downstream of N. In the wing pouch, Dl expression spans the cells abutting the DV boundary and is also expressed, parallel to the AP organizer, in the presumptive veins 3 and 4 (Fig. 1E). In haltere pouch, however, Dl is expressed in the DV cells of only the anterior compartment and is completely absent in cells abutting the AP boundary (corresponding to presumptive vein regions in the wing discs). HLHm8 is expressed in the D/V boundary of wing discs in both anterior and posterior compartments, whereas, in haltere discs, it is expressed only in the anterior compartment (Fig. 2B). Thus, repression of Dl and/or HLHm8 expression in D/V cells of the posterior compartment may explain the failure of activated N to turn on Wg expression in those cells. However, over-expression of Dl or HLHm8 using vg-GAL4 driver had no effect on Wg expression in the haltere pouch (Figs. 3D, E). We then examined the effect of Dl and HLHm8 over-expression in Ubx heterozygous animals. We hypothesized that over-expression in such a sensitized genetic background may override the effect of Ubx. Indeed, over-expression of HLHm8, although not of Dl, resulted in the activation of Wg expression in the posterior compartment of Ubx heterozygous haltere discs (Fig. 3F). Ubx may directly repress HLHm8 or affect a step downstream of Dl and upstream of HLHm8. Either way, down-regulation of HLHm8 in haltere discs appears to be critical to keep Wg repressed.

We also examined the effects of over-expression of Dl and HLHm8 on the cuticular phenotypes of adult haltere. Neither of them induced any haltere-to-wing transformations (data not shown), although over-expression of Dl, but not HLHm8, enhanced homoeotic transformations in Ubx heterozygous...
Activation of the pro-wing gene Vg requires both Wg-dependent and -independent events, and its over-expression can by-pass the requirement for Wg (Mohit et al., 2003). It is possible that Dl may activate both Wg-dependent and -independent events in the D/V boundary, while HLHm8 may activate only Wg-dependent events, thus explaining the distinct effects of over-expression of these N pathway genes.

A/P signaling

Our microarray results suggest that A/P signaling is also a target of Ubx function. Most, if not all, A/P patterning events are regulated by Hh signaling (reviewed in Aza-Blanc and Kornberg, 1999; Ingham and McMahon, 2001). DSRF and Kn, both components of the A/P pathway, have already been reported as targets of Ubx (Weatherbee et al., 1998; Galant et al., 2002; Hersh and Carroll, 2005). They are Dpp-independent targets of the Hh signaling pathway and function to specify the L3–L4 intervein region (Crozatier et al., 2002). Our microarray analysis suggests that Dpp pathway components tkv (a receptor of Dpp) and Dad (a negative regulator of Dpp signaling) are differentially expressed in wing and haltere discs (Figs. 2D, E). As Dad is a feedback regulator of Dpp, its repression in haltere discs may reflect down-regulation of an upstream event. Indeed, RNA in situ hybridization (Dpp is not part of the arrays we have examined so far) shows that expression of Dpp transcripts in the haltere A/P boundary is significantly lower than in wing discs (Fig. 4A). Taken together, Ubx appears to down-regulate both Dpp-dependent and -independent events along the A/P axis of the haltere disc. In addition, Sal, a short-range target of Dpp signaling, has been reported as a direct target of Ubx (Galant et al., 2002). Thus, within the Dpp pathway itself, Ubx acts at more than one level in the hierarchy of gene regulation.

Considering the critical function of Dpp in specifying wing shape, size and differentiation, we examined the effect of its over-expression in developing halteres. Over-expression of Dpp in, otherwise, wild type genetic background induced significant haltere-to-wing homeotic transformations (Fig. 4C). Interestingly, we observed this phenotype despite Sal being directly down-regulated by Ubx (Galant et al., 2002). Furthermore, Sal was not activated when Dpp was over-expressed in haltere discs (data not shown). Importantly, no homeotic transformations were observed when either Hh or Ci was over-expressed in haltere discs (data not shown), suggesting that, among the components of the A/P signaling pathway, down-regulation of Dpp is critical in specifying haltere fate.

Fig. 2. Validation of microarray positives for their differential expression between wing and haltere discs using enhancer-trap lacZ marker that reflect endogenous gene expression patterns. In each panel, expression pattern of a given gene is shown for both wing (left) and haltere (right) discs. (A–B) CG6383 (A) and CG8365 (B) are expressed along the D/V boundary of wing discs. In haltere discs, CG6383 is completely absent, while CG8365 is expressed only in the anterior compartment. (C) CG17998 is expressed in a narrow stripe along the A/P boundary of wing discs. In haltere discs, it is expressed only in the notum and is completely absent in the pouch. (D) CG14026 is not expressed in the central part of the wing pouch. In haltere discs, it is expressed at much lower levels, particularly, in the posterior compartment. (E) CG5201 is expressed in a broad domain along the A/P axis. Its expression is complementary to that of CG14026. In haltere discs, CG5201 expression is narrow, just limited to the A/P boundary. Interestingly, peripodial expression of both CG17998 and CG5201 is not differential between wing and haltere discs.
Combined over-expression of A/P and D/V components induce dramatic haltere-to-wing homeotic transformations

All reported haltere-to-wing homeotic transformations at the cuticle level are associated with loss of Ubx protein, with the exception of the partial homeotic transformation, induced by over-expressing Vg (Mohit et al., 2003; Fig. 4D). As mentioned above, over-expression of Dpp also caused considerable homeotic transformations in adult halteres (Fig. 4C). Thus, repression of Dpp expression in the A/P axis and that of Vg in the D/V axis may represent critical steps in the hierarchy of events regulated by Ubx during the specification of haltere fate.

We therefore examined the effect of combined over-expression of Dpp and Vg in developing halteres.

Over-expression of both Dpp and Vg using the omb-GAL4 driver resulted in stronger homeotic transformations than

Fig. 3. Ubx represses events downstream of Delta in Notch pathway. All discs in A–F are stained for Wg (as a read-out of N signaling) expression. (A–B) Wild type (A) and vg-GAL4/UAS-Dl (B) wing discs. Note ectopic Wg in wing pouch cells over-expressing Dl. (C) Wild type haltere discs. Wg is not expressed in the posterior compartment. Similar pattern of Wg expression is observed in Ubx+/background (data not shown). (D, E) vg-GAL4/UAS-Dl (D) and vg-GAL4/UAS-HLHm8 (E) haltere discs. No change in Wg expression pattern is observed. (F) vg-GAL4/UAS-HLHm8; Ubx+/haltere disc shows de-repression of Wg in the posterior compartment. No such de-repression was observed when Dl was over-expressed in Ubx+/background. (G) Adult haltere of wild type fly. (H) Adult halteres of Ubx-GAL4+/fly showing few wing-type bristles and trichomes. Ubx-GAL4 is a mutational insertion in Ubx and thus serves as a sensitized background for over-expression studies. (I) Adult halteres of Ubx-GAL4/UAS-Dl showing large number of wing-type bristles and trichomes. Higher magnification of a part of this haltere is shown on the right side. No such phenotype was observed when HLHm8 was over-expressed in using Ubx-GAL4 driver. In this figure and in Fig. 4, anterior is oriented up for all adult halteres.
expressing them individually. All haltere capitellum cells showed haltere-to-wing cell fate transformations (Fig. 4E). This phenotype was observed in all pharate adults (>30). Ubx protein levels in discs were unchanged (data not shown), confirming that the resultant phenotype is due to altering events downstream of Ubx.

Identification of novel mediators of A/P and D/V signaling

In addition to the identification of several genes of known function as potential targets of Ubx function, we have identified 9 genes whose expression patterns are modulated along the A/P or the D/V axis. Among these, Gprk2 is a previously characterized gene, required for oocyte morphogenesis and early embryonic development (Schneider and Spradling, 1997; Fan and Schneider, 2003), whose potential role in wing development has not been examined. We observed that Gprk2 expression is restricted exclusively to the A/P boundary of the wing discs (Fig. 2C) and is repressed in the haltere pouch, suggesting a possible role in wing patterning along the A/P axis.

As many as 8 genes with restricted expression along the D/V axis were identified in our microarray analysis. CG10990 and crb are expressed predominantly in the D/V boundary (Figs. 1H, 2A), while CG5119 is also expressed in non-D/V cells but restricted to the anterior sensory mother cells (Fig. 1C). Cyp310a1, CG17278, Glec, Stbm and cnc are also restricted to non-D/V cells of the pouch (Figs. 1A, B, D, F–G). Glec and Stbm are expressed in two rows of cells, one on each side of the D/V boundary (Figs. 1D, G), while cnc is expressed in a subset of non-D/V cells, mainly in the proximal region (Fig. 1F). Cyp310a1 and CG17278 are expressed in all non-D/V cells of the pouch (Figs. 1A, B). These observations are significant since several short- and long-range targets of D/V signaling show similar expression patterns. CG10990 and crb expression patterns are similar to that of Wg and Ct in the wing pouch. CG5119, which codes for a polyA binding protein, shows a pattern similar to that of Ac and Sca, while Glec and Stbm expression patterns resemble that of Dll. Finally, Cyp310a1 and CG17278 mimic the expression pattern of vg-QE.

Among these 8 genes, crb (epithelial cell polarity; Wodarz et al., 1995), Glec (a carbohydrate binding protein involved in cell adhesion; Tiemeyer and Goodman, 1996), Stbm (epithelial cell polarity; Rawls and Wolff, 2003) and cnc (Veraksa et al., 2000) have been studied before, but not in the context of wing development. Interestingly, cnc, which is expressed in the wing pouch, is a competitive repressor of homeotic function during embryonic development (particularly of Deformed, a homeotic gene of the ANTP complex; McGinnis et al., 1998). cnc expression in the wing pouch may ensure repression of homeotic gene activity since absence of homeotic function is a prerequisite for wing development (Carroll et al., 1995). The other four genes have not been characterized so far at the functional level.

Fig. 4. Homeotic transformations induced by the ectopic activation of A/P and/or D/V pathways in developing halteres. (A) Dpp RNA in situ hybridization on wild type wing (left) and haltere (right) imaginal discs. Note that Dpp expression is significantly lower in the haltere disc compared to the wing disc. (B) Wild type haltere. (C, D) omb-GAL4/+; UAS-Dpp/+ (C) and omb-GAL4/+; UAS-Vg/+ (D) halteres. Note the appearance of a large number of wing-like bristles and trichomes. (E) omb-GAL4/+; UAS-Dpp, UAS-Vg/+ haltere. Note the enhanced haltere-to-wing homeotic transformations compared to C and D. Almost all haltere cells of the capitellum are transformed to wing-type cells. It is possible that regions that do not express omb-Gal4 are masked by the homeotically transformed capitellum cells. Due to lethality (probably due to ectopic expression of Dpp and Vg elsewhere during development) at the pharate adult stage, the halteres are not properly unfolded and were of abnormal shape.
level (CG10990, Cyp310a1, CG17278 and CG5119). Functional characterization of these 9 genes may provide useful insights into the mechanism of A/P and D/V signaling pathways.

**Cyp310a1 and CG17278: putative regulators of Wingless pathway?**

Towards the functional characterization of newly identified A/P and D/V components, we selected Cyp310a1 and CG17278, which showed highly restricted expression patterns in wing discs (Figs. 1A, B). Cyp310a1 is a member of Cytochrome P450 family of proteins, whereas CG17278 codes for a Kazal-type serine protease inhibitor, but their function in non-D/V cells of the wing pouch is not apparent. Recently, the restricted expression pattern of these two genes has been shown to be Wg-dependent as inhibition of Wg results in their activation in D/V cells, while over-expression of Wg causes down-regulation in non-D/V cells (Butler et al., 2003).

We generated transgenic flies that express dsRNA against these two genes as well as transgenic flies to over-express them, both using GAL4-UAS system (Brand and Perrimon, 1993; Giordano et al., 2002). A large number of GAL4 drivers were used to drive the expression of these transgenes either in the entire wing disc or in a region of wing disc. However, no phenotypes were observed either in the discs or the adult cuticle, for any of the four transgenes (two dsRNA-expressing and two over-expressing transgenes). To address the possibility that the two genes (which share similar expression patterns in the wing disc) have redundant functions, we co-expressed dsRNA against both but still did not observe any phenotypes. Possibly, levels of dsRNA expression were not sufficient to down-regulate their function as both genes are expressed at relatively high levels in wing discs.

As expression patterns of Cyp310a1 and CG17278 are complementary to that of Wg (Figs. 1A, B) and these genes are known to be negatively regulated by Wg (Butler et al., 2003), these genes could play a role in maintaining the differences between D/V and non-D/V cells. Furthermore, similar to Wg, expression of both Cyp310a1 and CG17278 is down-regulated in Nts wing discs (data not shown). We therefore hypothesized that loss- and gain-of-function genetic studies on Cyp310a1 and CG17278 in genetic backgrounds where Wg signaling is compromised may provide clues to their potential function in wing development.

Over-expression of Wg or its transducer Dsh in the presumptive notum causes notum-to-wing transformations (Fig. 5A) since Wg is required to specify the wing pouch early during disc patterning (Ng et al., 1996). Co-expression of dsRNA against Cyp310a1 or CG17278 along with Dsh resulted in considerable enhancement of notum-to-wing transformation phenotypes (Figs. 5B, C). Conversely, over-expression of Cyp310a1 or CG17278 along with Dsh caused a considerable suppression of transformation (Figs. 5D, E).

In contrast to Dsh-induced phenotypes, over-expression of Shaggy/GSK-3β (Sgg) in D/V cells causes down-regulation of Wg expression (Fig. 5F). This phenotype was considerably suppressed when Sgg was over-expressed with dsRNA against Cyp310a1 (data not shown) or CG17278 (Fig. 5G). Interestingly,

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**Fig. 5.** Cyp310a1 and CG17278 are putative negative regulators of Wg signaling. All discs in this figure are stained for Wg expression. (A) *ptc*-GAL4/+; UAS-Dsh wing disc. Early activation of Wg signaling in the notum results in notum-to-wing pouch transformation. Note the ectopic D/V boundary in the presumptive notum. (B, C) *ptc*-GAL4/UAS-Cyp310a1

RNAi; UAS-Dsh (B) and *ptc*-GAL4/UAS-CG17278

RNAi; UAS-Dsh (C) wing discs. Note the enhanced notum-to-wing transformation induced by Dsh. The size and shape of the ectopic pouch and Wg expression in the ectopic D/V boundary are identical to that of the normal pouch. (D, E) *ptc*-GAL4/

UAS-Cyp310a1; UAS-Dsh (D) and *ptc*-GAL4/UAS-CG17278; UAS-Dsh (E) wing discs. Note the suppression of Dsh-induced notum-to-wing transformations. (F, G) *vg*-GAL4/+; UAS-Sgg (F) and *vg*-GAL4/UAS-CG17278

RNAi; UAS-Sgg (G) wing discs. Over-expression of Sgg causes loss of Wg expression in the D/V boundary and thereby causes reduction in the pouch size (F). This phenotype is suppressed when Cyp310a1

RNAi (data not shown) or CG17278

RNAi is co-expressed with Sgg (G). Over-expression of GFP along with Dsh or Sgg did not alter their respective phenotypes.
dsRNA against Cyp310a1 or CG17278 affected Dsh over-expression phenotype in the notum and Sgg over-expression phenotype in the D/V boundary, regions where Cyp310a1 and CG17278 are not normally expressed, suggesting a possible role as feedback negative regulators of Wg signaling. Taken together, the phenotypes of over-expressing and knocking down the differentially expressed genes Cyp310a1 and CG17278 in a sensitized background suggest that they may have a role in regulating the Wg signaling pathway.

Discussion

Suppression of wing fate and specification of haltere fate by Ubx is a classical example of Hox regulation, which has served as a paradigm for understanding the nature of homeotic gene function. Using microarray analyses and subsequent downstream validation by methods other than microarray, we have identified 18 potential targets of Ubx function during haltere specification. In addition, we have observed differential expression of Dpp at the transcriptional level between wing and haltere imaginal discs. Including previously known 13 targets, we now have as many as 32 well-established direct or indirect targets of Ubx function during haltere specification. Although Ubx may regulate additional downstream targets, the expression patterns of the genes we identified suggest that negative regulation of D/V and A/P signaling is one of the important mechanisms by which Ubx specifies haltere development.

The functional significance of down-regulation of these signaling pathways is confirmed by the dramatic homeotic transformations caused by ectopic activation of Dpp and/or Vg in developing haltere discs. These transformed halteres still lacked veins and wing margin bristles, indicating that Ubx specifies haltere development by additional mechanisms. Indeed, the EGFR pathway, which plays a significant role in specifying wing veins, is directly repressed by Ubx in haltere discs (SK Pallavi and LSS, unpublished observations). Furthermore, over-expression of Dad in wing discs does not cause any obvious wing-to-haltere transformation (Tsuneizumi et al., 1997) nor do dpp96/dpp112 wings show such phenotypes (data not shown). Thus, while over-expression of Dpp causes partial haltere-to-wing transformations, down-regulation of Dpp in wing discs has no such effect. Further investigation is needed to identify all the critical steps downstream of Ubx required to completely transform haltere to a wing or vice versa. Nevertheless, the dramatic homeotic transformations induced by the co-expression of just two genes (Dpp and Vg) suggest that down-regulation of these two steps by Ubx is critical to specify haltere fate.

Although both Vg and Dpp are known to induce growth, we believe that the observed homeotic transformation is due to re-patterning and trans-differentiation and not due to simple over-growth. Induction of over-growth in haltere leads to larger appendages, but not homeotic transformations (Shashidhara et al., 1999). Furthermore, a recent report suggests that changes in cell division patterns alone do not lead to cell fate changes (Berger et al., 2005). Thus, Dpp/Vg-induced homeosis is a specific mechanism that overrides the effect of Ubx and suggests an important mechanism for Ubx function during haltere specification. Interestingly, in the mouse, signaling molecules such as Bmp2, Bmp7 and Fgf8 are downstream targets of Hoxa13 during the development of limbs and genitalia (Morgan et al., 2003; Knosp et al., 2004). Thus, down-regulation of Dpp and Wnt/Wg signaling pathways in Drosophila and Bmp and Fgf in mouse suggest a common theme underlying Hox gene function during appendage specification and development.

The results presented in this report are significant in two ways. Firstly, they suggest a mechanism by which halteres may have evolved from hind wings of lepidopteran insects. Ubx protein itself has not evolved among the diverse insect groups, although there are significant differences in Ubx sequences between Drosophila and crustacean Arthropods (Galant and Carroll, 2002; Ronshaugen et al., 2002). Nevertheless, over-expression of Ubx derived from either a non-winged arthropod such as Onychophora or a four-winged insect such as Tribolium is sufficient to induce wing-to-haltere transformations in Drosophila (Grenier and Carroll, 2000). This suggests that, in the dipteran lineage, certain wing patterning genes have come under the regulation of Ubx (Weatherbee et al., 1999). In such a scenario, it is likely that only a small number of genes will have their cis-regulatory sequences modified (converging mutations) to enable their regulation by Ubx. Considering the gross morphological differences between lepidopteran hind wings and halteres, any new target of Ubx will have greater influence on the entire hind wing morphology. Indeed, over-expression of Dpp and/or Vg caused dramatic haltere-to-wing homeotic transformations. Since such transformations were not observed by over-expressing their upstream regulators such as Hh, Ci, N or Wg, it is likely that direct targets of Ubx would be closer to Dpp and Vg in the hierarchy of gene regulation. Currently, chromatin immunoprecipitation experiments using haltere extracts are underway to identify those target genes.

The second significant conclusion from the results described here is on the utility of differential development of wing and haltere as a good model system to identify additional components of both A/P and D/V signaling. We have identified 9 such genes, 8 of which show modulation of their expression patterns along the D/V axis. Based on restricted expression patterns and biochemical features of the encoded proteins, we predict their possible involvement in maintaining the integrity of the D/V boundary as well as differences between dorsal and ventral compartments. Indeed, preliminary characterization of two genes suggests their probable roles to restrict Wg expression to the D/V boundary.

A recent report has identified 16 potential genes downstream of mouse Hoxd cluster during the development of the most distal parts such as digits and genitalia (Cobb and Duboule, 2005). Most of them have not been previously implicated in the early stages of either limb or genital bud development or as components of the known signal transduction pathways. Considering tissue- and developmental stage-specific expression of those genes, it is possible that those targets too could be novel modulators of known signal transduction pathways.
Taken together, our results provide a framework for understanding the mechanisms by which Hox genes specify segment-specific developmental pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.12.022.

References


Morin, X., Daneman, R., Zavortink, M., Chia, W., 2001. A protein trap strategy


