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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.

Keywords: Microarray; Ultrabithorax; Notch; Delta; Decapentaplegic; Wingless; Vestigial

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-

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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.nih.gov/geo). GEO accession numbers for array elements are GPL1239 and GPL1240. GEO accession numbers for the raw data reported here are GSM23260 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 (Sturtevant et al., 1993). The following cDNA clones were used to generate antisense probes for RNA in situ hybridization: GH13232 (for Glec), 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-Strabismus (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 *Rpl32* 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 ($2^{-(-\Delta\Delta Ct)}$).

Fly stocks

Canton-S was used as the wild type strain. Other fly stocks used are *omb-GAL4* (M. Calleja, personal communication to FlyBase, 16 October 1996), *vg*-GAL4 (Simmonds et al., 1995), UAS-Dl (Jonsson and Knust, 1996), UAS-Dpp (Frasch, 1995), UAS-HLHm8 (Giebel and Campos-Ortega, 1997), UAS-Ubx (Castelli-Gair et al., 1994), Cbx^{Hm} (described in FlyBase), P{*PTT-GB*}$CG10990^{G93}$ (Morin et al., 2001), Dad^{J1E4}-*lacZ* (Tsuneizumi et al., 1997), P{*PZ*}$Gprk2^{06936}$ (Schneider and Spradling, 1997) and *P{m8-lacZ}* (Lecourtois and Schweisguth, 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-symp vector (Giordano et al., 2002). UAS-Cyp310a1 and UAS-CG17278 constructs were made by sub-cloning above mentioned cDNA clones into pUAST 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^{Hm} 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 *w*ing disc vs. wild type *h*altere disc is referred to as WH, (ii) wild type *w*ing disc vs. Cbx^{Hm} wing disc as WC and (iii) wild type *w*ing 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
Validated microarray positives

Sl. no.	Gene ID	Gene symbol	Gene ontology	Up in	NLR WH	NLR WC	NLR WV	Validation method	Results
1	*CG10391*	Cyp310a1	Cytochrome P450 enzyme	Wing		0.408		RNA in situ	Fig. 1A
2	*CG17278*	CG17278	Kazal-type serine protease inhibitor	Wing	0.246			RNA in situ	Fig. 1B
3	*CG5119*	pAbp	RNA binding	Wing		0.303	0.367	RNA in situ	Fig. 1C
4	*CG6575*	glec	Cell adhesion	Wing		0.361	0.444	RNA in situ	Fig. 1D
5	*CG3619*	Dl	Notch ligand	Wing		0.311	0.498	Antibody	Fig. 1E
6	*CG17894*	cnc	Transcription factor	Wing			0.344	Antibody	Fig. 1F
7	*CG8075*	Stbm	Cell polarity	Wing			0.48	Antibody	Fig. 1G
8	*CG10990*	CG10990	Nucleic acid binding	Wing	0.52			Protein trap	Fig. 1H
9	*CG13335*	CG13335	Unknown	Haltere			−0.26	Real-time RT-PCR	Table 2
10	*CG13670*	CG13670	Structural constituent of larval cuticle	Haltere			−0.394	Real-time RT-PCR	Table 2
11	*CG3479*	osp	Unknown	Haltere	−0.28			Real-time RT-PCR	Table 2
12	*CG13222*	CG13222	Structural constituent of larval cuticle	Haltere	−0.52	−0.23		Real-time RT-PCR	Table 2
13	*CG5171*	CG5171	Trehalose phosphatase	Haltere	−0.39		−0.352	Real-time RT-PCR	Table 2
14	*CG6383*	crb	Cell polarity	Wing			0.455	Enhancer trap	Fig. 2A
15	*CG8365*	HLHm8	Notch pathway	Wing			0.581	Enhancer trap	Fig. 2B
16	*CG17998*	Gprk2	G-protein signaling activity	Wing			0.446	Enhancer trap	Fig. 2C
17	*CG14026*	tkv	Dpp pathway			0.265		Enhancer trap	Fig. 2D
18	*CG5201*	Dad	Dpp pathway	Wing			0.348	Enhancer trap	Fig. 2E
19	*CG2956*	twi	Transcription factor	Wing	0.57			Expression known	Fernandez et al. (1994)
20	*CG10197*	kn	Transcription factor	Wing		0.296	0.328	Expression known	Galant et al. (2002)
21	*CG4889*	wg	Wnt-1 family, morphogen	Wing			0.449	Expression known	Weatherbee et al. (1998); Shashidhara et al. (1999)
22	*CG8354*	HLHm6	Notch pathway	Wing	0.51			Expression known	Lai et al. (2000)
23	*CG10704*	toe	Transcription factor	Haltere		−0.309		Expression known	Personal communication by N. Azpiazu
24	*CG3396*	Ocho	Notch pathway	Haltere	−0.31			Expression known	Lai et al. (2000)

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

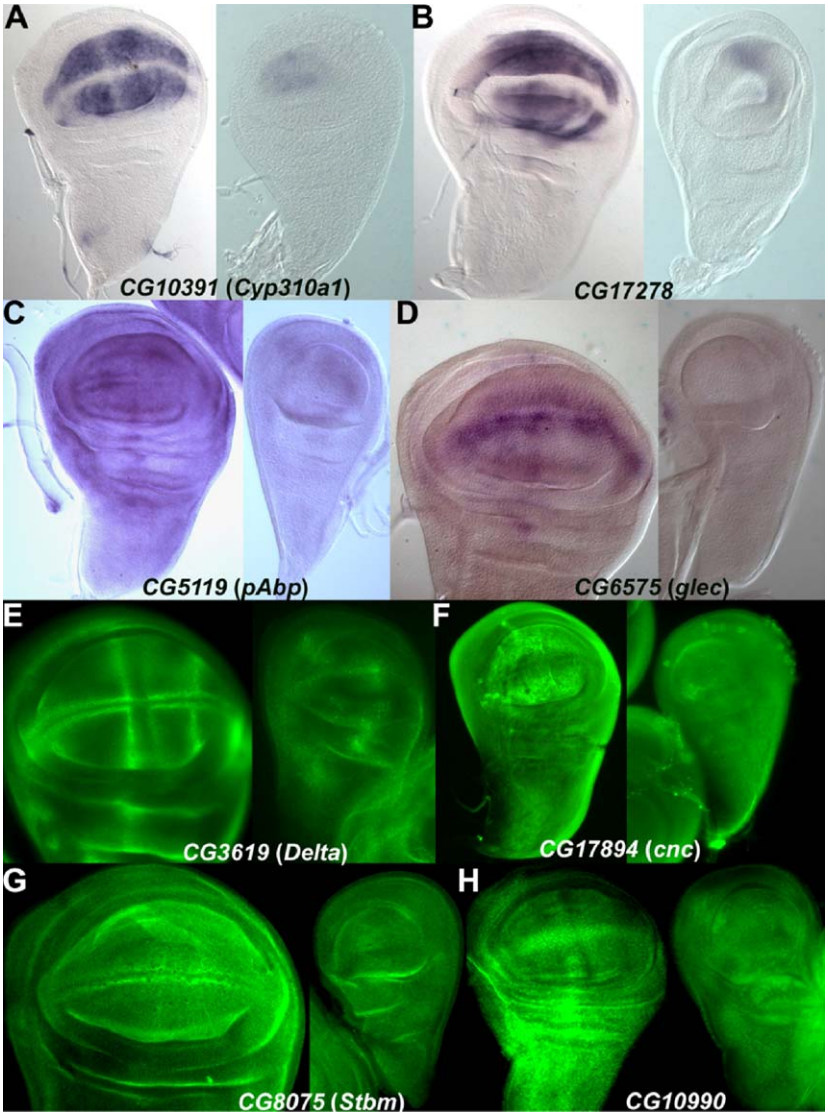

Fig. 1. Validation of microarray positives for their differential expression between wing and haltere discs by RNA in situ hybridization (A–D), immunohistochemistry (E–G) and by using protein-trap GFP marker (H). In each panel, expression pattern of a given gene is shown for both wing (left) and haltere (right) discs. *CG10391* (A) and *CG17278* (B) are expressed only in non-D/V cells of wing discs. Both genes are expressed at very low levels in the haltere disc. (C–D) *CG5119* (C) and *CG6575* (D) are expressed in dorsal and ventral rows of cells immediately adjacent to the D/V boundary of wing discs. However, *CG5119* expression is largely restricted to the anterior compartment of wing discs. Neither *CG5119* nor *CG6575* are expressed in haltere discs. (E) *CG3619* is expressed in the presumptive veins. In haltere discs, 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 *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.

Table 2
Validation of microarray positives for their differential expression between wing and haltere discs by using real-time RT-PCR

Sl. no.	Gene ID	Gene symbol	Up in	Fold difference in transcript levels in haltere discs compared to Wing discs ± SD
1	CG10197	kn	Wing	−2.645 ± 0.025
2	CG3830	vg	Wing	−2.809 ± 0.020
3	CG4889	wg	Wing	−1.721 ± 0.012
4	CG10388	Ubx	Haltere	20.086 ± 0.020
5	CG13335	CG13335	Haltere	3.498 ± 0.019
6	CG13670	CG13670	Haltere	2.538 ± 0.147
7	CG3479	osp	Haltere	2.391 ± 0.035
8	CG13222	CG13222	Haltere	15.232 ± 0.092
9	CG5171	CG5171	Haltere	5.023 ± 0.312

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.

genes, they all showed differences between wing and haltere discs (Figs. 2A–E).

Taken together, we have successfully validated 24 genes (of the 33 tested) identified by our microarray analysis (18 new genes + 6 previously reported), which are differentially expressed between wild type wing and haltere discs (Table 1). Interestingly, only 8 out of 24 validated genes were identified as differentially expressed between the WH sample pair, while 2 of those genes were common to WH and WC or WV sample pairs (Table 1). The remaining 16 were identified in WC and/or WV sample pairs. Furthermore, a majority (14 out of 16) of those genes showed highly restricted expression pattern in the wing pouch, demonstrating the utility of transformed wing discs in identifying targets of *Ubx*.

Ubx modulates the expression of components of D/V and A/P signaling pathways

Some of the major morphological differences between wings and halteres are size (wing blades are much larger than halteres), shape (wings are flat, whereas halteres are bulbous) and trichome organization (wing trichomes are flat and are well spaced, whereas haltere trichomes are cuboidal and densely arranged). Furthermore, wing blades have a characteristic venation pattern, and the anterior wing margin is marked with stout sensory bristles. Wing size is primarily regulated by the Notch (N) and Wg pathways from the D/V boundary and the Dpp pathway from the A/P boundary. Wing vein specification is dependent on Hh/Dpp, Notch and EGFR pathways, whereas wing margin specification is dependent on D/V signaling, particularly the Wg pathway.

Our microarray analysis shows that a number of components of both D/V and A/P signaling pathways are down-regulated by *Ubx* (Table 1), which is consistent with the morphological differences between wings and halteres.

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 disc). *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 homeotic transformations in *Ubx* heterozygous

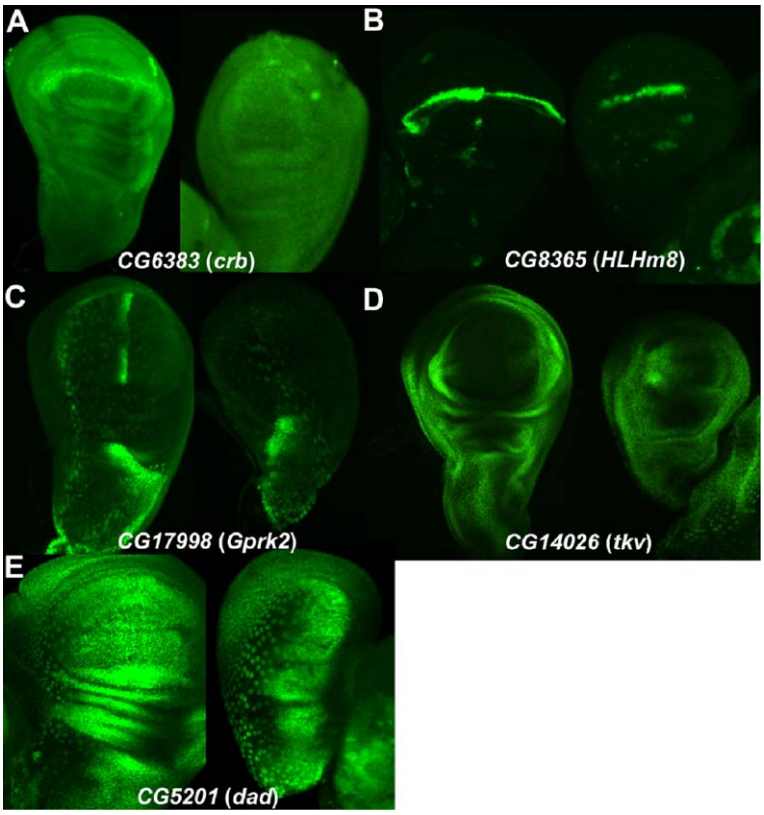

Fig. 2. Validation of microarray positives for their differential expression between wing and haltere discs using enhancer-trap lacZ marker that reflect endogenous gene expression patterns. In each panel, expression pattern of a given gene is shown for both wing (left) and haltere (right) discs. (A–B) *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.

background (Fig. 3I). 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.

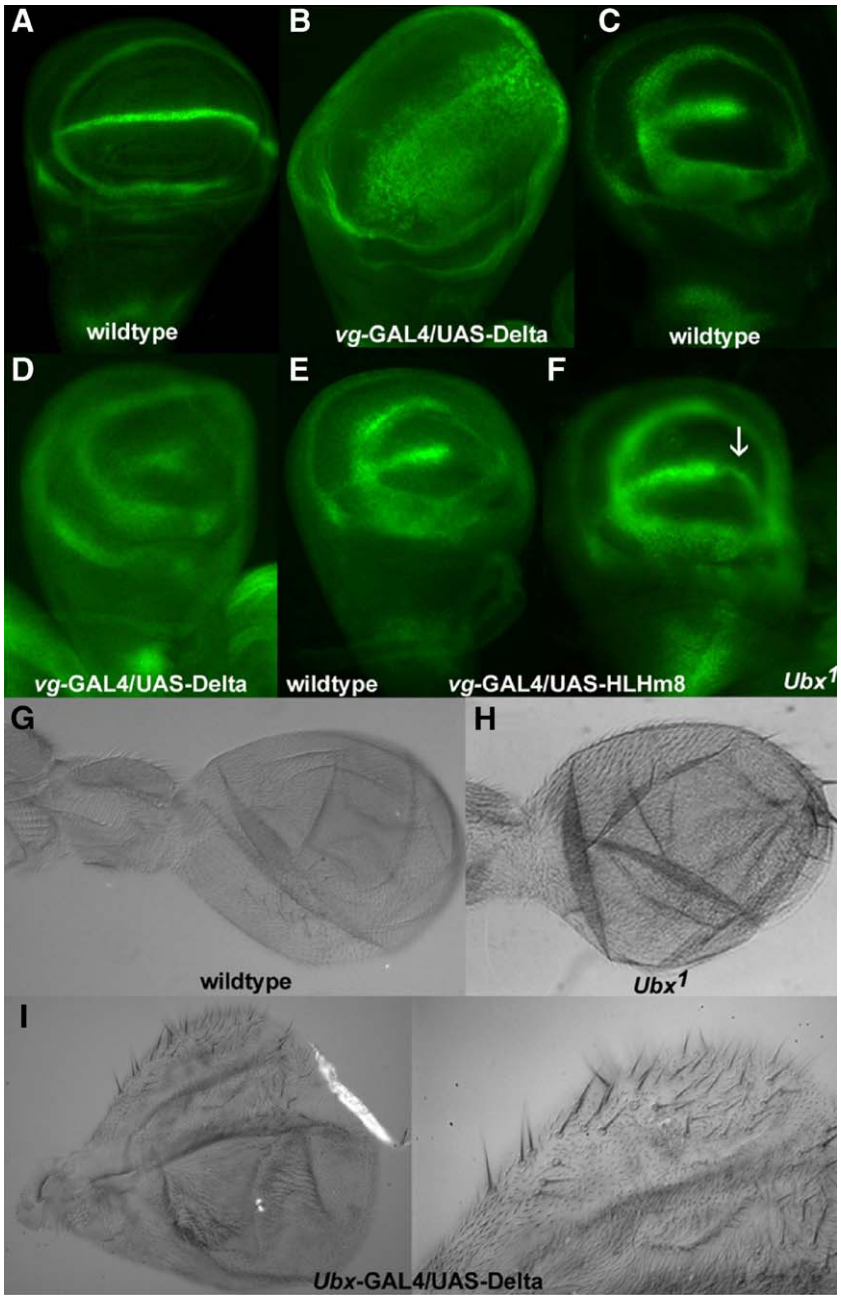

Fig. 3. *Ubx* represses events downstream of Delta in Notch pathway. All discs in A–F are stained for Wg (as a read-out of N signaling) expression. (A–B) Wild type (A) and *vg*-GAL4/UAS-Dl (B) wing discs. Note ectopic Wg in wing pouch cells over-expressing Dl. (C) Wild type haltere discs. Wg is not expressed in the posterior compartment. Similar pattern of Wg expression is observed in Ubx^1/+ 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^1/+ haltere disc shows de-repression of Wg in the posterior compartment. No such de-repression was observed when Dl was over-expressed in Ubx^1/+ 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.

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

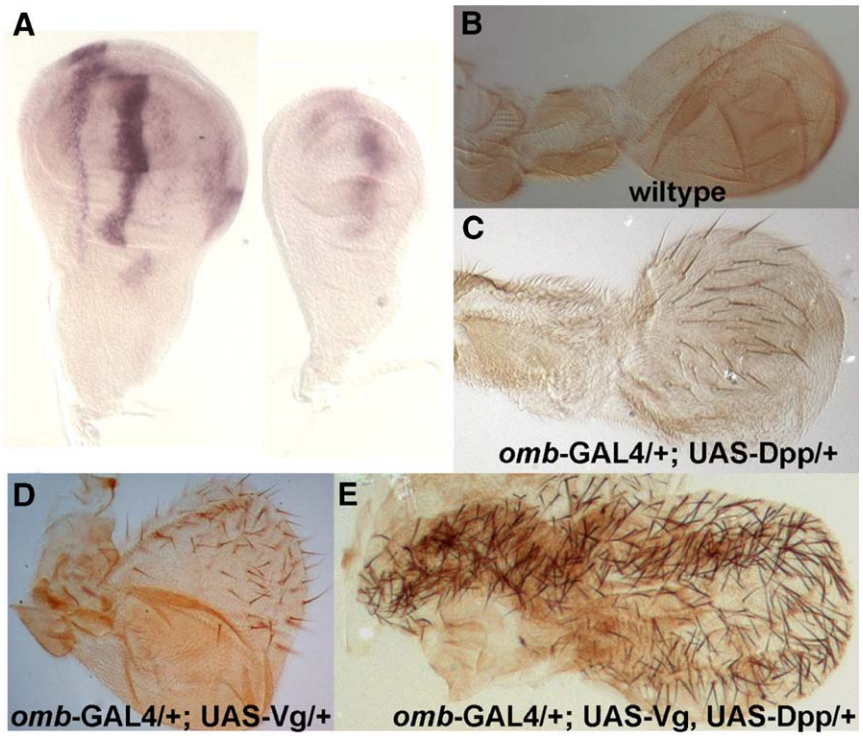

Fig. 4. Homeotic transformations induced by the ectopic activation of A/P and/or D/V pathways in developing halteres. (A) Dpp RNA in situ hybridization on wild type wing (left) and haltere (right) imaginal discs. Note that Dpp expression is significantly lower in the haltere disc compared to the wing 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.

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 Grpk2 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 pre-requisite for wing development (Carroll et al., 1995). The other four genes have not been characterized so far at the functional

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 N^{ts} 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,

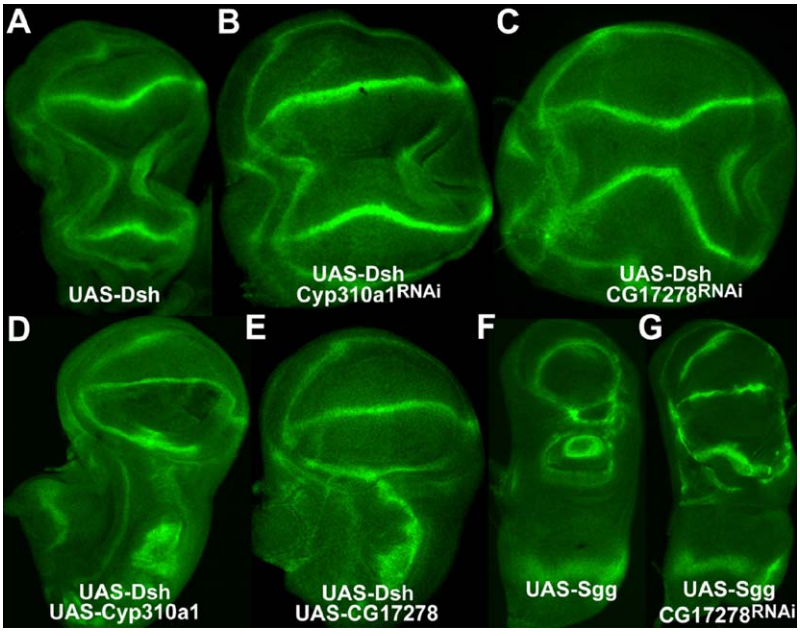

Fig. 5. Cyp310a1 and CG17278 are putative negative regulators of Wg signaling. All discs in this figure are stained for Wg expression. (A) *ptc*-GAL4/+; UAS-Dsh wing disc. Early activation of Wg signaling in the notum results in notum-to-wing pouch transformation. Note the ectopic D/V boundary in the 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 down-stream 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 dpp^{d6}/dpp^{d12} 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 genitals (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.

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