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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