Identification of a novel target of D/V signaling in Drosophila wing disc: Wg-independent function of the organizer

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Abstract

Growth and patterning during Drosophila wing development are mediated by signaling from its dorso-ventral (D/V) organizer. Wingless is expressed in the D/V boundary and functions as a morphogen to activate target genes at a distance. Wingless pathway and thereby D/V signaling is negatively regulated by the homeotic gene Ultrabithorax (Ubx) to mediate haltere development. In an enhancer–trap screen to identify genes that show differential expression between wing and haltere discs, we identified CG32062, which codes for a RNA-binding protein. In wing discs, CG32062 is expressed only in non-D/V cells. CG32062 expression in non-D/V cells is dependent on Notch-mediated signaling from the D/V boundary. However, CG32062 expression is independent of Wingless function, thus providing evidence for a second long-range signaling mechanism of the D/V organizer. In haltere discs, CG32062 is negatively regulated by Ubx. The non-cell autonomous nature of Ubx-mediated repression of CG32062 expression suggests that the novel component of D/V signaling is also negatively regulated during haltere specification.

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1. Introduction

In the fruitfly Drosophila melanogaster, wings and halteres are the dorsal appendages of the second and third thoracic segments, respectively. Growth and patterning during fly wing development are mediated by signaling from its dorso–ventral (D/V) organizer. Interactions between dorsal and ventral cells of the wing pouch set up the organizer by activating Notch (N) in the D/V boundary (Diaz-Benjumea and Cohen, 1993; Diaz-Benjumea and Cohen, 1995; Williams et al., 1994; Irvine and Wieschaus, 1994; Kim et al., 1995; de Celis et al., 1996). N, in turn, activates Wingless (Wg), Cut (Ct) and Vestigial (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). Wg is known to diffuse to non-D/V cells from the D/V boundary and acts as a morphogen (Zecca et al., 1996; Neumann and Cohen, 1997). High levels of Wg are required for activating Achaete (Ac), whereas moderate levels are enough to activate Distal-less (Dll) and low-levels to activate Vg (Neumann and Cohen, 1997). However, two lines of evidence suggest that Wg may not be the sole mediator of N signaling from the D/V boundary. First, over-expression of Wg in Ser− background (wherein N signaling is compromised) is not sufficient to activate downstream genes (Klein and Martinez-Arias, 1999). Second, ectopic expression of Wg or activated-Arm does not induce ectopic expression of vg-QE, the quadrant enhancer of Vg (Nagaraj et al., 1999).

In the third thoracic segment, wing development is suppressed by the homeotic selector gene Ultrabithorax (Ubx) to mediate haltere development (Lewis, 1978). Suppression of wing fate and specification of the haltere fate by Ubx is a classical example of Hox regulation of serial homology, which has served as a paradigm for understanding the nature of homeotic gene function. 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). Wg signaling is
down-regulated in haltere discs due to enhanced degradation of Armadillo (Arm), which in turn causes the repression of Vestigial 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. Over-expression of Vestigial in haltere discs is enough to override Ubx function and cause haltere-to-wing homeotic transformations, suggesting that negative regulation of D/V signaling by Ubx is a critical step during haltere specification (Mohit et al., 2003). The differential development of wings and halteres thus constitutes a good genetic system to study D/V signaling per se and to identify additional components of D/V signaling.

In an enhancer–trap screen to identify genes that show differential expression between wing and haltere discs, we identified CG32062, which codes for a RNA-binding protein. In wing discs, CG32062 is expressed exclusively in non-D/V cells. N signaling is required to activate CG32062 expression in non-D/V cells and for its repression in the D/V boundary. However, CG32062 is independent of Wg and Vg function in the D/V boundary, thus, providing a direct evidence for Wg-independent long-range signaling from the D/V boundary.

2. Results and discussion

2.1. Identification of an enhancer–trap marker differentially expressed between wing and haltere discs.

We employed an enhancer–trap based genetic approach to identify novel markers that are potential targets of homeotic gene control in Drosophila. A collection of ~ 800 GAL4 enhancer–trap lines were screened for segmentally modulated expression of the reporter gene. EN403-GAL4 was identified in this screen, which showed segmentally modulated pattern in its expression in the larval CNS (Fig. 1A). In the embryo too it is expressed in the CNS, although no specific pattern could be recognized (data not shown). The enhancer that is trapped is hereafter referred to as EN403.

In wing and haltere imaginal discs, EN403 is expressed in a subset of non-D/V cells of the pouch, on either side of the D/V boundary (Fig. 1B–E). EN403 is activated late during development after the establishment of Wg domain in the D/V boundary and the initiation of vg-QE expression in non-D/V cells (data not shown). Its expression in the wing pouch is more prominent in proximal than in distal cells. EN403 is also expressed in the peripodial cells of wing imaginal discs (Fig. 1F). Supplementary file (Video 1) carries a QuickTime movie on EN403 expression viewed from different angles in 3D, which shows that EN403 is expressed only in non-D/V cells of the wing pouch and in the peripodial membrane. EN403 is differentially expressed between wing and haltere discs. In wing discs, it is expressed in both anterior and posterior compartments (Fig. 1B), whereas in haltere discs it is expressed only in the posterior compartment (Fig. 1C). EN403 is also expressed in the presumptive distal segments of leg and antennal discs (Fig. 1G,H) and in the morphogenetic furrow of eye discs (Fig. 1H).

Interestingly, at the levels of both the expression pattern and differential expression between wing and haltere discs, EN403 is complementary to Wg and Ct. The latter two are expressed in D/V cells, whereas EN403 is restricted to non-D/V cells. In haltere discs, Wg and Ct are not expressed in the posterior compartment (Weatherbee et al., 1998; Shashidhara et al., 1999), whereas EN403 expression is absent from the anterior compartment. The only other non-D/V marker that shows differential expression between wing and haltere discs is vg-QE, whose expression overlaps with that of EN403 (Fig. 1I). However, no expression of vg-QE is observed in haltere discs, either in the anterior or posterior compartment (Weatherbee et al., 1998; Shashidhara et al., 1999), whereas EN403 expression is robust in the posterior compartment.

2.2. EN403 is an enhancer–trap of CG32062

Polytene in situ suggested EN403-GAL4 is mapped to 67E region (Fig. 1K). We further isolated flanking genomic DNA by plasmid rescue. The sequence analysis of this DNA fragment refined the mapping of EN403-GAL4 to 67E2-E3, within the 50 kb-long 2nd intron of CG32062 (Fig. 1J). This gene codes for a RNA-binding protein. Its closest homologue in vertebrates, at the level of RNA binding motif, is human Ataxin 2 binding protein. RNA in situ using LD15974, an EST mapped to CG32062 suggests that its expression pattern is very similar to that of EN403. In wing imaginal discs, it is expressed in non-D/V cells of the pouch (Fig. 2A,B), although RNA in situ pattern is more uniform than the EN403 expression along the proximo-distal axis of the wing pouch. In haltere discs, CG32062 is restricted to the posterior compartment (Fig. 2C). In leg and eye imaginal discs too, CG32062 expression pattern is identical to that of EN403 (compare Fig. 2D,E). These observations suggest that EN403 is an enhancer trap of CG32062.

For further studies on the regulation of CG32062 expression, we generated a lacZ enhancer trap by targeted P-conversion approach (Sepp and Auld, 1999). The newly generated EN403-lacZ showed an expression pattern identical to that of EN403-GAL4 in wing discs (Fig. 2F) and in all other tissues (data not shown). Hereafter CG32062 refers to both EN403-GAL4 and EN403-lacZ, unless specified otherwise. See figure legends for the actual method (by RNA in situ or by using EN403-GAL4/UAS-GFP or EN403-lacZ) by which CG32062 expression was determined in different experiments.

2.3. Non-cell autonomous regulation of CG32062 by Ultrabithorax in haltere discs

To determine whether Ubx regulates CG32062 in the haltere disc, we analyzed its expression in Ubx mutant
backgrounds. Unlike vg-QE, CG32062 expression is not sensitive to Ubx haploinsufficiency. vg-QE expression is partially de-repressed in haltere discs in Ubx heterozygous background (Fig. 3A), whereas CG32062 expression remained repressed in the anterior compartment (Fig. 3B). In 4-winged flies (Ubx/abx px bx<sup>3</sup>), CG32062 is de-repressed in the anterior compartment, thus its expression pattern in the transformed haltere disc is indistinguishable from that of the wing disc (Fig. 3C). Conversely, in the Contrabithorax (Cbx<sup>him</sup>) allele, CG32062 expression was repressed in the anterior compartment of the wing disc (Fig. 3D).

To determine the cell-autonomy of Ubx-mediated repression of CG32062 in haltere discs, we generated Ubx<sup>−</sup> mitotic clones. Clonal removal of Ubx<sup>−</sup> (at mid-2nd instar stages) did not affect CG32062 expression in the posterior compartment of haltere discs (Fig. 3E). This suggests that CG32062 expression in that compartment is independent of Ubx function. More importantly, Ubx<sup>−</sup> clones (n >= 50 haltere discs) in the anterior compartment did not result in the activation of CG32062 expression (Fig. 3F,G). As mentioned above, CG32062 expression is activated in the anterior
compartment of $Ubx^{1/abx\ \ pbx\ bx}$ haltere discs (Fig. 3C).
Together, these results suggest non-cell autonomous regulation of $CG32062$ by $Ubx$.

Homoeotic genes function as selector genes, which means presence or absence of their function within a cell confers a specific developmental fate. $Ubx$ may exert a non-cell autonomous effect since it down regulates long distance signaling activity of the D/V boundary (Shashidhara et al., 1999). A more direct genetic evidence for non-cell autonomy of $Ubx$ function was elusive, since $Ubx$ functions at multiple levels in the hierarchy of wing development (Weatherbee et al., 1998; Shashidhara et al., 1999; Galant et al., 2002; Mohit et al., 2003). Our results described above on the regulation of $CG32062$ provide strong evidence for non-cell autonomous function of $Ubx$.

As described above, $CG32062$ expression is completely repressed in the anterior compartment of $Ubx^{Hm}$ wing discs. In those wing discs, $Ubx$ is mis-expressed in the entire wing pouch (Casares et al., 1997). However, mitotic clonal analysis of $Ubx$ suggested non-cell autonomous regulation of $CG32062$. We therefore, tested spatial requirement for $Ubx$ function in this process by mis-expressing $Ubx$ in specific domains of wing discs using different GAL4 drivers. We used (i) $vg$-GAL4, which is specific to the D/V boundary, at least, in the third instar larval stages (Fig. 4A), (ii) $EN403$-GAL4 itself, which is specific to non-D/V cells (Fig. 4B) and (iii) $EN426$-GAL4, which is specific to the presumptive hinge (Fig. 4C). These GAL4 drivers were combined with $EN403$-lacZ before crossing to UAS-$Ubx$. Ectopic expression of $Ubx$ using $vg$-GAL4 driver caused complete down regulation of $CG32062$ expression in the anterior compartment of wing discs (Fig. 4D). However, mis-expression of $Ubx$ using other two GAL4 drivers did not affect $CG32062$ expression (Fig. 4E,F).

Although early during wing patterning $vg$-GAL4 is expressed in the progenitors of both D/V and non-D/V cells (Vegh and Basler, 2003), by early third instar stage it is specifically expressed in the D/V boundary. At the antibody staining level, no $Ubx$ protein is observed in non-D/V cells of $vg$-GAL4/UAS-$Ubx$ wing discs (Shashidhara et al., 1999). It is still possible that $vg$-GAL4-driven expression of $Ubx$ early during development may trigger a cascade of events in non-D/V cells that may repress $CG32062$, although at later stages $Ubx$ is not present in those cells. We therefore, examined the effect of removing $Ubx$ from the D/V boundary. In seven out of eight halteres with $Ubx^{-}$ clone/s in the D/V boundary, we observed $CG32062$ expression in non-D/V cells of the anterior compartment (Fig. 4G). More significantly, even $Ubx^{+}$ non-D/V cells showed $CG32062$ activation in response to removal of $Ubx$ from the D/V boundary. Taken together, we
2.4. CG32062 expression is dependent on D/V organizer function

To directly test if the activation of CG32062 is indeed dependent on the D/V organizer function, we analyzed its expression in temperature sensitive N (N°) mutants. When larvae were grown at non-permissive temperatures, there was partial to complete loss of CG32062 expression in wing discs (Fig. 5B). The degree of repression of CG32062 in N° mutant discs, its over-expression in non-D/V cells, its expression in non-D/V cells was partial to complete. When we over-expressed activated N (N°) in the D/V boundary, we observed similar repression of CG32062, when we over-expressed activated N (N°) using omb-GAL4 (Fig. 5D,E).

2.5. CG32062 expression is independent of Wingless function

CG32062 is expressed in the posterior compartment of haltere discs. In this compartment, no Wg expression is observed in the D/V boundary. Ectopic expression of Ubx in the wing disc causes down regulation of CG32062 expression in the anterior compartment, although it does not affect Wg expression in the D/V boundary of that compartment. Interestingly, ectopic Ubx down regulates Wg expression in the posterior compartment of wing discs (Shashidhara et al., 1999), wherein it does not affect CG32062 expression. This suggests that CG32062 expression in non-D/V cells is independent of Wg function. We experimentally tested this hypothesis as follows.

Over-expression of Wg, Dsh or activated Arm using vg-, omb- or ptc-GAL4 drivers did not affect CG32062 expression pattern in wing and haltere discs (data not shown), suggesting that Wg signaling may not have any input either on activation or repression of CG32062 expression. To further test the requirement for Wg to activate CG32062 expression in non-D/V cells, we employed genetic backgrounds that remove Wg function in the D/V boundary and activation of CG32062 even in Ubx+ cells (arrow).

In addition to conferring long-range signaling activity to the D/V boundary cells, N inhibits D/V cells taking the fate of non-D/V cells. For example, although N function in the D/V boundary is required to activate vg-QE in non-D/V cells, its over-expression in non-D/V cells inhibits vg-QE expression (Klein and Martinez-Arias, 1999). We observed similar repression of CG32062, when we over-expressed activated N (N°) using omb-GAL4 (Fig. 5D,E).
ectopic CG32062 in wing discs nor they activated CG32062 in anterior haltere discs. In addition, we over-expressed GPI-linked Dfz2, which acts as dominant negative for all Wnt proteins (Nusse, 1997). There was no effect on CG32062 expression pattern in wing discs, suggesting that CG32062 expression is not dependent on any member of the Wnt family of genes.

2.6. Attempts to identify new D/V signal

N-dependent and Wg-independent expression of CG32062 in non-D/V cells suggest the presence of a second long-range signaling molecule at the D/V organizer. Other than Wg signaling, EGFR/Ras signaling is known to function in the D/V axis of the wing pouch (Nagaraj et al., 1999). We therefore, examined if EGFR/Ras signaling is involved in the regulation of CG32062 expression. We expressed both positive (activated EGFR and activated Ras) and negative regulators of EGFR pathway (dominant negative EGFR and dominant negative Ras) using vg- and omb-GAL4 drivers. CG32062 expression was not affected in any of the combination, which rules out EGFR/Ras being the regulator of CG32062 expression.

A gain of function EP screen (Rorth, 1996) was initiated to identify and characterize the genes that can regulate CG32062 expression. We used modified EP screen strategy, known as the Gene Search System, in which inserted P-element has two UAS sequences in opposite directions (Toba et al., 1999). This enables the expression of trapped gene irrespective of the orientation of the P-insertion. In the first round of screen on 400 GS insertions, we identified two GS lines that cell-autonomously repress CG32062.
expression (data not shown). However, further analysis showed that this is by the activation of N (NS, unpublished observations). We did not identify any GS insertion that acts downstream of N to activate or repress CG32062.

Further work is in progress to identify the novel component of D/V signaling, which is different and independent of Wg, but dependent on N activity.

3. Conclusion

To conclude, the regulation of CG32062 expression is dependent on the function of N in the D/V boundary. However, CG32062 expression is entirely independent of Wg signaling pathway, thus suggesting the presence of a second signal molecule at the D/V boundary. Recently, similar conclusions have been drawn in a study employing an entirely different approach (Giraldez and Cohen, 2003). In their study, to understand the mechanism of cell survival and control of cell proliferation, the authors have concluded that Wg accounts partially for the effects of Notch. Based on the ability of wing pouch cells to respond to N signals, even in the absence of Wg, they have concluded that Notch also acts through another relay signal. There are many parallels between Wg signaling and the predicted second signal. The latter is also activated by N and, in turn, activates target gene (CG32062) expression at a distance. Similar to Wg signaling (Mohit et al., 2003), the novel D/V signaling mechanism is also a target of Ubx function during haltere development. While Ubx represses Wg in the posterior compartment, it may repress this putative signal molecule in the anterior compartment.

3.1. Experimental procedure

Recombinant chromosomes and combinations of GAL4 drivers, UAS lines, different mutations and/or markers were by standard genetic techniques. \( \text{vg}^1, N^{ts}, wg^P \) and \( \text{spd}^{fs} \) alleles are listed in Flybase. GAL4-UAS system (Brand and Perrimon, 1993) was used for targeted mis-expression of gene products. FLP-FRT method (Xu and Rubin, 1993) was used for generating mitotic clones of Ubx gene products. FLP-FRT method (Xu and Rubin, 1993) was used for targeted mis-expression of alleles are listed in Flybase. GAL4-UAS system (Brand and Perrimon, 1993) was used for targeted mis-expression of alleles are listed in Flybase.

3.2. Histology

X-gal and immuno-histochemical staining was essentially as described by Ghysen and O’Kane (1989) and Patel et al. (1989), respectively. RNA in situ was according to Manoukian and Krause (1992). LD15974, a cDNA representing CG32062, was used as the probe. The primary antibodies used are anti-Ct (Blochlinger et al., 1993), anti-En (Patel et al., 1989), anti-Wg (Brook and Cohen, 1996) and anti-\( \beta \)-galactosidase (Sigma, St. Louis, USA). Monoclonal anti-En and anti-Wg antibodies were obtained from the Development Studies Hybridoma Bank, University of Iowa, USA. Fluorescence images were obtained either on a Zeiss Apotome \( ^\text{TM} \) microscope or on Zeiss LSM/Meta. Control and experimental images were digitized always at identical fluorescence microscope and camera settings. QuickTime movie on EN403: Wing discs were imaged on a Zeiss Apotome \( ^\text{TM} \) microscope with motorized stage with 0.5 \( \mu \) step size. 3D imaging and subsequent conversion of the optical sections to QuickTime movie was done using the de-convolution technology with AxioVision4 image analysis software.

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References


