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Interaction between Ataxin-2 Binding Protein 1 and Cubitus-interruptus during wing development in *Drosophila*

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ABSTRACT

Animal growth and development is dependent on reiterative use of key signaling pathways such as Hedgehog (Hh) pathway. It is widely believed that Cubitus-interruptus (Ci) mediates all functions of Hh pathway. Here we report that CG32062, the *Drosophila* homologue of Ataxin-2 Binding Protein 1 (dA2BP1), functions as a cofactor of Ci to specify intervein region between L3 and L4 veins of the adult wing. Specifically, Ci-mediated transactivation of *knot/collier* (*kn*) in this region of the developing wing imaginal disc is dependent on dA2BP1 function. Protein interaction studies and chromatin-immunoprecipiation experiments suggest that Ci helps dA2BP1 to bind *kn* promoter, which in turn may help Ci to activate *kn* expression. These results suggest a mechanism by which Ci may activate targets such as *kn*, which do not have classical Ci/Gli-binding sites.

Introduction

Growth and patterning during fly wing development are mediated by signaling from its anterior-posterior (A/P) and dorsal-ventral (D/V) organizers. The A/P compartmentalization is controlled by the expression of *engrailed* (*en*; Lawrence and Morata, 1976), a selector gene that specifies posterior identity (Kornberg et al., 1985). En activates *hedgehog* (*Hh*), which is a short-range morphogen that diffuses to the anterior compartment, and activates Smoothened (Smo) by releasing it from Patched (Ptc). In the presumptive A/P boundary, Smo activates Cubitus-interruptus (Ci) by stabilizing its full-length isoform, which in turn activates targets of Hh, particularly Decapentaplegic (Dpp) and Knot/Collier (Kn). Ci also activates Ptc and thus, a higher concentration of Ptc receptor is maintained at the AP boundary (reviewed by Aza-Blanc et al., 1997).

A combination of repressor activity of 75 kDa form and activator activity of 155-kDa form of Ci regulate the expression of different sets of target genes (Methot and Basler, 2001; Wang and Holmgren, 1999). This, however, is thought to be mediated by Ci binding to similar binding sites (Muller and Basler, 2000). Targets of Ci such as *ptc, dpp, wg* possess well characterized binding sites on their promoters (Alexandre et al., 1996; Hidalgo and Ingham, 1990; Muller and Basler, 2000; Von Ohlen et al., 1997). However, classical Gli/Ci-binding sites are not present on *kn* promoter (Hersh and Carroll, 2005). This suggests that the mechanism of Ci-mediated activation of *kn* may be different from the mechanism activating *ptc* or *dpp* expression.

Previously, we reported the identification of *CG32062* in an enhancer-trap screen to identify genes that show differential expression between wing and haltere discs (Bajpai et al., 2004). Here we report functional characterization of *CG32062*. Sequence analyses suggest that protein encoded by *CG32062* (hereafter referred to as *dA2BP1*) is the closest homologue of human Ataxin-2 Binding Protein 1 (A2BP1). In mammals including human, expansion of polyglutamine repeats in Ataxin-2 causes Spinocerebellar ataxia type 2 (SCA2), a neuro-degenerative disease. The Ataxin-2 activity is modulated by A2BP1, which binds to the C-terminus of the former (Shibata et al., 2000).

dA2BP1 shows a dynamic expression pattern; both spatially and temporally. The protein is predominantly nuclear with traces of the protein in the cytoplasm. Removal of *dA2BP1* function causes formation of ectopic veins and loss of intervein region, a phenotype similar to those caused by loss-of-function alleles of *kn*. Epistasis experiments suggested *dA2BP1* interacts with Ci to activate the expression of *kn*. Protein interaction studies and chromatin-immunoprecipiation experiments suggest that Ci helps dA2BP1 to bind *kn* promoter, which in turn may facilitate Ci to activate *kn* expression. Results presented here suggest that dA2BP1 may facilitate activation of those targets of Ci such as *kn*, which do not have classical Ci/Gli-binding sites.

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Materials and Methods

Genetics

Recombinant chromosomes and combinations of GAL4 drivers and UAS lines, generation of different mutations and markers, molecular cloning and associated nucleic acid and protein techniques (expression, purification, immunoprecipitation etc) were as per the standard genetic and molecular procedures. The LD15974 (Berkeley *Drosophila* Genome Project), the cDNA representing full-length CG32062-RE isoform, was used to express dA2BP1 in *E. coli* (bacterially expressed protein was used to raise polyclonal antibodies against dA2BP1) and to generate transgenic UAS-dA2BP1. Cloning strategies, sequence of PCR primers, details of GST-pull down assay and immunoprecipitation experiments are described in the supplement text.

Several flystocks were obtained from various sources: GAL4 strains used are Actin5c>CD2>GAL4 (Pignoni and Zipursky, 1997), *C96*-GAL4 (Gustafson and Boulianne, 1996), *ap*-GAL4 (Calleja et al., 1996), *EN403*-GAL4 (Bajpai et al., 2004), *MS1096*-GAL4 (Capdevila et al., 1994), *omb*-GAL4 (personal communication to Flybase, *Calleja, 1996.10.16*), *ptc*-GAL4 (Brand and Perrimon, 1993) and *vg*-GAL4 (Simmonds et al., 1995). Other fly-strains used: UAS-Ci (Aza-Blanc et al., 1997), UAS-Ci-HA (Wang and Holmgren, 1999), $dpp^{BS3.0}$-lacz (Blackman et al., 1991), UAS-Dsh (Neumann and Cohen, 1996), *EN403*-lacZ (Bajpai et al., 2004), UAS-mCD8::GFP (Lee and Luo, 1999), *hs*-FLP (Xu and Rubin, 1993), UAS-Kn (Mohler et al., 2000), *kn*-lacZ (Hersh and Carroll, 2005)and UAS-$Notch^{intra}$ (Fortini et al., 1993). The P-insertion $y^1\ w^{67c23}$; *P{EPgy2}EY01049/TM3,* $Sb^1\ Ser^1$ (Bloomington Stock # 15489) in dA2BP1 is described in Flybase.

Histology

RNA in situ and immunochemical staining on embryos and imaginal discs were performed as described earlier Patel et al. (1989) and Tautz and Pfeifle (1989). LD15974 and RE20611 were used as templates to generate DIG-labelled probes for RNA in situ for *dA2BP1* and *dpp*, respectively. Antibodies used were, anti-dA2BP1 (1:1), monoclonal anti-Cut (1:10; Blochlinger et al., 1993), anti-β-Galactosidase and anti-GFP (Molecular Probes), anti-HA (1:1000; Roche), anti-DSRF (1:100; Montagne et al., 1996), anti-dpERK1/2 (1:50; Sigma, USA), anti-Ci (raised in rat; 1:5; Motzny and Holmgren, 1995), anti-Caspase 3 (1:300; Cell Signaling), monoclonal anti-Wg (1:500; Brook and Cohen, 1996), anti-Delta (1:100; Qi et al., 1999)) anti-Ubx (1:30; White and Wilcox, 1984); anti-Ptc (1:50; Capdevila et al., 1994), anti-Hh (1:1000; Tabata and Kornberg, 1994), anti-En (1;200; Patel et al., 1989). Antibodies against Wg and Dl were obtained from Developmental Studies Hybridoma Bank (DSHB), Iowa, USA. All secondary antibodies conjugated to Alkaline Phosphatase or Horse Radish Peroxidase were purchased from Jackson Immuno Research Laboratories, USA and those conjugated to fluorophores were purchased from Molecular Probes, USA. Protein A Agarose (Amersham) or Protein G Agarose (Upstate) were used for immunoprecipitation experiments.

Chromatin immunoprecipitation (ChIP) experiments

The ChIP protocol used is modified from Papp and Muller (2006) and Sanchez-Elsner et al. (2006). Chromatin of 500 bp-2 kb was isolated from wing discs of larvae of desired genotype and subjected ChIP using goat anti-Ci (Santacruz, USA) or anti-dA2BP1 antibodies. The immunoprecipitated DNA was subjected to PCR amplification with primers designed to amplify those regions of *kn* and *dpp* genes, which harbour Ci-binding motifs (Hersh and Carroll, 2005; Muller and Basler, 2000). Primers against a region of *kn* with no known binding sites for Ci, which is more than 20 kb away from the region with Ci binding site, was also included in all experiments. ChIP experiments for a region of 3rd exon of *Rpl32* served as negative control and the same was used to normalize the differences between mock and test of a given experiment. Details of the procedure and sequences of primers used are given in the Supplement Text.

Electromobility shift assays

The ability of purified dA2BP1 (bacterially expressed) to bind *kn* promoter was examined using electromobility shift assays as described earlier (Pallavi et al., 2006). The protein + DNA mix was electrophoretically resolved on 5% polyacrylamide gels. Probes used: (1) PCR amplified *kn* and *dpp* cis-regulatory regions using the same primers as used for ChIP. (2) a 60-mer oligo comprising Ci-binding sites on *kn* and its mutated version. Sequences of the two oligomers used are shown in the Supplement Table 1.

Results

Loss of dA2BP1 causes loss of intervein tissue in the adult wing

The *dA2BP1* gene in *Drosophila*, is a complex locus spanning more than 80 kb of the genomic region located at 67E1-2 polytene position (Bajpai et al., 2004). Based on the analyses of ESTs for this gene, *dA2BP1* is predicted to have 17 exons coding for 5 isoforms (http://www.flybase.org). In addition to a predicted RNA-recognition motif, all isoforms of *dA2BP1* have 2 long poly-Q domains (Suppl. Fig. 1).

To address the role of this gene in the growth and patterning, we started by examining the expression pattern of this gene in developing tissues. First, we raised antibodies against a variant of dA2BP1 protein containing all the predicted functional domains of dA2BP1 (Suppl. Fig. 1). We examined the expression of dA2BP1 during embryonic development and in the larval imaginal discs. In embryos, expression of dA2BP1 was primarily in the central and peripheral nervous systems (Suppl. Fig. 2A, B). Expression was also noted in embryonic pericaridial cells (Suppl. Fig. 2B).

In the 3rd instar larva, expression of dA2BP1 was observed in all imaginal discs. In wing imaginal discs, expression was seen in the pouch region as well as the notum. Within the pouch region, cells away from the dorsal-ventral (D/V) compartment boundary showed relatively higher levels of expression as compared to the cells at the D/V boundary (Fig. 1A). This is similar to the RNA in situ pattern reported for this gene (Bajpai et al., 2004). The expression of dA2BP1 in the notum appeared to be in myoblasts; this was confirmed by its co-localization with the transcription factor Cut, which marks these cells (Fig. 1A). In addition to the pouch and notum regions, dA2BP1 expression was also observed in peripodial cells of the wing disc (data not shown).

As mentioned earlier, *dA2BP1* was identified in an enhancer trap screen. The enhancer trap line *EN403-lacZ* is inserted within the 49 kb long intron of the *dA2BP1* gene (Bajpai et al., 2004). To determine if the anti-dA2BP1 staining pattern in the wing discs is in the same cells that express *EN403-lacZ*, we carried out a double staining with anti-dA2BP1 and anti-βgal antibodies on *EN403-lacZ* wing discs. dA2BP1 and β-gal were found colocalized in the wing pouch (Fig. 1B), suggesting that the anti-A2BP1 antibodies recognize the gene product trapped by *EN403*-lacZ.

In addition to the wing disc, expression of dA2BP1 was also detected in other imaginal discs. In the leg and antennal discs, staining was observed in the central region (data not shown); in the eye imaginal discs, the protein was detected predominantly in cells posterior to the morphogenetic furrow (Suppl. Fig. 2E). In all cases, the protein appeared to be nuclear (Fig. 1A; Suppl. Fig. 2).

We had previously shown that expression of dA2BP1 is dependent on Notch signaling at the D/V boundary (Bajpai et al., 2004). However, the functional role of dA2BP1 in wing patterning was not explored. To address this question, we attempted to generate loss-of-function

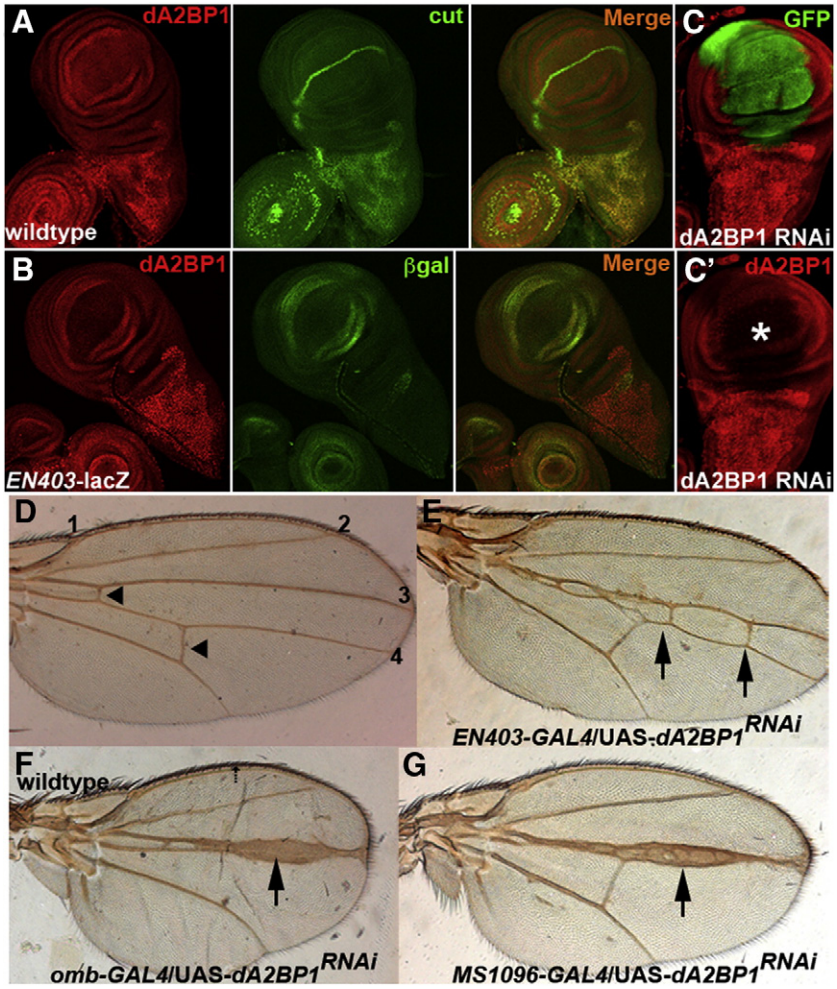

Fig. 1. dA2BP1 is required for the specification of the intervein region in the wing. (A) Wild type wing discs stained with anti-dA2BP1 (red) and anti-Cut (green). dA2BP1 is expressed in the wing pouch (asterisk) and myoblast cells in the notum (arrow) as determined by the expression of Cut. (B) Expression of dA2BP1 in the wing pouch is identical to the enhancer trap line *EN403*-LacZ (green). The expression is relatively higher in non-D/V cells than in D/V cells. (C) *omb*-GAL4/UAS-*dA2BP1*^{RNAi}; UAS-GFP/+ wing discs stained with anti-dA2BP1 (red) and anti-GFP (green) antibodies (B). Note loss of dA2BP1 in *omb*-GAL4 domains (asterisk). The large nuclei that are stained in this domain are of peripodial cells and not in the disc proper cells. (D) Wildtype wing blade. Five distinct veins (numbered) with two cross veins between veins 3 and 4 and between 4 and 5 (arrowheads) are marked. (E) *EN403*-GAL4>*dA2BP1*^{RNAi} wing blade. Multiple crossveins develop between veins 3 and 4. Also note loss of intervein in that region. (F-G) Expression of *dA2BP1*^{RNAi} using *omb*-GAL4 (F) and *MS1096*-GAL4 (G) leads to loss of the intervein region between veins 3 and 4 and formation of excess vein tissue (arrows in F and G).

alleles of *dA2BP1* by trying to mobilize the P-element in *EN403*-lacZ and *P{EPgy2}EY01049*). Several attempts to generate loss-of-function alleles of *dA2BP1* failed (see supplement text for details) and, in the absence of any deficiency stocks for this region, we employed RNAi-mediated gene knock-down approach to examine the effect of down-regulation of *dA2BP1*.

We designed and generated *dA2BP1*^{RNAi} transgenes (UAS-*dA2BP1*^{RNAi}) that would generate dsRNA against the RNA binding domain. As this domain is common to all isoforms of *dA2BP1*, we predicted that the RNAi transgene would knock-down expression of all isoforms. In addition, this was the only domain that predicted not to generate any possible off-target effects. To test the efficacy of these RNAi lines, we crossed them to different GAL4 drivers such as *omb*- and *ptc*-GAL4, which express in the pouch region of the wing imaginal disc and carried out immunohistochemistry using anti-dA2BP1 antibodies. Expression of UAS-dA2BP1^{RNAi} resulted in a considerable decrease in the expression of dA2BP1 in *omb*- and *ptc*-GAL4 domains (Fig. 1C; Suppl. Fig. 3A). RNAi-mediated knock-down of dA2BP1 using ubiquitous GAL4 drivers such as *tubulin*-GAL4, *hs*-GAL4 resulted in early larval lethal, suggesting vital functions for dA2BP1 during early development.

To examine the effect of knock-down of dA2BP1 on wing morphology, we first expressed dA2BP1^{RNAi} using *EN403*-GAL4 whose expression reflects the endogenous expression pattern of the gene (Bajpai et al., 2004). Interestingly, all the adult flies that emerged had only one specific phenotype viz., loss of intervein region and ectopic cross veins between L3 and L4 of the adult wing blade (Fig. 1E; penetrance - 85%; n>75). As mentioned earlier, D/V boundary cells express lower levels of dA2BP1 as compared to cells away from the boundary. Down-regulation of dA2BP1 (using *vg*-GAL4 and *C96*-GAL4 drivers) in these cells did not result in any wing phenotype and the progeny were fully viable (n>75).

Next, we examined the effect of knock-down of dA2BP1 using different GAL4 drivers, which are expressed in different regions of the wing disc. *omb*-GAL4 expresses in the central region of the wing pouch; *ap*-GAL4 and *MS1096*-GAL4 express in the dorsal compartment of the disc, and *ptc*-GAL4 is expressed only in the anterior cells immediately adjacent to the A/P boundary (hereafter referred to as AB cells). When crossed to these GAL4 drivers, UAS-*dA2BP1*^{RNAi} caused loss of intervein region and fusion of veins L3 and L4. In some cases, the amount of vein material appeared to be more suggesting the formation of ectopic vein tissue (Fig. 1F, G; Suppl. Fig. 3C, D; penetrance 85-100%; n>75 for all crosses).

Clonal induction of *dA2BP1*^{RNAi} expression also caused ectopic veins in the wing blade (n=85), while majority (n=60) of flies displayed ectopic vein between L3 and L4 (Suppl. Fig. 3E-G). When

UAS-*dA2BP1*^{RNAi} was crossed to *MS1096*-GAL4 in the presence of one copy of P-insertion in *dA2BP1* (*P{EPgy2}EY01049*), we observed enhanced L3/L4 vein phenotype: fusion of L3 and L4 accompanied by thickening of the fused vein was observed. In addition, large patches of ectopic veins in regions beyond L5 were observed (Suppl. Fig. 3H). These results suggest that while dA2BP1 may have a role in the specification of the entire wing blade, L3-L4 vein/intervein specification is more sensitive to small perturbations in the levels of dA2BP1.

dA2BP1 regulates Knot/Collier expression

To test if this phenotype was caused by cell death of intervein specifying cells, we stained RNAi expressing wing discs with anti-Caspase3, Acridine-orange and DAPI. However, no indication of cell death was observed (data not shown). Furthermore, co-expression of UAS-P35 or UAS-CycE did not rescue UAS-*dA2BP1*^{RNAi}-induced wing phenotype suggesting that knock-down of dA2BP1 interferes with the specification of intervein region. We, therefore, examined the effect of *dA2BP1*^{RNAi} on the EGFR/Ras and Notch signaling pathways which are known to specify vein and intervein regions (reviewed in de Celis, 2003). Components of EGFR/Ras pathway such as dpERK1/2 (transducer of the EGFR/Ras signal) and Argos (negative feed-back regulator) are expressed in the presumptive vein regions (Suppl. Fig. 4A, C). Dl (the ligand of Notch) is also expressed in the presumptive veins (Suppl. Fig. 4E), but activates Notch pathway in adjacent intervein regions. Upon expression of dA2BP1 dsRNA, we observed fusion of the two stripes expressing dpERK1 and Argos, which represent presumptive L3 and L4 veins (Suppl. Fig. 4B, D) and, loss of Dl staining in those cells (Suppl. Fig. 4F). Fusion of dpERK1 and Argos stripes and loss of Dl reflect the observed fusion of L3 and L4 veins and loss of the intervein region in adult wings.

In addition to EGFR/Ras and Notch pathways, DSRF (a target of Hh pathway; Nussbaumer et al., 2000) is expressed (Suppl. Fig. 4G) and required in all intervein regions. Consistent with L3-L4 intervein region being very sensitive to knock-down of dA2BP1, we observed loss of DSRF only in L3-L4 intervein region, although its expression overlaps with that of dA2BP1 in 4 stripes, representing four major intervein regions (Suppl. Fig. 4H).

The transcription factor Kn functions by activating DSRF and repressing EGFR/Ras signaling (Vervoort et al., 1999; Crozatier et al., 2002; Mohler et al., 2000; for a review see de Celis, 2003). Loss of *kn* results in the loss of L3-L4 intervein region, a phenotype identical to the one observed by knocking-down dA2BP1(Crozatier et al., 2002; Mohler et al., 2000). Conversely, over-expression of Kn results in loss of vein tissue (Mohler et al., 2000). Given the strong similarity in the loss-of-function phenotypes of *kn* and *dA2BP1*, we examined the possibility of an interaction between these two genes.

When Kn was over-expressed in a background compromised for dA2BP1 (*omb*-GAL4 driven expression of both UAS-Kn and UAS-*dA2BP1*^{RNAi} transgenes), the loss of vein tissue caused by Kn over-expression was dominant over loss of intervein tissue due to knock-down of dA2BP1 (data not shown).

Rescue of UAS-*dA2BP1*^{RNAi}-induced wing phenotype by the co-expression of Kn indicated that *dA2BP1* might function upstream of *kn*. We examined the effect of knock-down of dA2BP1 on *kn* expression. We examined the effect of knock-down of dA2BP1 on *kn* expression by both RNA in situ and by monitoring the expression of its reporter gene *kn*-lacZ. Expression of UAS-dA2BP1^{RNAi} in the wing pouch lead to a significant reduction in the *kn* levels (Fig. 2B, F). Conversely, over-expression of dA2BP1 resulted in increased levels of *kn* expression (Fig. 2G).

In the absence of a null allele for *dA2BP1*, we examined the specificity and cell autonomy of RNAi-mediated phenotypes as follows: (1) dA2BP1 levels were reduced using *MS1096*-GAL4 driver, which expresses predominantly, but not exclusively, in the dorsal compartment of the wing pouch. We observed a correspondingly enhanced reduction (Fig. 2F) or increase (Fig. 2G) in *kn* expression in the dorsal compartment when dA2BP1 levels were reduced or increased, respectively. *kn* expression in the ventral compartment remained relatively unaffected. (2) *dA2BP1*^{RNAi} transgene was expressed in a background of high levels of Dicer2 to ensure near complete knock-down of dA2BP1. We observed severe reduction in the levels of *kn* (Fig. 2H; effect was visible even in the ventral compartment) suggesting a dosage-dependent effect. We, however, could not observe corresponding wing phenotypes as these animals died at early pupal stages. (3) Next, we carried out simultaneous expression of both, UAS-dA2BP1 and UAS-*dA2BP1*^{RNAi} transgenes. Similar to the overexpression of UAS-dA2BP1 alone, combined expression of UAS-dA2BP1 and UAS-*dA2BP1*^{RNAi} transgenes lead to larval lethality. However, in the wing disc level, we observed a rescue of *kn* expression levels suggesting that dA2BP1 indeed regulates *kn* expression (Fig. 2I).

The Vienna *Drosophila* RNAi collection has listed two RNAi transgenes for dA2BP1: ID 34046 and ID 34047. Both are predicted to have as many as 500 off targets. As expected, crossing these two lines with MS1096-GAL4 driver resulted in pupal lethality. However, despite the presence of a large number of off targets, we observed a reduction in *kn* expression in the wing disc (data not shown).

dA2BP1 functions as a potential cofactor of Cubitus-interruptus in regulating kn expression

Next, we examined the effect of modulating dA2BP1 levels on the expression of genes upstream of *kn* such as Ci, Ptc, Hh and En. We did not observe any significant change in the expression of these genes (Fig 3A-D; Suppl. Fig. 5). This suggests that dA2BP1 may function downstream of or in parallel to Ci which is known to regulate *kn* expression (Hersh and Carroll, 2005).

As mentioned above, when over-expressed in the entire dorsal compartment, dA2BP1 causes an increase in *kn* expression levels only in AB cells. These cells are known to express the stabilized 155 kDa form of Ci. In contrast, over-expression of Ci using *MS1096*-GAL4 driver causes ectopic expression of *kn* in the entire dorsal compartment of the wing pouch (Fig. 3E). This effect of Ci over-expression was suppressed, albeit partially, when UAS-Ci was co-expressed with UAS-*dA2BP1*^{RNAi} (Fig. 3F). As a control, we also examined the expression of *dpp*-lacZ in wing discs over expressing Ci. Similar to *kn*, over expression of Ci lead to ectopic expression of *dpp* in the entire dorsal compartment (Fig. 3G). But, unlike *kn*, the expression of *dpp* was unaffected by dA2BP1^{RNAi} (Fig. 3H). These results suggest specific interactions between dA2BP1and Ci to regulate *kn* expression in the wing disc. In these and subsequent sets of experiments, UAS-GFP was used as a control in combination with other UAS lines (UAS-*dA2BP1*^{RNAi}, UAS-dA2BP1, UAS-Ci etc) to rule out the possibility of any dilution effect of the GAL4.

dA2BP1 interacts with Ci through its C-terminal domain

Next, we examined if dA2BP1 physically interacts with Ci to regulate *kn* expression. Immunoprecipitation of dA2BP1 from nuclear extract of wildtype third instar larval wing discs using anti-dA2BP1 polyclonal antibodies resulted in co-precipitation of full-length (155 kDa) Ci (Fig. 4A). As the anti-Ci antibodies used can detect only 155 kDa protein, this experiment did not confirm if dA2BP1 can also bind the 75 kDa form of Ci protein.

Several attempts to do the reverse experiment using anti-Ci antibodies to immunoprecipitate dA2BP1 failed. This was probably due to the poor efficacy of the antibodies used in these experiments. We therefore, used a transgene that expresses full-length Ci tagged with HA (Ci-HA; Wang and Holmgren, 1999). Ci-HA was expressed in the wing pouch using *MS1096*-GAL4 driver. Nuclear extract of third

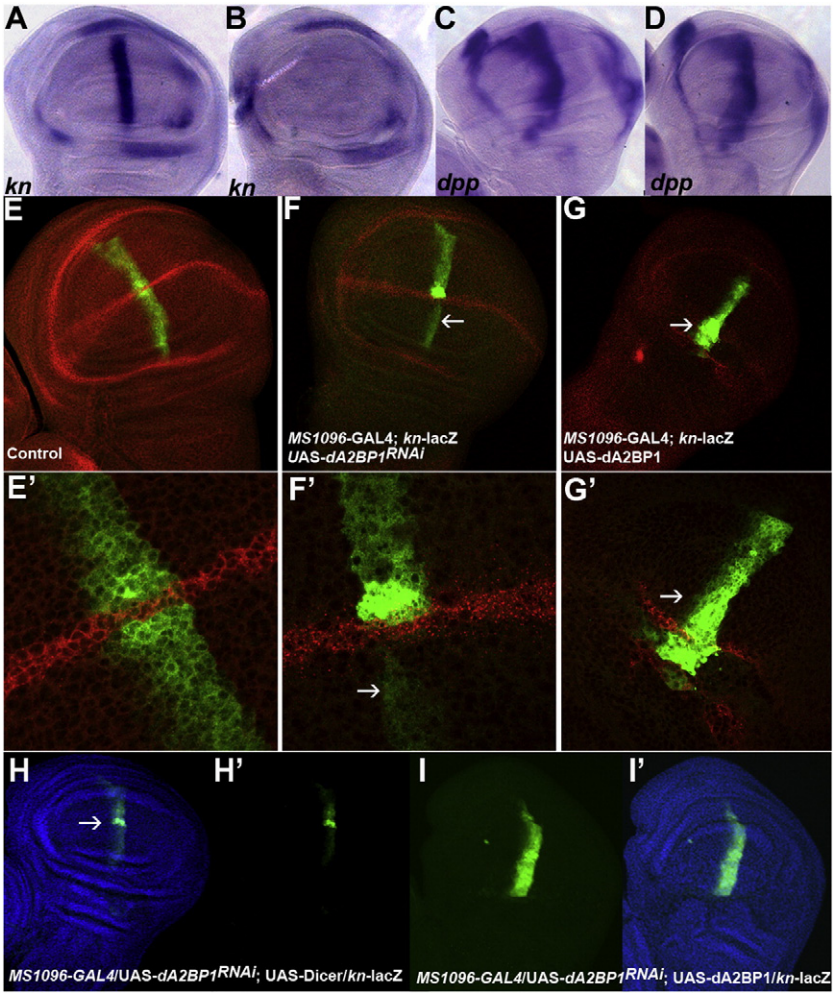

Fig. 2. dA2BP1 regulates *kn* expression in the Antero-posterior boundary cells. (A-B) RNA in situ for *kn* on wild type (A) and UAS-*dA2BP1*^{RNAi}/+; *ptc*-GAL4/+ (B) wing discs. Note *kn* expression pattern is severely compromised when dA2BP1 is knocked-down. (C-D) RNA in situ for *dpp* on wild type (C) and UAS-*dA2BP1*^{RNAi}/+; *ptc*-GAL4/+ (D) wing discs. Note *dpp* expression pattern is unaffected when dA2BP1 is knocked-down. (E-G) *kn*-lacZ (E), UAS-*dA2BP1*^{RNAi}/*MS1096*-GAL4; *kn*-lacZ/+ (F) and *MS1096*-GAL4/+; UAS-dA2BP1/*kn*-lacZ (G) wing discs stained with anti-β-galactosidase (green) and Wg (red) antibodies. E'-G' show higher magnification images of E-F, respectively. Expression of *dA2BP1*^{RNAi} causes downregulation of *kn*-lacZ expression (arrows in F and F'); over-expression of dA2BP1 leads to enhanced *kn*-lacZ expression (arrows in G and G'). (H and H') *MS1096*-GAL4-mediated expression of UAS-Dicer along with UAS-*dA2BP1*^{RNAi} leads to further decrease in the expression of *kn-lacZ* as monitored by anti-βgal staining. (I and I') *MS1096*-GAL4-mediated co-expression of UAS-dA2BP1 and UAS-*dA2BP1*^{RNAi} does not cause any decrease in *kn*-lacZ expression.

instar larval wing discs were subjected to immunoprecipitation using anti-HA monoclonal antibodies or anti-dA2BP1 polyclonal antibodies. Immunoprecipitation of Ci-HA using anti-HA antibodies was confirmed by Western blot using anti-Ci antibodies (Fig. 4B). Precipitation using anti-HA antibodies also lead to co-precipitation of dA2BP1 (Fig. 4C). Immunoprecipitation of dA2BP1 from Ci- dA2BP1 expressing wing discs using anti-dA2BP1 polyclonal antibodies also resulted in the co-precipitation of Ci-HA (Fig. 4D).

To identify the domain in dA2BP1 which is essential for its interaction with Ci, we cloned and expressed GST tagged truncated proteins corresponding to amino acids 1-517 of the N-terminus; residues 395-476 of the RNA-binding motif and, amino acids 478-865 of the C-terminal domain (Fig. 4E, F). The purified GST-tagged fusion proteins were challenged with wing disc lysate and immunoprecipitated using anti-GST antibodies. Western blot analyses with anti-Ci antibodies revealed that only the construct with amino acids 478-865 binds to Ci (Fig. 4G). This suggests that a region between amino acids 518-865 of dA2BP1 is critical for its binding to Ci.

dA2BP1 binds directly to the kn promoter to regulate its expression

The wing-enhancer element of *kn* is known to harbor, at least, three Ci-binding sites, although only one site (at Chr2R: 10699592-10699600) is shown to be functional (Hersh and Carroll, 2005). To understand the mechanism by which these two proteins interact to regulate *kn* expression, we asked if dA2BP1 binds directly to the *kn* promoter. We first carried out electromobility shift assays to determine if dA2BP1 could independently bind to DNA. Varying amounts of purified dA2BP1 protein was incubated with radio-labeled *kn* or *dpp* probes, which have known Ci binding sites (see Suppl. text). A clear shift in mobility was observed for the *kn* probe, but not for *dpp* probe (Fig. 5A). This binding could be competed out by the addition of varying amounts of cold unlabelled probe (Fig.5B). We also compared the binding of dA2BP1 to *kn* probes that had intact Ci binding site and to those which had mutated Ci binding site. In both cases, we were able to detect shifts in mobility (Fig. 5C) indicating that, in-vitro, dA2BP1 may bind to regions other than Ci-binding region of the *kn* promoter. dA2BP1 did not bind to *dpp* sequences, suggesting sequence specificity in its binding properties. DNA-binding properties have been reported earlier for at least one RNA-binding protein; TIAR (Suswam et al., 2005). Furthermore, they have observed that the RNA recognition motifs of TIAR themselves show DNA binding activity. However, the predicted RNA-binding domain of dA2BP1 (395-476aa) alone failed to bind *kn* DNA in our electromobility shift assays (data not shown). Understanding of the mechanism by which dA2BP1 binds *kn* promoter needs further investigation involving precise molecular experiments.

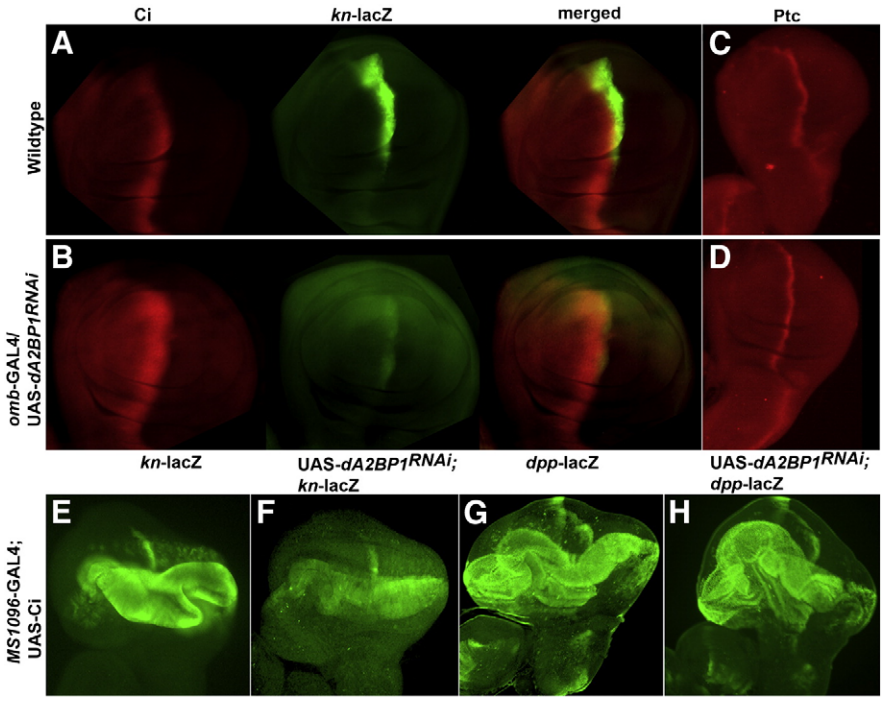

Fig. 3. Genetic interaction between Ci and dA2BP1 to regulate *kn* expression. (A-D) wildtype (A,C) and *omb*-GAL4/UAS- UAS-*dA2BP1*^{RNAi} (B,D) wing discs stained for Ci (A-B) and Ptc (C-D). Knock-down of dA2BP1 has no effect on the expression of either of the two proteins and other upstream regulators of Ci viz., En and Hh (see suppl. Fig). (E-H) *MS1096*-GAL4/+; UAS-Ci/*kn*-lacZ (E), *MS1096*-GAL4/UAS-*dA2BP1*^{RNAi}; UAS-Ci/*kn*-lacZ (F), *MS1096*-GAL4/+; UAS-Ci/*dpp*-lacZ (G) and *MS1096*-GAL4/ UAS-*dA2BP1*^{RNAi}; UAS-Ci/*dpp*-lacZ (H) wing discs stained with anti-β-galactosidase antibodies. *MS1096*-GAL4 driven ectopic activation of Ci causes ectopic expression of both *kn*-lacZ and *dpp*-lacZ in the entire dorsal compartment. However, when Ci is expressed in animals with compromised dA2BP1 function, the ectopic expression of *kn*-LacZ is considerably suppressed (F) with little or no effect on the expression of *dpp*-lacZ (H).

To test if dA2BP1 binds *kn* promoter in-vivo, we carried out Chromatin immunoprecipitation experiments (ChIP) on chromatin isolated from wildtype wing discs using antibodies against dA2BP1. Subsequently, PCR was carried out on the precipitated DNA using primers designed to a region (*kn*$^{+}$; Fig. 6) within the regulatory region used in *kn*-lacZ (Hersh and Carroll, 2005). This region includes the only known functional Ci-binding site (at Chr2R: 10699592-10699600). As described above, the expression of this reporter is under the regulation by dA2BP1 (Fig. 2B). In addition to *kn*$^{+}$ sequences, a region of *dpp*, known to harbor functional Ci-binding sites was also tested for the binding of dA2BP1. A region in the 3rd exon of *Rpl32* was used for normalizing ChIP data and a region in the 3′ end of *kn*, which doesn't not have any known or predicted Ci binding sites (*kn*$^{-}$) was used as negative control (Fig. 6). ChIP carried out using dA2BP1antibodies showed that the protein binds to *kn*$^{+}$ sequences (Fig. 6A; arrow). We did not observe binding of dA2BP1 to the 3′ region of *kn* nor to the *dpp* promoter.

As dA2BP1 has characteristic RNA recognition motif, it is possible that dA2BP1 binds to *kn* RNA at the site of transcription itself and enhances *kn* transcription by processing *kn* RNA co-transcriptionally. Such mode of gene regulation has been reported for few RNA binding proteins (Swinburne et al., 2006). This is unlikely for dA2BP1 as its effect on the regulation of *kn* expression was observed using a *kn*-lacZ reporter construct, which carries just a short enhancer fragment from a region that is nearly 5 kb away from the transcription initiation site of *kn* (Hersh and Carroll, 2005). In addition, overexpression of Kn did not affect *kn*-lacZ expression (data not shown), supporting previous reports that ruled out auto-regulation of *kn* (Crozatier et al., 2002).

dA2BP1 can suppress the dominant negative effect of Ci^{75}

ChIP was also carried out using anti-Ci antibodies, which as expected, showed specific binding to *kn*$^{+}$ and *dpp* sequences (Fig. 6B, arrows). However, knock-down of dA2BP1 did not affect the ability of Ci to bind to the promoter of *kn* (Fig. 6C). To investigate precise interactions between dA2BP1 and Ci, we expressed various deletion constructs of Ci and examined the ability of dA2BP1 to suppress the effects of over-expressed Ci on *kn* expression.

We examined the ability of UAS-*dA2BP1*^{RNAi} or UAS-dA2BP1 to modulate phenotypes caused by the over-expression of constitutively active or dominant negative forms of Ci, respectively. This is based on the assumption that they would modulate only if the two proteins interact. Ci protein has several distinct functional domains such as N-terminal repressor domain, DNA-binding Zn finger domain, proteolytic site, phosphorylation (by PKA) site and C-terminal dCBP-binding activator domain (Suppl. Fig. 6). Including wildtype Ci, we expressed as many as 10 different Ci constructs (Suppl. Fig. 6; Suppl. Table 3) and examined their effect on *kn* expression. All these constructs have been previously reported and few of them act as constitutively active Ci, while others manifest as dominant negative to endogenous Ci (Suppl. Table 3). Transgenes Ci$^{\Delta C1}$ and Ci$^{\Delta NC1}$, which have intact Zn-finger domain and dCBP-binding domain, activated *kn* expression in the entire dorsal compartment when expressed using *MS1096*-GAL4 driver (Suppl. Fig. 7). Ci^{GA1} and Ci^{GA2}, which have intact Zn finger domain fused to GAL4 activator domain (in the absence of dCBP-binding domain), also activated *kn* expression, although only in the posterior compartment (Fig. 7B). Since ectopic activation of *kn* expression by wildtype Ci is dependent on dA2BP1 function (Fig. 5F), we examined if the activity of this constitutively active Ci form is subject to modulation by dA2BP1 function. RNAi-mediated down-regulation of dA2BP1 reduced ectopic activation of *kn* caused by Ci^{GA2} suggesting that the two proteins can still interact with each other and this interaction is essential for the ability of Ci to activate *kn* expression (Fig. 7B'; Suppl. Fig. 7; Suppl. Table 3).

Ci constructs, with dCBP-binding domain deleted and with various additional deletions, did not cause ectopic activation of *kn* expression when expressed using MS1096-GAL4 driver. They, in fact, suppressed endogenous levels of *kn* expression in AB cells suggesting dominant

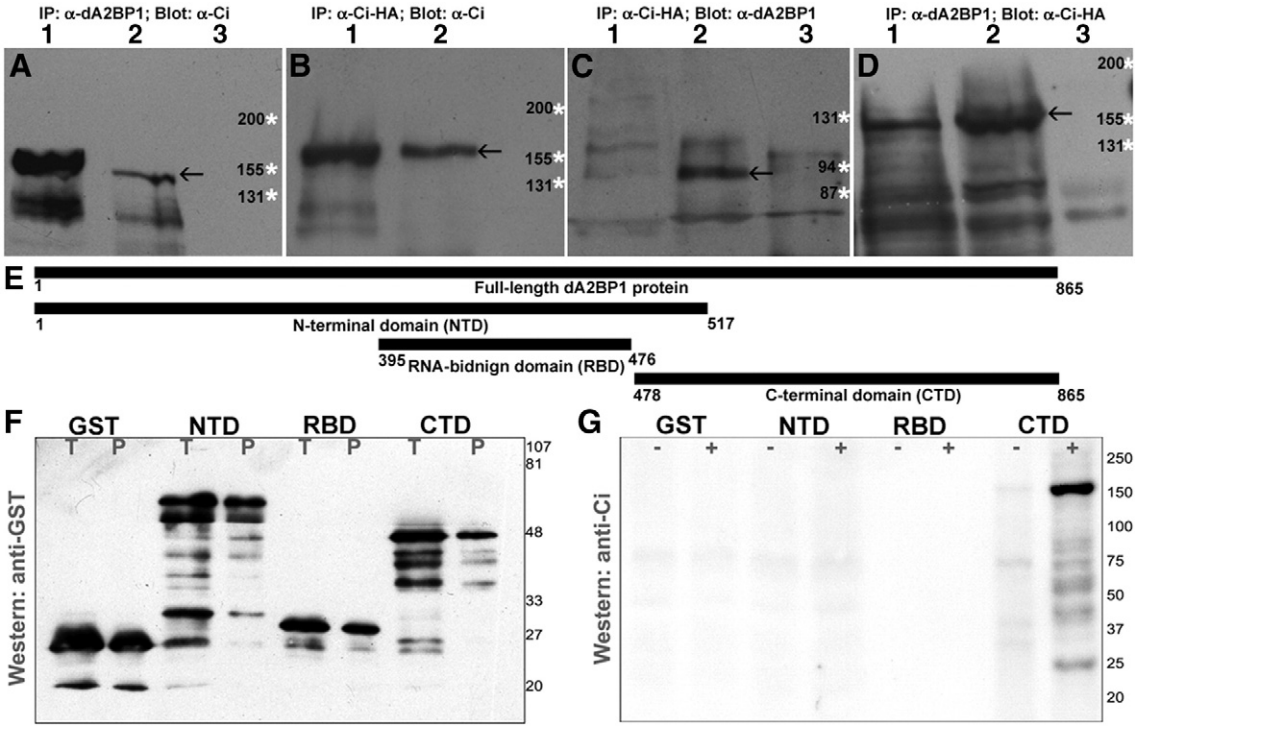

Fig. 4. dA2BP1 complexes with Ci through its C-terminal domain to regulate *kn* expression. (A-D) Results of immunoprecipitation experiments using antibodies against either dA2BP1 or Ci followed by Western blot analyses for either Ci or dA2BP1. Lane 1: Protein lysates from wing discs of appropriate genotype; Lane 2: product of immunoprecipitation experiment; Lane 3: product of mock experiment in which no primary antibodies were used. White asterix mark molecular weight markers used. (A) Western blot analyses done with anti-Ci antibodies on immunoprecipitation carried out on wing disc lysates using anti-dA2BP1 antibodies. Note co-precipitation of 155 kDa form of Ci. (B) Immunoprecipitation carried out on wing disc lysates from *omb*-GAL4/+; UAS-Ci-HA/+ larvae using anti-HA antibodies followed by Western blot analyses using anti-Ci antibodies. Anti-HA can be used to effectively to precipitate Ci-HA. (C) Immunoprecipitation using anti-HA antibodies on lysates from *omb*-GAL4/+; UAS-Ci-HA/+ larvae followed by Western blot analysis using anti-dA2BP1 antibodies. A 91 kDa band corresponding to dA2BP1 was observed. (D) Immunoprecipitation on lysates from *omb*-GAL4/+; UAS-Ci-HA/+ wing discs using anti-dA2BP1 antibodies followed by Western analysis using anti-HA antibodies. The 155 kDa form of Ci-HA was observed in the precipitate (E-G). Results of GST-pull down assays. (E) Schematic showing different constructs (not to scale) of dA2BP1 used in this experiment. dA2BP1 full-length protein (which is not used here in GST-pulled down experiments) is 865 amino-acid long. The N-terminal domain (NTD; amino acids 1-517), RNA-binding domain (RBD; amino acids 395-476) and C-terminal domain (CTD; amino acids 478-865) were expressed in *E.coli* and purified. (F) Western blot with anti-GST antibodies done to test expression of the different GST fused domains (N and C terminal domains and the RNA binding domains) of dA2BP1. T indicates total protein; P indicates purified protein. (G) Purified protein samples from (F) were immunoprecipitated using anti-GST antibodies either directly (-) or after incubation with wing disc lysate (+). The immunoprecipitated products were subjected to Western blot analyses using anti-Ci antibodies. Co-precipitation of Ci was observed only with C-terminal domain, suggesting that this domain is essential for binding to Ci.

negative effect of these constructs on endogenous Ci. Amongst these, Ci^{75} had strongest dominant negative effect, while Ci^{Cell} had minimal dominant negative effect (Fig. 7D; Suppl. Fig. 7; Suppl. Fig. 8; Suppl. Table 3). We examined if over-expression of dA2BP1 could rescue these phenotypes. Co-expression of dA2BP1 with $Ci^{\Delta C3}$, Ci^{75} or Ci^{Cell} resulted in the restoration of *kn* expression in AB cells (Suppl. Fig. 7E; Suppl. Fig. 7, Suppl. Fig. 8; Suppl. Table 3). It is likely that dA2BP1 binds to these dominant negative forms of Ci and sequester them from *kn* promoter, thereby allowing endogenous Ci to activate *kn*. However, dA2BP1 had less significant effect on the dominant negative activity of two constructs, Ci^{NZn} and $Ci^{NZn\Delta NLS}$; the latter has a partial deletion of Zn finger domain in addition to the entire C-terminal dCBP-binding domain (Fig. 7F-G; Suppl. Fig. 7, Suppl. Fig. 8; Suppl. Table 3). It is possible that these constructs, while retaining some ability to bind regulatory sequences of *kn*, have lost the domains essential for interaction with dA2BP1. Based on above-described observations with different forms of Ci, we conclude that residues 441-609aa of Ci are required for binding to the regulatory sequences of *kn* and residues 616-649 are required for interacting with dA2BP1.

Supporting this, ChIP experiments for dA2BP1, done using chromatin isolated from wing discs expressing Ci^{75}, did not affect dA2BP binding to kn^{+} (Fig. 6D). However, when $Ci^{NZ\Delta NLS}$ was expressed, binding of dA2BP1 to kn^{+} was abolished (Fig. 6E).

This was further confirmed by immunoprecipitation experiments where Ci^{GA2} and $Ci^{NZn\Delta NLS}$ were tested for their ability to bind to dA2BP1. While we were able to co-precipitate Ci^{GA2} with dA2BP1 (Fig 8A), we were unable to pull down $Ci^{NZn\Delta NLS}$ through precipitation of dA2BP1 (Fig. 8C), suggesting that residues 616-649 are indeed required for binding to dA2BP1.

Taken together, these results show that while dA2BP1 can bind to "naked" DNA in-vitro, its interaction with Ci is important for it to bind to *kn* promoter and thereby regulate *kn* expression in the wing disc.

Discussion

Hedgehog (Hh) is an important signaling molecule regulating a variety of events throughout development as well as adult life of all higher eukaryotes. All functions of Hh are mediated by Ci (Methot and Basler, 2001). This raises the question of how spatio-temporal diversity in the output of Hh pathway is established. It is generally assumed that tissue-specific factors act to regulate Ci function. So far, the only report of such factors is of Teashirt (Tsh) and Armadillo (Arm). It is believed that these two proteins complex with Ci to specify naked cuticle at distinct positions along the antero-posterior axis of *Drosophila* embryos (Angelats et al., 2002). However, it is still unclear how they might influence Ci function.

In this report, we demonstrate a role for dA2BP1 as a co-factor of Ci necessary for regulating *kn* expression during wing development. We identified dA2BP1, an ortholog of Ataxin2 Binding Protein or Fox-1, in a previous study involving identification of genes with differential expression in wing and haltere discs (Bajpai et al., 2004). We show that loss of dA2BP1 leads to loss of intervein region between L3-L4 veins of

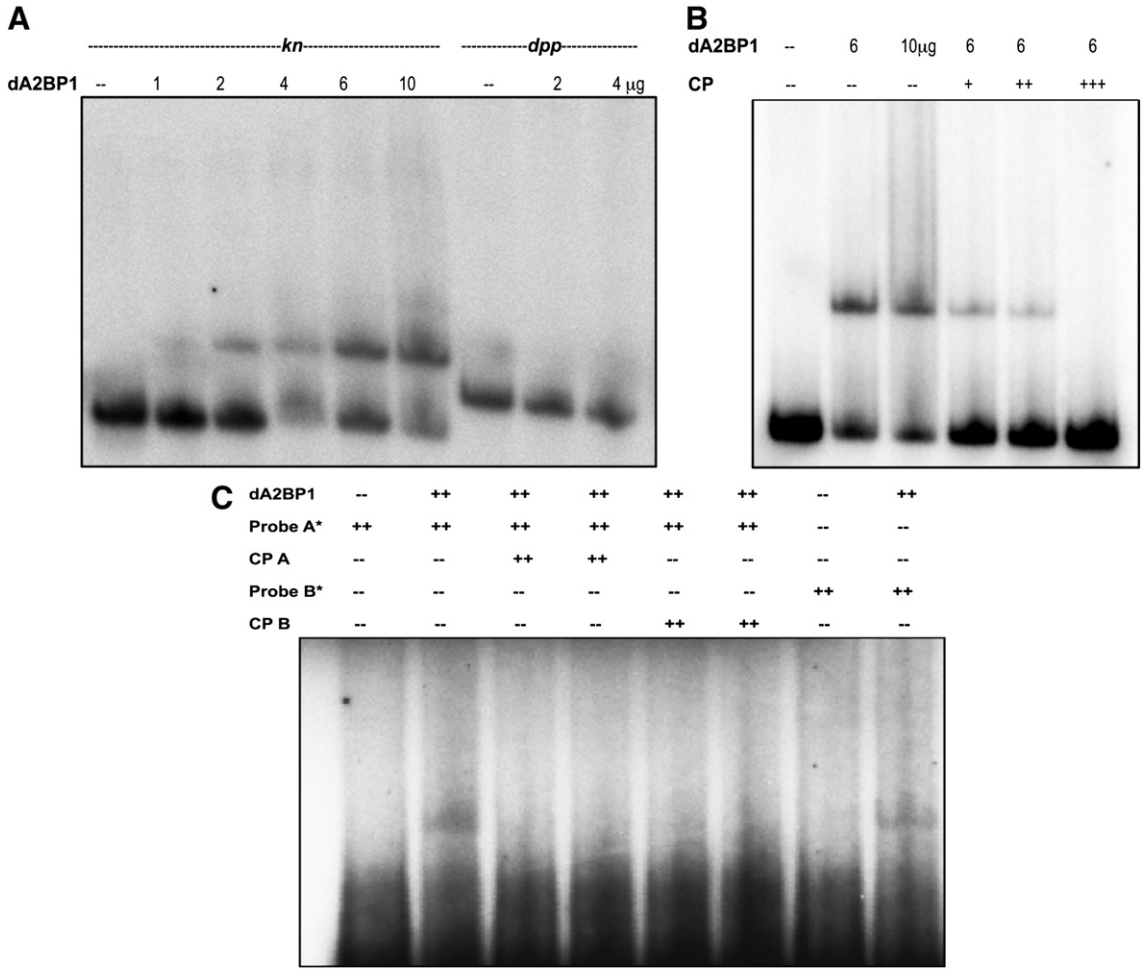

Fig. 5. dA2BP1 has DNA-binding ability. Electromobility shift assays carried out to examine DNA-binding ability of dA2BP1. (A) Different amounts of purified dA2BP1 was incubated with radio-labelled *kn* or *dpp* probes (144 bp and 191 bp long fragments as described in Suppl. Table 1), both with known Ci-binding motifs. Note, mobility shift is observed only for *kn* probe. (B) The binding of dA2BP1 to radio-labeled kn probe could be competed out using increased amount of unlabelled kn probe. + 1:50, ++ 1:100 and +++ 1:200 dilutions of labeled to cold probes. (C) dA2BP1 causes shift in the mobility of radio-labeled oligo-probes of *kn*, both with intact (Probe A) and mutated Ci-binding sites (Probe B). The binding of dA2BP1 to Probe A can be competed with cold Probe B suggesting that dABP1 may bind to regions outside the Ci-binding regions. See Suppl. Text for details of the probes used here.

adult wings- a phenotype similar to *kn* mutants. Furthermore, down regulation of dA2BP1 leads to a complete loss of *kn* RNA expression.

The role of Hh in patterning the vein and intervein regions is well established (reviewed in de Celis, 2003). *kn* has been shown to be a direct target of Hh signaling in patterning the intervein region between veins 3 and 4. Results from our experiments show that while over-expression of Ci leads to upregulation of *kn*, down-regulation of dA2BP1 can suppress this effect, suggesting that dA2BP1 is necessary for Ci-mediated *kn* expression. Immunoprecipitation experiments suggest interactions at the protein levels (which could be either direct or indirect) between dA2BP1 and the 155 kDa activator form of Ci. In-vitro, Ci (Hersh and Carroll, 2005) and dA2BP1 (this study) can independently bind to upstream sequence of *kn*; ChIP experiments done using truncated versions of Ci suggest that in-vivo binding of dA2BP1 on *kn* enhancer is dependent on Ci but not vice-versa. Thus, dA2BP1 appears to be a factor recruited by Ci to bring about transcription of *kn*. This appears to be context (or promoter-) dependent since, in the same cells that express *kn*, Ci is also known to regulate the expression of *dpp* and, this function is independent of dA2BP1.

In vivo genetic interaction studies followed by immunoprecipitation experiments with different forms of Ci suggest interactions between dA2BP1 and repressor forms of Ci too. However, it is likely that interaction between dA2BP1 and full-length activator form of Ci is important during wing morphogenesis.

The promoter-specific role of dA2BP1 leads to questions on the nature of interactions between dA2BP1 and Ci to activate *kn*, but not for the activation of *dpp*. It is possible that dA2BP1 may enhance the stability of binding and transcriptional activator function of Ci on *kn* promoter. In this context, it is to be noted that dA2BP1 binds to the Zinc finger domain of Ci, which is the main DNA binding domain. As dA2BP1 has DNA-binding properties (based on in vitro evidence), we suggest that once bound to Ci, dA2BP1 may anchor on DNA and this helps stabilizing binding of Ci to *kn* promoter. As binding of dA2BP1 on *kn*-promoter is also sequence dependent (based on the results of in vitro electromobility shift experiments: dA2BP1 binds sequences from *kn* promoter and not those of *dpp*), this property may also help in better stabilizing Ci-dA2BP1 complex on *kn* promoter, which does not have classical Ci/Gli-binding sites. Alternatively, when both Ci and dA2BP1 together bind to *kn* sequences, dA2BP1 may recruit additional factors on to the chromatin. This local chromatin modulation may help in regulating *kn* expression (Fig. 9A, B). Identification of other proteins, if any, that are part of dA2BP1-Ci complex may help us in understanding precise molecular mechanism by which dA2BP1 modulates Ci function.

dA2BP1 appears to be a complex protein with a predicted RNA-binding motif and poly-glutamine domains. RNAi-mediated knock-down of dA2BP1 using ubiquitous GAL4 drivers such as *tubulin*-GAL4, *hs*-GAL4 etc were early larval lethal, suggesting that dA2BP1 has vital

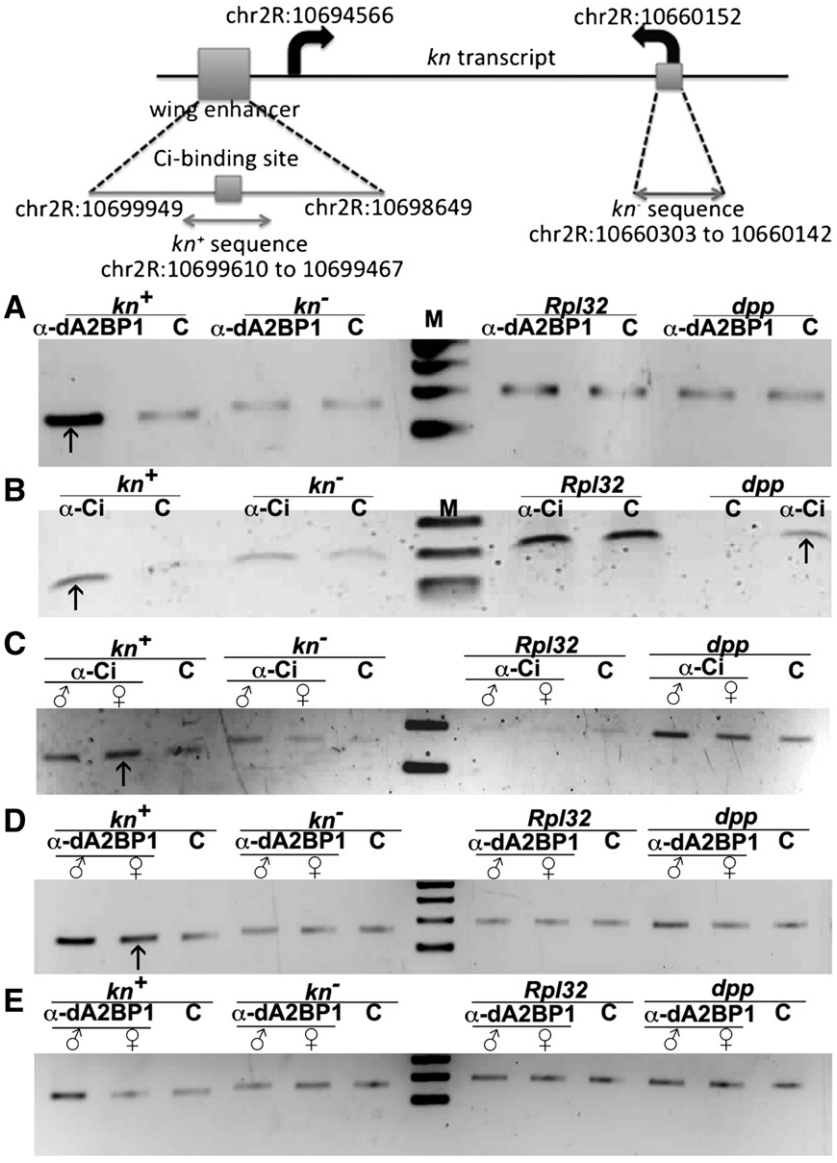

Fig. 6. Binding of dA2BP1 to kn regulatory sequences is dependent on Ci. The top panel shows kn gene region with various positions indicated. kn^+ refers to regulatory sequences of *kn* with known functional Ci binding site (at Chr2R: 10699592-10699600; Hersh and Carroll, 2005). The regions that do not have any Ci binding sites are referred to as kn^-. Regulatory sequences of *dpp* with known Ci binding sites were used as a positive control, and those of *Rpl32* were used as negative control. See Suppl. Table 1 for details of the primer sequences used. C indicated in all panels represents mock ChIP carried out in the absence of any antibodies. (A) A representative ChIP experiment using anti-dA2BP1 antibodies, on chromatin isolated from wildtype wing discs. dA2BP1 binds specifically to the kn^+ regulatory sequences (arrow), and not to kn^-, *Rpl32* or *dpp* sequences. This suggests specificity of dA2BP1 to bind to regulatory sequences of *kn* with known Ci binding sites. The results of quantitative analyses of these ChIP experiments are shown in Suppl. Table 2. (B) ChIP experiments using anti-Ci antibodies on chromatin isolated from wildtype wingdiscs. Note that Ci binds to both kn^+ sequence and *dpp* sequences. It does not bind to kn^- sequence suggesting the specificity of ChIP experiments. (C) ChIP experiments using anti-Ci antibodies on chromatin isolated from *omb*-GAL4 X UAS-$dA2BP1^{RNAi}$ wingdiscs. Since both the transgenes are on X-chromosome, dA2BP1 is knocked down only in the female progeny (♀); male (♂) larvae are normal and serve as internal controls. Reduction in the levels of dA2BP1 does not affect binding of Ci to kn^+ sequences (arrows). (D-E) ChIP experiments with anti-dA2BP1 antibodies on chromatin isolated from wing discs of *MS1096*-GAL4/+; UAS-Ci^{75}/+ (D) and *MS1096*-GAL4/+; UAS-$Ci^{NZn\ NLS}$/+ (E) larvae. The UAS transgene is expressed only in females; the male discs are used as positive control. Note binding of dA2BP1 to the kn^+ region even in the presence of dominant negative *Ci75* (D, arrow); it does not bind to kn^+ region in the presence of $Ci^{NZn\ NLS}$ (E, arrow) indicating that residues 610-1377 on Ci are important for dA2BP1 binding on the kn^+ sequence.

function during development. Its dynamic expression pattern too indicates the same. Here we have analyzed just one part of its function. It is likely that it interacts with different proteins/RNA in different tissues generating diversity in its functions.

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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.2010.02.039.

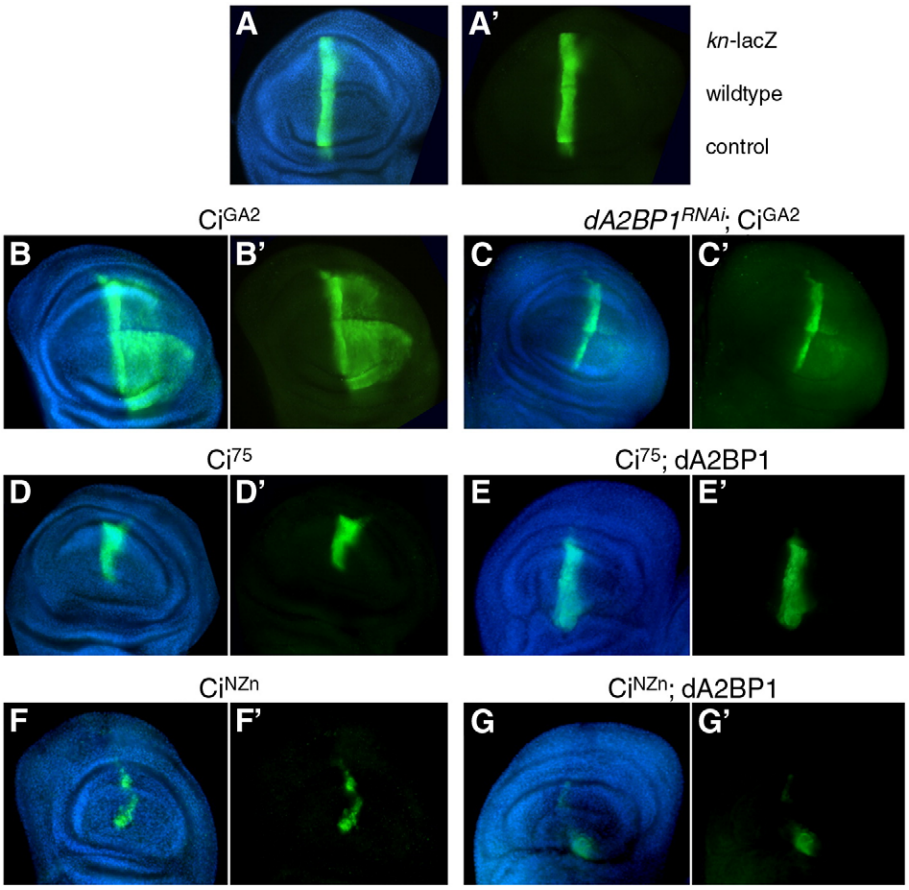

Fig. 7. dA2BP1 interacts with the C-terminal domain of Ci. All discs are stained for *kn*-lacZ (green) and DAPI (blue). (A-A') Wildtype wing disc. (B-B') Expression of a truncated form of Ci (Ci^{GA2}), which lacks the N-terminal repressor domain, but has intact Zn finger domain, causes ectopic activation of *kn*-lacZ expression, albeit only in the posterior compartment. (C-C') Activation of *kn*-lacZ caused by expression of Ci^{GA2} can be relieved by the over-expression of dA2BP1. (D-D') Expression of Ci^{75}, which lacks the entire C-terminal region, but has intact N-terminal repressor and Zn finger domains, causes repression of *kn*-lacZ expression. (E-E') Repression of *kn*-lacZ caused by expression of Ci^{75} can be relieved by the over-expression of dA2BP1. (F-F') Wing discs expressing Ci^{NZn}, which lacks the part of the Zn finger domain and the entire C-terminal domain of Ci. Expression of *kn*-lacZ is downregulated. (G-G') Co-expression of dA2BP1 fails to restore *kn*-lacZ expression in Ci^{NZn} expressing wing discs. This suggests that the non-overlapping region between Ci^{75} and Ci^{NZn} is required for binding to dA2BP1.

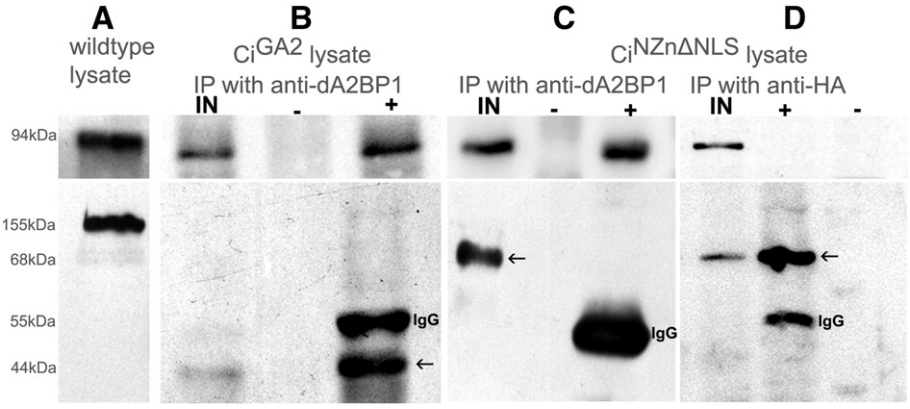

Fig. 8. Amino acids 616-649 in Ci are required for its interaction with dA2BP1. dA2BP1 was immunoprecipitated from wildtype wing discs (A), wing discs over-expressing Ci^{GA2} (B) or $Ci^{NZn\ NLS}$ (C) followed by Western blot analysis using anti-Ci antibodies. For wing discs over-expressing $Ci^{NZn\ NLS}$ immunoprecipitation was also done using anti-HA antibodies (D, $Ci^{NZn\ NLS}$ is HA-tagged) followed by Western blot analysis using anti-dA2BP1 antibodies. Top panel shows Western blot analyses using anti-dA2BP1 antibodies and bottom panel shows Western blot analyses using polyclonal anti-Ci antibodies. "IN" indicates whole protein extract of wing discs of appropriate genotype loaded directly for Western analyses; + indicates immunoprecipitation experiment done with the appropriate antibodies; - indicates a mock IP experiment done without the addition of the primary antibodies. (A) Control disc lysates showing precipitation of dA2BP1 (top panel) and co-precipitation of the 155kD Ci protein (bottom panel). (B) Wingdiscs overexpressing Ci^{GA2}- a truncated form of Ci. This form of Ci is precipitated along with dA2BP1 (arrow, bottom panel). (C) Immunoprecipitation with anti-dA2BP1 on lysates from wing discs overexpressing $Ci^{NZ\Delta NLS}$. No precipitation of Ci is observed even though the protein is detected in the "INPUT" (arrow, bottom panel). (D) Immunoprecipitation on $Ci^{NZ\Delta NLS}$ lysates using anti-HA antibodies. Co-precipitation of dA2BP1 (top panel) is not observed. However, the HA-tagged $Ci^{NZ\Delta NLS}$ protein is detected by anti-Ci antibodies (arrow, lower panel).

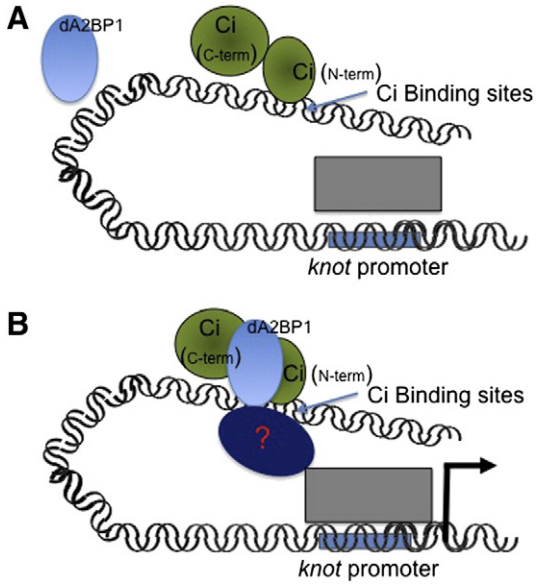

Fig. 9. A hypothetical model for dA2BP1 and Ci interactions to regulate *kn* expression. (A) dA2BP1 may directly or indirectly bind the Zinc finger domain of Ci, which is the main DNA binding domain. In the absence of dA2BP1, Ci may still bind enhancer elements of *kn*, but is unable not transactivate *kn* expression. (B) Ci would bring dA2BP1 on to the DNA and this may help stabilize binding of Ci to enhancer elements of *kn*. Alternatively, when both Ci and dA2BP1 together bind to *kn* sequences, dA2BP1 may recruit additional factors on to the chromatin. This local chromatin modulation may activate *kn* expression.

References

Alexandre, C., Jacinto, A., Ingham, P.W., 1996. Transcriptional activation of *hedgehog* target genes in *Drosophila* is mediated directly by the *cubitus interruptus* protein, a member of the GLI family of zinc finger DNA-binding proteins. Genes Dev. 10, 2003–2013.

Angelats, C., Gallet, A., Therond, P., Fasano, L., Kerridge, S., 2002. Cubitus interruptus acts to specify *naked cuticle* in the trunk of *Drosophila* embryos. Dev. Biol. 241, 132–144.

Aza-Blanc, P., Ramirez-Weber, F.A., Laget, M.P., Schwartz, C., Kornberg, T.B., 1997. Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. Cell 89, 1043–1053.

Bajpai, R., Sambrani, N., Stadelmayer, B., Shashidhara, L.S., 2004. Identification of a novel target of D/V signaling in *Drosophila* wing disc: Wg-independent function of the organizer. Gene Expr. Patterns 5, 113–121.

Blackman, R.K., Sanicola, M., Raftery, L.A., Gillevet, T., Gelbart, W.M., 1991. An extensive 3′ cis-regulatory region directs the imaginal disk expression of *decapentaplegic*, a member of the TGF-beta family in *Drosophila*. Development 111, 657–666.

Blochlinger, K., Jan, L.Y., Jan, Y.N., 1993. Postembryonic patterns of expression of *cut*, a locus regulating sensory organ identity in *Drosophila*. Development 117, 441–450.

Brand, A.H., Perrimon, N., 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118, 401–415.

Brook, W.J., Cohen, S.M., 1996. Antagonistic interactions between *wingless* and *decapentaplegic* responsible for dorsal-ventral pattern in the *Drosophila* Leg. Science 273, 1373–1377.

Calleja, M., Moreno, E., Pelaz, S., Morata, G., 1996. Visualization of gene expression in living adult *Drosophila*. Science 274, 252–255.

Capdevila, J., Pariente, F., Sampedro, J., Alonso, J.L., Guerrero, I., 1994. Subcellular localization of the segment polarity protein patched suggests an interaction with the wingless reception complex in *Drosophila* embryos. Development 120, 987–998.

Crozatier, M., Glise, B., Vincent, A., 2002. Connecting Hh, Dpp and EGF signalling in patterning of the *Drosophila* wing; the pivotal role of *collier*/*knot* in the AP organiser. Development 129, 4261–4269.

De Celis, J.F., 2003. Pattern formation in the *Drosophila* wing: The development of the veins. Bioessays 25, 443–451.

Fortini, M.E., Rebay, I., Caron, L.A., Artavanis-Tsakonas, S., 1993. An activated Notch receptor blocks cell-fate commitment in the developing *Drosophila* eye. Nature 365, 555–557.

Gustafson, K., Boulianne, G.L., 1996. Distinct expression patterns detected within individual tissues by the GAL4 enhancer trap technique. Genome 39, 174–182.

Hersh, B.M., Carroll, S.B., 2005. Direct regulation of *knot* gene expression by Ultrabithorax and the evolution of cis-regulatory elements in *Drosophila*. Development 132, 1567–1577.

Hidalgo, A., Ingham, P., 1990. Cell patterning in the *Drosophila* segment: spatial regulation of the segment polarity gene patched. Development 110, 291–301.

Kornberg, T., Siden, I., O'Farrell, P., Simon, M., 1985. The *engrailed* locus of *Drosophila*: *in situ* localization of transcripts reveals compartment-specific expression. Cell 40, 45–53.

Lawrence, P.A., Morata, G., 1976. Compartments in the wing of *Drosophila*, a study of the engrailed gene. Dev. Biol. 50, 321–337.

Lee, T., Luo, L., 1999. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. Neuron 22, 451–461.

Methot, N., Basler, K., 2001. An absolute requirement for Cubitus interruptus in Hedgehog signaling. Development 128, 733–742.

Mohler, J., Seecoomar, M., Agarwal, S., Bier, E., Hsai, J., 2000. Activation of *knot* (*kn*) specifies the 3-4 intervein region in the *Drosophila* wing. Development 127, 55–63.

Montagne, J., Groppe, J., Guillemin, K., Krasnow, M.A., Gehring, W.J., Affolter, M., 1996. The *Drosophila* Serum Response Factor gene is required for the formation of intervein tissue of the wing and is allelic to blistered. Development 122, 2589–2597.

Motzny, C.K., Holmgren, R., 1995. The *Drosophila* Cubitus interruptus protein and its role in the wingless and hedgehog signal transduction pathways. Mech. Dev. 52, 137–150.

Muller, B., Basler, K., 2000. The repressor and activator forms of Cubitus interruptus control Hedgehog target genes through common generic gli-binding sites. Development 127, 2999–3007.

Nussbaumer, U., Halder, G., Groppe, J., Affolter, M., Montagne, J., 2000. Expression of the blistered/DSRF gene is controlled by different morphogens during Drosophila trachea and wing development. Mech. Dev. 96, 27–36.

Neumann, C.J., Cohen, S.M., 1996. A hierarchy of cross-regulation involving Notch, wingless, vestigial and cut organizes the dorsal/ventral axis of the *Drosophila* wing. Development 122, 3477–3485.

Pallavi, S.K., Kannan, R., Shashidhara, L.S., 2006. Negative regulation of Egfr/Ras pathway by Ultrabithorax during haltere development in *Drosophila*. Dev. Biol. 296, 340–352.

Papp, B., Muller, J., 2006. Histone trimethylation and the maintenance of transcriptional ON and OFF states by trxG and PcG proteins. Genes Dev. 20, 2041–2054.

Patel, N.H., Martin-Blanco, E., Coleman, K.G., Poole, S.J., Ellis, M.C., Kornberg, T.B., Goodman, C.S., 1989. Expression of Engrailed proteins in arthropods, annelids, and chordates. Cell 58, 955–968.

Pignoni, F., Zipursky, S.L., 1997. Induction of *Drosophila* eye development by decapentaplegic. Development 124, 271–278.

Qi, H., Rand, M.D., Wu, X., Sestan, N., Wang, W., Rakic, P., Xu, T., Artavanis-Tsakonas, S., 1999. Processing of the notch ligand delta by the metalloprotease Kuzbanian. Science 283, 91–94.

Sanchez-Elsner, T., Gou, D., Kremmer, E., Sauer, F., 2006. Noncoding RNAs of trithorax response elements recruit *Drosophila* Ash1 to Ultrabithorax. Science 311, 1118–1123.

Shibata, H., Huynh, D.P., Pulst, S.M., 2000. A novel protein with RNA-binding motifs interacts with ataxin-2. Hum. Mol. Genet. 9, 1303–1313.

Simmonds, A.J., Brook, W.J., Cohen, S.M., Bell, J.B., 1995. Distinguishable functions for engrailed and invected in anterior-posterior patterning in the *Drosophila* wing. Nature 376, 424–427.

Suswam, E.A., Li, Y.Y., Mahtani, H., King, P.H., 2005. Novel DNA-binding properties of the RNA-binding protein TIAR. Nucleic Acids Res. 33, 4507–4518.

Swinburne, I.A., Meyer, C.A., Liu, X.S., Silver, P.A., Brodsky, A.S., 2006. Genomic localization of RNA binding proteins reveals links between pre-mRNA processing and transcription. Genome Res. 16, 912–921.

Tabata, T., Kornberg, T.B., 1994. Hedgehog is a signaling protein with a key role in patterning *Drosophila* imaginal discs. Cell 76, 89–102.

Tautz, D., Pfeifle, C., 1989. A non-radioactive *in situ hybridization* method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. Chromosoma 98, 81–85.

Vervoort, M., Crozatier, M., Valle, D., Vincent, A., 1999. The COE transcription factor Collier is a mediator of short-range Hedgehog-induced patterning of the Drosophila wing. Curr. Biol. 9, 632–639.

Von Ohlen, T., Lessing, D., Nusse, R., Hooper, J.E., 1997. Hedgehog signaling regulates transcription through cubitus interruptus, a sequence-specific DNA binding protein. Proc. Natl. Acad. Sci. U. S. A. 94, 2404–2409.

Wang, Q.T., Holmgren, R.A., 1999. The subcellular localization and activity of *Drosophila* cubitus interruptus are regulated at multiple levels. Development 126, 5097–5106.

White, R.A., Wilcox, M., 1984. Protein products of the *bithorax* complex in *Drosophila*. Cell 39, 163–171.

Xu, T., Rubin, G.M., 1993. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. Development 117, 1223–1237.