The *ash2* gene is involved in *Drosophila* wing development

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**ABSTRACT** The absent small and homeotic (*ash2*) gene is a member of the trithorax group of positive transcriptional regulators of the homeotic genes. In this work we present evidence that loss-of-function of *ash2* results in patterning alterations in the developing wing. Homozygous adults of the weak allele *ash2* develop extra cross-veins. However, clonal analysis of a stronger allele, *ash2*¹, shows that this allele results in reduction of intervein tissue and increase of longitudinal veins and cross-vein tissue in the wing except the region between vein L3 and L4. These results suggest that *ash2* function is required for both activation of intervein tissue and repression of vein tissue. Moreover, we have found that cross-vein development can be rescued in the absence of crossevenless-2 when the levels of *ash2* were reduced.

**KEY WORDS:** *ash2*, imaginal disc, vein, *Drosophila*

The non-neural wing tissue of *Drosophila* is organized in vein and intervein domains (Bier, 2000). Intervein domains are flattened cells separated by six longitudinal veins (L1-L6) and two transversal veins, the anterior and the posterior cross vein, ACV and PCV respectively. Vein development requires the EGFR/MAPK pathway to specify vein-competent domains of cells (Sturtevant et al., 1993; Sturtevant and Bier, 1995) and then restrict the domain using Notch-Delta signalling interactions, even though longitudinal veins and cross-veins form at different times during development (Marcus, 2001). Moreover, the ventral components of both longitudinal veins and cross-veins appear to require an inductive signal from the dorsal wing epithelium and both types of veins also require BMP-like signalling in order to form normally (Posakony et al., 1990; de Celis, 1997; Markus, 2001).

In this work we have studied the *I(3)112411* mutant, isolated from a third chromosomal collection of *PlacW* insertional lines (Deák et al., 1997). In this mutant the transposable element *PlacW* is inserted in a previously described gene that belongs to the trithorax group of genes (trx-G), *absent*, *small* or *homeotic discs2* (*ash2*), and is required for imaginal discs pattern formation (Shearn et al., 1987; Adamson and Shearn, 1995). Genes of the trx-G appear to be required for the maintenance and expression of homeotic selector genes of the Antennapedia and Bithorax complexes. Maintenance of the pattern of homeotic gene expression along the anterior/posterior axis requires ubiquitous expression of the Polycomb group (Pc-G) and the trithorax group (trx-G) genes and loss of function mutations in genes of either complex result in a wide array of homeotic transformation phenotypes. This suggests that the genes of the Pc-G and trx-G are transacting factors that maintain the differential expression of the Antennapedia and Bithorax complexes. Although weak homeotic transformations are evident in our mutant as described for other *ash2* alleles (Shearn et al., 1987; Lajeunesse and Shearn, 1995), in this report we present evidence suggesting a role of *ash2* in vein-intervein patterning.

In the *I(3)112411* mutant a single *PlacW* transposon was inserted in the region 96A11-16, as detected by *in situ* hybridisation on polyten chromosomes (Deák et al., 1997). Only around 12% of homozygous individuals developed normally up to sterile adults and survived for two days. These flies showed anomalous pattern of differentiation of appendages, often associated to homeotic transformations such as transformation of the first to the second thoracic segment as observed by a reduction of the sex comb teeth, a partial transformation of the haltere to wing, though with a very low frequency, and interaction between *ash2* and *Antennapedia* as observed by enhancement of the transformation phenotype of the *Antp* gain-of-function allele (results not shown). However, the most striking phenotype observed was that the wings contain supernumery cross-veins (from 1 to 5), in addition to the anterior and posterior cross-veins, with 95% penetrance (Fig. 1A-C). Differentiation elements such as longitudinal veins were normally distributed although an overall reduction of wing size was also observed. The formation of the extra cross-veins occurred in two specific territories of the wing (Fig. 1D). One was the proximal region between vein L2 and vein L3.
and the other occupied the proximal region between L4 and L5. The strongest wing phenotype was sometimes observed and consisted of notches along the posterior margin, wider veins L3 and L5 and irregularly distorted vein L2, in addition of reduction of wing size and extra cross-veins (Fig. 1C).

Confirmation that PlacW insertion caused the mutant phenotype was obtained by reversion of the mutant phenotype after the remobilisation of the PlacW transposon as described by Török et al. (1993). Viable revertants representing precise excision of PlacW were obtained showing normal morphology and viability, indicating that the PlacW insertion itself was responsible for the mutant phenotype observed in l(3)112411 homozygotes.

Three non-viable revertants were also isolated. The allele I1 is early pupal lethal whereas the alleles I2 and I3 are semilethals. Heteroallelic combinations of l(3)112411/I1 individuals were pupal lethals. However, some individuals (frequency <1%) of the allelic combination l(3)112411/I1 reached adult/pharate stage. In those flies, extra cross-veins were found, wing size was reduced and vein L2 distorted (data not shown).

DNA flanking both sides of the PlacW element were cloned by plasmid rescue and by direct sequencing of the recovered DNA fragments we identified that the transposon was inserted in the fourth intron of the ash2 gene and molecular alterations detected in the ash2 allele.

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absent, small or homeotic discs2 (ash2) gene (Fig. 1E). For the rest of this work we used l(3)112411 (hence renamed ash2172411) as a weak allele and the non-viable revertant I1 (renamed ash211) as the strongest allele.

This ash2 allele fails to complement the previously described allele ash2 (Shearn, 1989). The combination of the PlacW insertion ash2 and the ash2 allele also fails to complement, although some individuals reached adult stage with extra cross-veins located in the same regions shown in Fig. 1D. Similarly we found that the combination of ash2 and Df(3R)XS, a deficiency covering the ash2 locus, results in wings with extra cross-veins and a weak reduction in size.

In order to identify the alteration present in the ash2 allele, we sequenced the entire genomic region encompassing the coding part of the ash2 gene and the only changes detected were a two base pair deletion (GT) and an insertion of 5 nucleotides (TTAGG) in the fourth intron (Fig. 1E). Although this small insertion may create a putative new acceptor splice site in ash2, we cannot discard other modifications in the promoter region. However, the alterations observed in the intron of ash2 allele were not found after sequencing the equivalent region of the original l(3)112411 line, suggesting that they are caused by the excision of the PlacW.

Because of the pronounced extra-cross-vein phenotype, we tested genetic interactions with a mutant that specifically removes cross-veins to study suppression or enhancement of the phenotype. The crossveinless class of mutations in Drosophila is characterised by the lack of cross-veins (García-Bellido and de Celis, 1992). The crossveinless 2 gene (cv-2) belongs to the loss of veins phenotypic group of mutants that affect the wing vein patterning. We used one allele of this second chromosome crossveinless-2 gene (Conley et al., 2000) to test for genetic interactions with our ash2 alleles. Adult wings cv2/ cv2; ash2172411/TM6c showed rescue of the normal cross-vein pattern (Fig. 2A,B). Wings were normal sized and normal ACV and PCV link veins L3 and L4 and L4 and L5 respectively, as found in the wild type. Similarly cv2/ cv2; ash211/ TM6c restored normal venation and wing size was normal (Fig. 2C). Moreover, wings of cv2/ cv2; ash2172411 / ash2112411 individuals showed extra cross-veins and
reduction of wing size (Fig. 2D). The experiments shown here demonstrate that cross-vein development can be rescued in the absence of cv-2 when the levels of ash2 are reduced. These results suggest that ash2 acts as a repressor, directly or indirectly, of normal cross-vein formation in the wing territories where cross-veins can be formed.

To investigate whether the role of ash2 in wing development is restricted to cross-vein formation or alternatively to a more generalized role on vein-intervein patterning, we have generated large clones of the lethal ash2 allele by mitotic recombination in a Minute background. In our conditions these clones cover extensive vein and intervein territories, therefore allowing us to compare the effects on both tissues. Clones generated at 60±12h AEL (n=18) can cover most of a compartment (Fig. 3A) and clones generated at 80h±12 AEL (n=40) can partially cover a vein and adjacent intervein domains (Fig. 3E). The wings bearing these clones were distorted, show blistering and the intervein region is strongly reduced (Fig. 3 A-E). In early clones that cross the dorso-ventral boundary, both the dorsal and ventral intervein regions were reduced (Fig. 4A). In clones located in either dorsal or ventral regions, the clone-bearing intervein region was reduced whereas the opposite intervein region showed a ballooned shape. This reduction of the mutant region was evident in spite of the proliferative advantage that have the mutant cells over the Minute background and suggests a failure in proliferation and adhesion between the dorsal and ventral layers (Fig. 3B). Before mounting, this wing showed a large blister, possibly due to the reduction of the dorsal compartment, where the clone runs, and therefore affecting the dorsal-ventral apposition of the wing tissue.

In addition to that, the ash2 clones resulted in thickening of the vein tissue when the mutant cells run over a vein (Fig. 3). In these clones, the corrugation of the veins (i.e. ventral or dorsal bulging characteristic of each vein) was arranged as in the wild type and resulted in a severe thickening of the vein tissue at the same wing surface where the clone was located. Veins were enlarged with the exception of vein L4, which even in the presence of a clone on the corresponding corrugation side, was not affected (data not shown). Clones that cross the dorso-ventral boundary disrupted the morphology of border cells, as detected by anomalous arrangement of bristles of the wing margin (Fig. 3 A-C). In few cases, ectopic vein tissue developed in wild type cells adjacent to mutant clones, suggesting a non-autonomous effect (Fig. 3D). We also have observed extra cross-veins connecting normal and ectopic longitudinal veins (Fig. 3E).

In contrast to the above results, those clones located in the intervein region between veins L3 and L4 differentiated in intervein tissue, did not form blisters and at the most slight reduction of the area was observed (data not shown). It has been shown that his zone is resistant to ectopic vein formation in some excess vein mutants, but not in mutants of the knot group (Diaz-Benjumea and Garcia-Bellido, 1990). This region is under the control of the organizer specific genes that respond to hedgehog signalling (Tabata and Kornberg, 1994; Zecca et al., 1995). One of the hedgehog responsive genes is knot, which specifies the L3-L4 intervein region and suppresses expression of EGF receptor (Mohler et al., 2000). The expression of knot in the region between L3 and L4 specifies a vein free zone and the L3 and L4 veins are formed outside this zone. Therefore, we propose that in the L3-L4 region intervein development and repression of vein initiation genes does not depend on ash2 function. In contrast ash2 would operate in wing patterning of regions beyond the L3-L4. Early function of dpp in the central region of the wing triggers the expression of target genes that position the interveins and longitudinal veins (de Celis and Barrio, 2000). Therefore it is also possible that besides a role in intervein/vein differentiation ash2 has an early

**Fig. 3. Clonal analysis of the ash2 allele in a Minute background.** (A) Anterior clone, which covers most of the anterior compartment, in both dorsal and ventral sides. Note the thickness of the vein tissue at the same wing surface where the clone was located. Veins were enlarged with the exception of vein L4, which even in the presence of a clone on the corresponding corrugation side, was not affected (data not shown). Clones that cross the dorso-ventral boundary disrupted the morphology of border cells, as detected by anomalous arrangement of bristles of the wing margin (Fig. 3 A,C). In few cases, ectopic vein tissue developed in wild type cells adjacent to mutant clones, suggesting a non-autonomous effect (Fig. 3D). We also have observed extra cross-veins connecting normal and ectopic longitudinal veins (Fig. 3E).

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role in the regulation of some of those dpp target genes and therefore in the commitment of the intervein/vein patterning. In summary, ash2\textsuperscript{2f} mutant clones do not only result in extra cross-veins but also more efficiently result in reduction of the wing size and transformation of intervein cells in vein tissue. From this work we conclude that ash2 has a role in vein-intervein patterning. The extra cross-veins of the ash2\textsuperscript{112417} insertion line may be a consequence of this weak allele being able to trigger differentiation of vein tissue in regions that enable cross-vein formation, although this residual ash2 function may be enough to avoid more severe vein phenotypes.

**Experimental Procedures**

**Drosophila Stocks and Clonal Analysis**

Wild type and mutant strains were maintained on standard medium and all experiments were performed at 25°C. The I(3)1124/11 mutant line was obtained from a collection resulting from a PlacW insertional mutagenesis on the third chromosome (Deak et al., 1997). PlacW revertants were induced using the \( \Delta^2 \)-2 transposase. The ash2\textsuperscript{2f} allele was kindly provided by Dr. A.Shearn. The cell marker forked (\( f^{R+}, 1-56.7 \)) was used for clonal analysis. We used \( f^{R+}, mwh t^{(O)M(3)j\alpha 124}/ash2^{2f} \) to generate Minuter clones. Mitotic recombination was induced by X-rays from a Philips X-ray source operating at a dose of 10 Gy (100 kV, 15 mA, 2 mm Al filter). Larvae were timed in hours after egg laying (AEL) and clones were recovered by plasmid rescue (Pirrotta 1986). DNA from heterozygous \( l(3)112411/TM6B \) ash2\textsuperscript{1} insertion were recovered by plasmid rescue (Pirrotta 1986). DNA isolation and analysis were performed according to standard protocols. Genomic DNA fragment representing both sides of the PlacW insertion were recovered by plasmid rescue (Pirrotta 1986). DNA from heterozygous \( l(3)112411/TM6B \) flies was digested with EcoR1 or BamH1, ligated and transformed into Ecorpiricus Coli XL-2 Blue ultra competent cells. The insertion site of the PlacW in the genomic sequence was determined in separate experiments at 60 h AEL. Adult flies of appropriate genotype were dissected out and their wings mounted in lactic acid/ethanol (1:1) for microscopy.

**Nucleic Acid Procedures**

DNA isolation and analysis were performed according to standard protocols. Genomic DNA fragment representing both sides of the PlacW insertion were recovered by plasmid rescue (Pirrotta 1986). DNA from heterozygous \( l(3)112411/TM6B \) flies was digested with EcoR1 or BamH1, ligated and transformed into Ecorpiricus Coli XL-2 Blue ultra competent cells. The rescued clones were analysed by restriction digestion and direct sequencing of the clones that contain PlacW sequences and flanking DNA was performed using a PlacW inverted repeat probe (28 mer) as a primer. The insertion site of the PlacW in the genomic sequence was determined after sequencing the rescued plasmids with two oligonucleotide primers located inside the \( \text{PlacW} \): 5’TACACTGCGCACTTATGCA AGCAGACG’3 for the EcoR1 rescue and 5’ACACAACCTTCTCTCAACAA’3 for the BamH1 rescue. All sequencing reactions were performed using a Perkin Elmer sequencing kit in an automated sequencer ABI PRISM 377. Data analysis was performed using the Sturtevant\textsuperscript{53}: a practical approach. IRL Press, 334-351.

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**References**


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