Activation and repression activities of *ash2* in *Drosophila* wing imaginal discs

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Summary

Polycomb (PcG) and trithorax (trxG) group genes are chromatin regulators involved in the maintenance of developmental decisions. Although their function as transcriptional regulators of homeotic genes has been well documented, little is known about their effect on other target genes or their role in other developmental processes. In this study, we have used the patterning of veins and interveins in the wing as a model with which to understand the function of the trxG gene *ash2* (*absent, small or homeotic discs 2*). We show that *ash2* is required to sustain the activation of the intervein-promoting genes *net* and *blistered* (*bs*) and to repress *rhomboid* (*rho*), a component of the EGF receptor (*Egfr*) pathway. Moreover, loss-of-function phenotypes of the *Egfr* pathway are suppressed by *ash2* mutants, while gain-of-function phenotypes are enhanced. Our results also show that *ash2* acts as a repressor of the vein L2-organising gene *knirps* (*kni*), whose expression is upregulated throughout the whole wing imaginal disc in *ash2* mutants and mitotic clones. Furthermore, *ash2*-mediated inhibition of *kni* is independent of *spalt-major* and *spalt-related*. Together, these experiments indicate that *ash2* plays a role in two processes during wing development: (1) maintaining intervein cell fate, either by activation of intervein genes or inhibition of vein differentiation genes; and (2) keeping *kni* in an off state in tissues beyond the L2 vein. We propose that the Ash2 complex provides a molecular framework for a mechanism required to maintain cellular identities in the wing development.

Key words: *ash2*, Cellular memory, Imaginal disc, *knirps*, *Drosophila*

Introduction

Differential gene expression results in cell diversity, although how different cell identities are established early in development and maintained throughout life is still poorly understood. Most of the transcription factors required for early developmental decisions are expressed transiently, but the gene expression patterns they trigger are maintained during cell division and inherited by daughter cells. Actively dividing cells must preserve individual genes in an on or off expression state after an initial commitment is made, especially given that some regulators disassemble from promoters during DNA replication or mitosis. Thus, developmental decisions may be maintained by the ability to deposit epigenetic marks involving chromatin-modifying complexes to control the cellular memory of gene activity states (Francis and Kingston, 2001; Simon and Tamkun, 2002).

Genes of the Polycomb (PcG) and trithorax group (trxG) encode proteins that are engaged in the regulation of cellular memory (Orlando, 2003). In early *Drosophila* embryonic development, Hox gene expression is controlled by a genetic cascade that includes the segmentation genes (Simon, 1995). When the segmentation proteins decay, Hox expression is maintained in the correct spatiotemporal pattern by the action of PcG and trxG genes, which often act as transcriptional repressor- and activator-chromatin complexes (Francis and Kingston, 2001; Simon and Tamkun, 2002). In addition to Hox genes, PcG/trxG also act on other target genes (Beltran et al., 2003; Francis and Kingston, 2001). In a genome-wide prediction of PcG/trxG response elements (PRE/TRE) in *Drosophila*, more than 100 elements were identified that mapped to genes involved in development and cell proliferation (Ringrose et al., 2003). However, epigenetic marks are not only restricted to embryonic stages. At later stages, developmental fates are also frozen and inherited by the repressor and activator activities of PcG/trxG complexes. The *Drosophila* wing imaginal disc has proven to be a useful model with which to study how these complexes act to maintain cell identities, as shown for *wingless* (*wg*) and *hedgehog* (*hh*) pathways (Collins and Treisman, 2000; Maurange and Paro, 2002).

The non-neural tissues of the *Drosophila* wing are organised into two types: the A-E intervein regions and the L1-L6 veins (Fig. 1A). The specification of veins in the wing imaginal disc occurs during larval and pupal stages, and is controlled by a network of cell-to-cell interactions, including the *Egfr* signalling pathway (Diaz-Benjumea and Hafen, 1994). The *rho* gene, which encodes a seven-pass transmembrane serine protease, is an activator of *Egfr* (Bier et al., 1990; Sturtevant et al., 1993). *rho* is expressed in rows of cells coinciding with vein primordia and is required for vein formation, as indicated by the observation that the loss-of-function allele rho^{ve} displays truncated veins (Diaz-Benjumea and Garcia-Bellido, 1990; Sturtevant et al., 1993). Localised expression of *rho* and *vein* (*vn*), which encodes a diffusible neuregulin class of

ligands, activates the Ras/MAPK signalling cascade necessary for vein differentiation (Sturtevant et al., 1993; Schnepp et al., 1996). By contrast, inhibition of Egfr signalling by the transcription factors *blistered* (*bs*) and *net* is responsible for intervein specification. *bs*, the *Drosophila* homologue of the Serum Response Factor, is expressed in a pattern associated with intervein regions and is required for the organisation and differentiation of intervein cells (Fristrom et al., 1994; Montagne et al., 1996). During disc proliferation, *bs* expression is independent of *rho*, but during the pupal period *bs* and *rho* expression become mutually exclusive (Roch et al., 1998). The *net* gene, which encodes a basic helix-loop-helix (bHLH) protein, is also expressed in the intervein regions (Brentrup et al., 2000). In contrast to *bs*, *net* and *rho* expression is mutually exclusive in the wing discs of third instar larvae. Lack of *net* activity causes *rho* expression to expand, and vice versa. Furthermore, ectopic *rho* expression results in repression of *net*, thus generating wings with ectopic vein tissue (Brentrup et al., 2000).

The refined localisation of the L2-L5 veins in the wing depends on positional cues established along the AP axis of the wing imaginal disc (Biehs et al., 1998; de Celis et al., 1996; Sturtevant et al., 1997; Sturtevant and Bier, 1995). The posterior compartment cells express *engrailed* (*en*), which activates *hh*. Hh diffuses through a few cell rows of the anterior compartment, activating *vn*, *knot* (*kn*) and *decapentaplegic* (*dpp*), which form the AP organising centre (Bier, 2000). Vn activates *Egfr* in the borders of the organiser, giving rise to the L3 and L4 vein primordia, while Kn prevents the domain between L3 and L4 responding to vein differentiation, ensuring the intervein fate of this region (Crozatier et al., 2002; Mohler et al., 2000). Dpp diffuses from the AP boundary and activates target genes in a threshold-dependent fashion (Lawrence and Struhl, 1996). The development of L5 is dependent upon the two abutting Dpp target genes, *optomotor-blind* (*omb*) and *brinker* (*brk*) (Cook et al., 2004). Another Dpp target is the *spalt-major* (*salm*)*/spalt-related* (*salr*) complex (*sal-C*) of zinc-finger transcription factors (de Celis et al., 1996; Lecuit et al., 1996; Nellen et al., 1996), which is expressed in the central domain of the wing. The anterior low *sal-C*-expressing domain promotes the activation of the *knirps* (*kni*)*/knirps-related* (*knrl*) complex (*kni-C*), which results in L2 specification (Lunde et al., 1998).

Ash2 is a trxG protein that belongs to a 0.5 MDa complex thought to be involved in chromatin remodelling (Papoulas et al., 1998). Ash2 accumulates uniformly in imaginal discs, fat body cells and salivary glands (Adamson and Shearn, 1996). Loss-of-function alleles of this gene cause homeotic transformations (LaJeunesse and Shearn, 1995; Shearn, 1974; Shearn et al., 1987; Shearn, 1989; Shearn et al., 1971) and downregulation of Hox genes (Beltran et al., 2003; LaJeunesse and Shearn, 1995), in addition to severe abnormalities in the wing, such as reduction of intervein and enhancement of vein tissues (Adamson and Shearn, 1996; Amorós et al., 2002). To gain more insight into the function of *ash2*, we examined whether vein- and intervein-specific genes and vein positioning genes act as putative targets of *ash2* function. We found that *ash2* is involved in activating intervein-promoting genes and downregulating the Egfr pathway. Moreover, *ash2* also acts as a *kni* repressor independently of *sal-C*. These results strongly support a role for *ash2* in maintaining vein/intervein developmental decisions and vein patterning in the developing wing.

Materials and methods

Drosophila strains

We used the following genetic strains as *ash2* alleles: *yw; ash2^{112411}/TM6C* (Deak et al., 1997; Amorós et al., 2002) and *yw; ash2^{I1}/TM6C* (Amorós et al., 2002; Beltran et al., 2003*). Canton S* was used as a wild-type strain. To study interactions between *ash2* and *Egfr* signalling pathway elements, we tested the hypomorphic combination *rho^{ve}vn^{1}*, *top^{1}/top^{3C81}* (Clifford and Schupbach, 1989; Diaz-Benjumea and Garcia-Bellido, 1990), the gain-of-function allele of Egfr *Elp^{B1}/CyO* and the *rolled* gain-of-function mutation *D-Raf^{C110}; rl^{Sem}* (provided by A. García-Bellido). In order to eliminate the *D-Raf^{C110}* allele, crosses were designed to score the male *+/Y; rl^{Sem}/+; ash2^{I1}/+* progeny. The alleles *bs^{03267}/CyO* (provided by M. Affolter), *E(spl)mβ-lacZ* (provided by S. Bray) and *net^{1}* were used as intervein markers; the stock *w; h kni^{ri-1}* was used to analyse L2 development. To study the effects of *ash2* on *kni* expression, the minimal L2-enhancer element *EX-lacZ*, a 1.4 kb fragment that contains an activation and repression domain of the *kni* gene (Lunde et al., 2003) was provided by E. Bier. For ectopic expression of *sal-C*, we used the *UAS-sal^{64d}* transgenic, on the first chromosome, the *UAS-salr^{8}* transgenic, on the second chromosome, and the *nubbin-Gal4* insertion line (provided by J. F. de Celis). We also analysed whether *ash2* regulates *brk* and *scalloped* (*sd*) expression by using the *brk^{X47}-lacZ* transgene (provided by G. Morata) and the *sd^{EXT4}* stock. The stocks *Elp^{B1}/Cyo*, *net^{1}*, *w; h kni^{ri-1}* and *sd^{EXT4}* mentioned above were obtained from the Bloomington Stock Center.

Genetic mosaics

Clones mutant for *ash2^{I1}* were obtained by mitotic recombination using the *FLP/FRT* technique (Xu and Rubin, 1993). *yw;FRT82Bash2^{I1}/TM6C* flies were crossed with *ywhsflp;FRT82BGFP/TM6B* and wing imaginal discs from third instar Tubby^{+} larvae and pupae were dissected. Heat shock was carried out for 30 minutes at 37°C [52±4 hours after egg laying (AEL)] to induce clone formation. To monitor *EX-lacZ* (Lunde et al., 2003) expression, a *yw; EX-lacZ; FRT82Bash2^{I1}/TM6C* stock was created and clones were induced as above.

Overexpression of *sal-C* was obtained by crossing *UAS-Sal^{64d}; FRT82Bash2^{I1}/+* males with *ywhsflp; nubbin-Gal4; FRT82BGFP/TM6B* females. Tubby^{+} female larvae were dissected. To monitor *brk* and *sd* expression, *brk^{X47}-lacZ; FRT82Bash2^{I1}/+* males and *sd^{EXT4}-lacZ; FRT82Bash2^{I1}/+* males were crossed to *ywhsflp;FRT82BGFP/TM6B* and Tubby^{+} female larvae were dissected. In both cases, only 50% of the progeny contained *ash2^{I1}* clones.

To obtain *Minute*$^{+}$ clones the stock used was *yw; FRT82B arm-lacZ M(3)/TM6C* and heat shock was carried out for 7 minutes at 34°C (110±4 hours AEL). Adult *ash2* mutant *FLP/FRT M*$^{+}$ clones marked with the forked mutation were analysed in males with the following genotype: *ywhsflpf^{36a}; FRT82BP[f^{+}]87DM(3)w[124]/ FRT82Bash2^{I1}*. The heat shock was carried out for 10 minutes at 37°C (80±12 hours AEL).

Larvae and pupae of the appropriate genotypes were cultured at 25°C and timed in hours AEL or after puparium formation (APF).

Immunohistochemistry

Immunohistochemistry was performed according to standard protocols. Primary antibodies used were: guinea pig anti-Kni (1/50), provided by J. Reinitz; rabbit anti-Salm (1/500), provided by R. Barrio; rat anti-Bs (1/200), provided by M. Affolter; rabbit anti-Plexus (1/1000), provided by H. Matakatsu; rabbit anti-Vestigial (1/20), provided by S. Carroll; mouse anti-En (1/25) from the Developmental

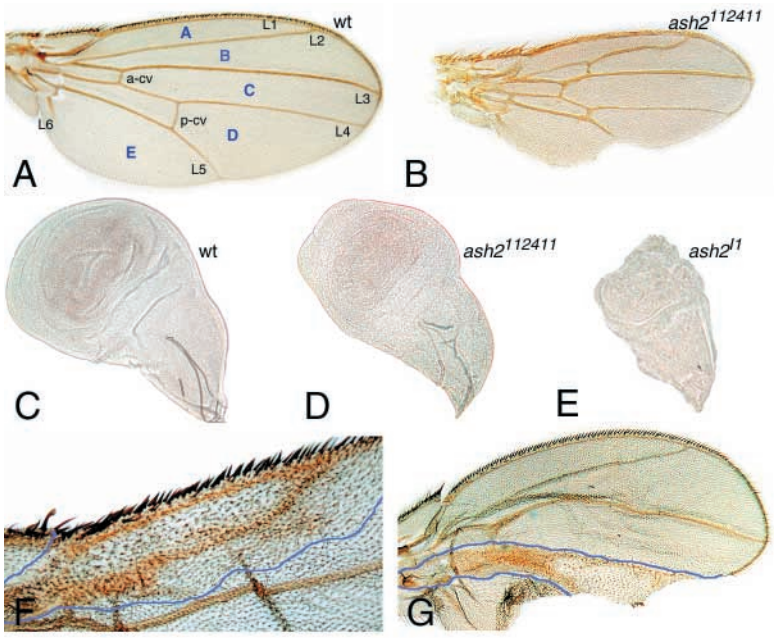

Fig. 1. Wing phenotypes of *ash2* alleles. (A) Wild-type (wt) adult wing. Intervein regions (A-E, in blue), longitudinal veins (L1-L6) and anterior (a-cv) and posterior (p-cv) crossveins are marked. (B) Adult wing of $ash2^{112411}$ homozygous mutant at the same magnification as A, showing anomalous L2 in which the distal half is crooked, ectopic crossvein tissue between L2-L3 and L4-L5, and a notch in the posterior margin. (C) Wing disc of wild-type third instar. (D) Wing disc of homozygous $ash2^{112411}$ third instar. (E) Wing disc of homozygous $ash2^{I1}$ third instar. (F,G) Mitotic clones homozygous for $ash2^{I1}$ in *Minute* background. (F) Ventral clone where L2 appears thickened and intervein region A is reduced. (G) Dorsal clone showing ectopic vein tissue between the proximal region of L4-L5.

Studies Hybridoma Bank of the University of Iowa; and rabbit anti-β-galactosidase (Cappel) (1/1000). Kni, Salm and Vestigial antibodies were pre-absorbed before use.

Secondary antibodies were obtained from Jackson Immuno Research and include: donkey anti-rat-Rhodamine Red (1/200), donkey anti-guinea pig-Cy5 (1/400), goat anti-mouse-FITC (1/200) and donkey anti-rabbit-Rhodamine Red (1/200). Propidium Iodide (Molecular Probes) was used as a nuclear marker after RNAase treatment. Fluorescence was visualised with a Leica TCS confocal microscope.

Whole-mount in situ hybridisation with larval wing discs and pupal wings

In situ hybridisation using digoxigenin-labelled antisense RNA probes was carried out essentially as described previously (Sturtevant et al., 1993; Brentrup et al., 2000).

DIG-labelled riboprobes for *net* RNA were synthesised using a 2.2 kb insert of netcelΔ922 (gift of M. Noll) linearised with *Eco*R1, and for *rho* from a *rho* cDNA clone (gift of E. Bier) linearised with *Hin*dIII. Sense RNA probes for *net* and *rho* did not show detectable signal.

RT-PCR

Total RNA from wild-type and $ash2^{I1}$ homozygous larvae was extracted using Trizol (GibcoBRL) and a poly(dT)-24 primer was used for cDNA synthesis. The reaction was carried out in a final volume of 25 μl with five units of avian myeloblastosis virus-RT (Promega) and 200 units of Moloney murine leukaemia virus RT (Gibco). One microlitre of the RT reaction was used for PCR. The specific primers used [5′-cgccgccctgcccttcttc-3′ (forward) and 5′gggctgctgctagtcggagtggt 3′ (reverse)] were designed to amplify a 369 bp product of the *kni* gene.

Results

ash2 is required to downregulate Egfr activity

The $ash2^{112411}$ mutation is a single *PlacW* insertion (Deak et al., 1997) in the fourth intron of the *ash2* gene (Amorós et al., 2002) that causes pharate lethality. Homozygous flies that reach the adult stage (12% at 25°C) are sterile and show reduced wing size, crooked L2 and ectopic vein tissue, mainly extra crossveins and thicker veins (Fig. 1A,B). The $ash2^{I1}$ allele was generated after an imprecise excision of the $ash2^{112411}$ insertion and is lethal in late third instar/early pupae. Molecular analysis of the $ash2^{I1}$ mutation has shown that it comprises a 2 bp deletion and a 5 bp insertion that result in the absence of the full-length 2 kb transcript (Beltran et al., 2003). Imaginal discs of both alleles are reduced in size, $ash2^{I1}$ being smaller than $ash2^{112411}$ (Fig. 1C-E). The smaller size and abnormal shape suggests that the $ash2^{I1}$ mutation alters proliferation and patterning. Clones homozygous for $ash2^{I1}$ exhibit impaired proliferation, intervein reduction and extra vein tissue, preferentially close to the normal veins, which appear thickened (Amoros et al., 2002) (Fig. 1F,G). This phenotype is a consequence of intervein cells acquiring morphological features of vein cells, which are typically smaller, more pigmented and with shorter and thicker trichomes than wild-type intervein cells. This extra vein phenotype led us to question whether *ash2* functions as a negative regulator of vein differentiation in intervein territories. To test this hypothesis, we assessed genetic interactions with alleles of genes involved in vein/intervein development.

We perturbed the Ras/MAPK signalling pathway in the wing using mutants of genes required for Egfr activation. We first analysed loss-of-function mutants of the pathway. In flies mutant for the hypomorphic viable combination rho^{ve} vn^{1}, activation of the MAPK pathway in presumptive vein cells is prevented and, as a consequence, veins fail to differentiate. By contrast, the triple mutant rho^{ve} vn^{1} $ash2^{112411}$ develops veins (Fig. 2A,B). We observed varying degrees of rescue, ranging from wings that develop only L2 to wings that develop veins almost completely, even with extra crossvein tissue or proximal vein fusions between L2-L3 and L4-L5. Rescue of L2 and L5 is more pronounced than L3 and L4, which are never distally complete, and many wings show notches in the posterior wing margin (77% of cases, n=75 wings; Fig. 2B). In 25% of these cases, wings show a tube-like shape, possibly owing to

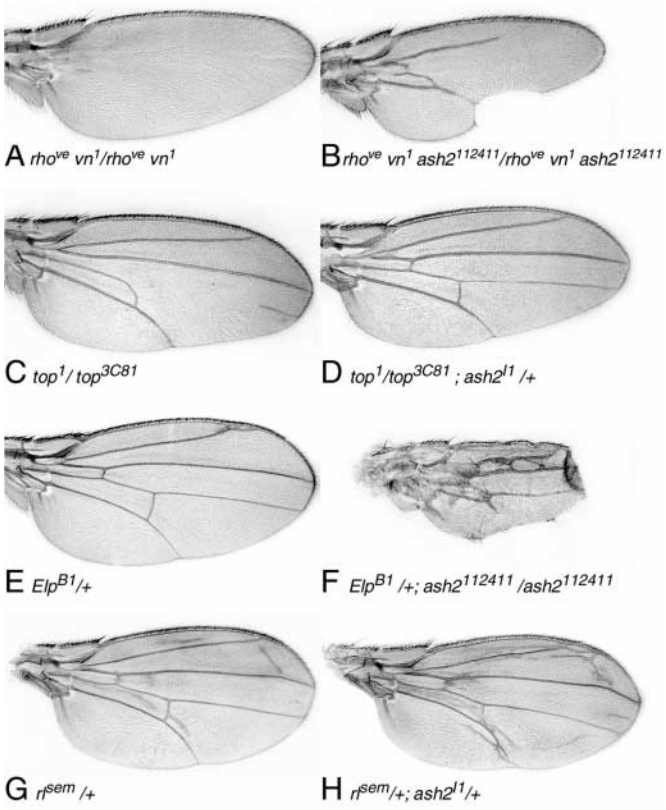

Fig. 2. *ash2* downregulates the Egfr pathway during vein development. (A) Lack of wing veins in rho^{ve} vn^{1} homozygous flies. (B) In the triple mutant rho^{ve} vn^{1} $ash2^{112411}$, the loss of veins is rescued. (C) top^{1}/top^{3C81} adult wing that lacks the a-cv and the middle part of L4. (D) The combination top^{1}/top^{3C81} with one copy of the $ash2^{I1}$ allele restores the wild-type vein pattern. (E) The gain-of-function Elp^{B1} results in ectopic vein tissue in distal L2. (F) Elp^{B1}; $ash2^{112411}$ individuals show a reduction in wing size, with notches appearing in the posterior margin and extra vein tissue (extra crossveins and a proximal L2-L3 fusion). (G) rl^{Sem}/+ flies develop wings with ectopic branches of veins. (H) Ectopic vein tissue is enhanced in rl^{Sem}; $ash2^{I1}$ transheterozygous flies. All wings are shown at the same magnification.

detachment of the dorsal and ventral cell layers. This variety of phenotypes is probably due to the variable expressivity found in the $ash2^{112411}$ allele. The top^{1}/top^{3C81} allelic combination is a hypomorphic mutation of Egfr (*top*) in which wings lack the anterior crossvein (a-cv) and a segment of vein L4 (Fig. 2C). Rescue of missing vein tissues is observed in top^{1}/top^{3C81}; $ash2^{I1}$/+ flies, as shown by the presence of a-cv (58% of the cases, *n*=93), a complete L4 (4%), or restoration of both a-cv and L4 (28%; Fig. 2D).

We also tested whether *ash2* alleles enhance gain-of-function phenotypes of the Egfr pathway. The $Ellipse^{B1}$ (Elp^{B1}) allele is an activated form of Egfr. In addition to other phenotypes, Elp^{B1} individuals consistently develop wings with ectopic vein tissue in L2 (Fig. 2E). In Elp^{B1}; $ash2^{I1}$/+ no enhancement was found (data not shown), but Elp^{B1}/+; $ash2^{112411}/ash2^{112411}$ individuals had reduced wings with ectopic vein tissue (46%, *n*=50; Fig. 2F), including vein fusion of the proximal regions and extra crossveins between L2 and L3, and between L4 and L5, but never between L3 and L4. In this case, a graded enhancement was also found, ranging from a wing similar to that of $ash2^{112411}$ to ectopic vein tissue covering most of the wing. Similarly, the MAPK gain-of-function allele, rl^{sem}, generates ectopic vein tissue, a phenotype that is enhanced in $ash2^{I1}$ combinations (61%, *n*=70; Fig. 2G,H).

As these results indicate that *ash2* antagonises the Egfr pathway, we tested whether *rho* expression is affected. In situ hybridisation for *rho* mRNA in third instar $ash2^{I1}$ discs showed either no expression at all or expression in only a few scattered cells (Fig. 3A,B), possibly owing to a strong perturbation of patterning in these discs. However, in $ash2^{112411}$ homozygous pupal wings, *rho* is expressed and organised in veins, but the domains of *rho* expression are larger (Fig. 3C-F).

ash2 is required for the differentiation of intervein tissue

As several genes that promote intervein specification are antagonists of the Egfr signalling pathway, we tested whether the phenotypes described above are associated with loss of intervein gene activity. During larval stages, *net* is the key gene involved in intervein development and acts as an antagonist of *rho* (Brentrup et al., 2000). In third instar wing discs, *net* transcripts are confined to broad domains corresponding to prospective interveins, in a pattern complementary to *rho* (Brentrup et al., 2000). *net* RNA expression is considerably reduced in *ash2* mutants, although in $ash2^{112411}$, some expression is still found in the central domain of the wing pouch (Fig. 4A-C). Likewise, homozygous flies for the net^{1}/net^{1} allele develop extra vein tissue, which is preferentially associated with transverse connections between L2 and L3 and between L4 and L5 (Fig. 4D), resulting in wing blades with an increased number of cells (Diaz-Benjumea and Garcia-Bellido, 1990; Garcia-Bellido and de Celis, 1992). The mutant combination net^{1}/net^{1}; $ash2^{I1}$/+ results in extra vein connections along the proximal and distal L2-L3 region, thickening of veins, blistering and a lanceolated shape to the wing. In addition, the intervein tissue is strongly reduced between veins L2-L3 and L4-L5, and to a lesser extent in A and E intervein regions (Fig. 4E). A more extreme phenotype (75%, *n*=20 wings; Fig. 4F) was obtained in net^{1}/net^{1}; $ash2^{112411}/ash2^{112411}$ wings, in which the intervein areas between L2-L3 and L4-L5 are totally missing and result in thicker and fused veins (Fig. 4G). The region between L3 and L4 did not show extra vein tissue and was much less affected.

We also generated double mutant flies to test whether *ash2* interacts with the intervein-promoting gene *bs*. The loss-of-function allele bs^{03267}, a *PlacZ* insertion within the *bs* gene, is not viable in homozygosis (Karpen and Spradling, 1992). In pharate bs^{03267} homozygotes, the whole wing blade is transformed into corrugated vein tissue, whilst the hinge region is almost intact. Heterozygous bs^{03267} flies display small patches of ectopic veins in the proximity of L2 and L5 (Roch et al., 1998) (Fig. 5A). However, flies that are homozygous for $ash2^{112411}$ and heterozygous for bs^{03267} exhibit blistering (Fig. 5B), reduced wing size associated with localised reduction of the intervein tissue (Fig. 5B,C) and development of extra crossveins (Fig. 5C,D). Veins L2 and L3 are thicker and totally or partially fused in the proximal region. Similarly, the D-intervein region between veins L4 and L5 is reduced and these

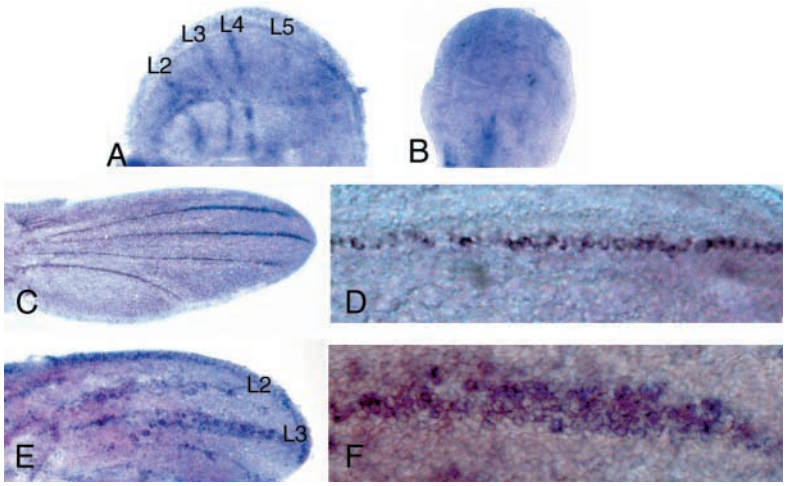

Fig. 3. In situ hybridisation for *rho* in discs and pupal wings. (A) Wild-type disc. (B) *rho* organisation is lost in $ash2^{I1}$ homozygous discs. (C) Wild-type pupal wing. (D) High magnification of a wild-type vein. (E) $ash2^{112411}$ homozygous pupal wing. Veins L2 and L3 are wider. (F) High magnification of a severe $ash2^{112411}$ wing showing vein thickening.

veins are partially fused and thicker. Moreover, in pupal $bs^{03267}/+$; $ash2^{112411}/ash2^{112411}$ wings, *rho* is expressed in broader domains (Fig. 5E,F). Interestingly, the C-intervein region is much less affected. This region is smaller than the corresponding wild type, probably owing to the overall wing reduction. However, as for *net*, neither ectopic vein tissue in the C-intervein region nor fusion of L3-L4 was found (Fig. 5C).

To determine whether *ash2* function is necessary for transcriptional activation of *bs*, we used genetic mosaics. In wild-type third instar imaginal wings, *bs* is expressed in stripes corresponding to the intervein regions (Fig. 5G). When $ash2^{I1}$ mutant clones fall within intervein tissue, *bs* expression is reduced. The most severe cases correspond to those clones located in B or D regions, where Bs expression is completely eliminated. Downregulated expression of *bs* is weaker in clones located in A, C and E regions (Fig. 5H-J). *bs* and *rho* expression is only mutually exclusive during pupal development, when *bs* promotes intervein differentiation and is expressed in intervein regions with abrupt borders with veins (Roch et al., 1998). In $ash2^{112411}$ pupal wings, *bs* is also expressed in interveins, although the size of the expression domains is smaller than the corresponding wild type. These *bs*-expressing domains are separated by wide stripes, corresponding to veins (Fig. 5K-M). The pattern of *bs* expression in $ash2^{112411}$ interveins is non-uniform, as indicated by the presence of negatively stained cells spread throughout the wing. Accordingly, pupal $ash2^{I1}$ clones result in a clearer downregulation of *bs* (Fig. 5N-S).

Enhancer of split [*E(spl)mβ*], a gene downstream of Notch, is also expressed in the wing pouch in broad domains that correspond to most interveins (de Celis et al., 1997). However, when we generated clones of $ash2^{I1}$, we observed no effect on either *E(spl)mβ* or *plexus* (*px*), another intervein-associated gene (Matakatsu et al., 1999) (data not shown).

As the phenotypes of double mutants in *ash2* and either *net* or *bs* are reminiscent of mutants with reduced Dpp signalling (de Celis et al., 1996), we investigated whether the expression pattern of the Dpp target gene *salm* is altered in these flies or in *ash2* single mutants. We found that in $ash2^{I1}/ash2^{I1}$ discs *salm* is slightly downregulated (Fig. 6A,B). In addition, $ash2^{I1}$ clones resulted in weak cell-autonomous downregulation of

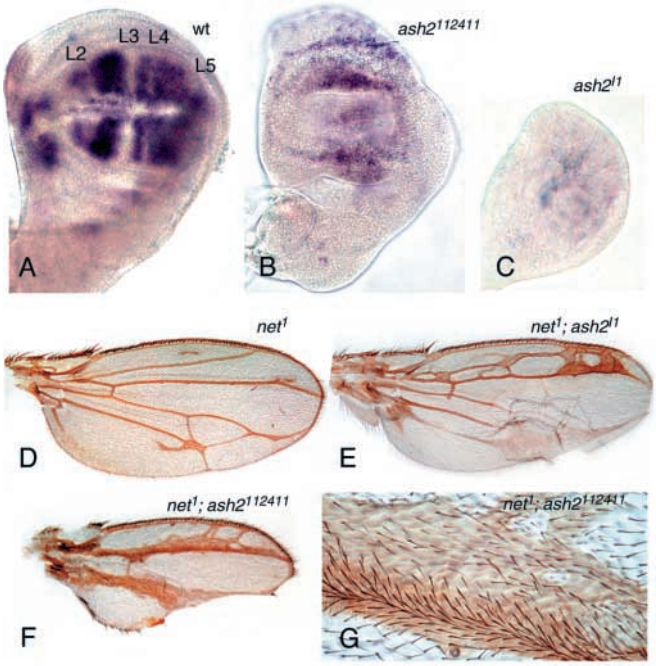

Fig. 4. *ash2* is required for *net* activation. (A-C) Expression of *net* mRNA in third instar wing discs. (A) Wild-type discs showing *net* transcripts in intervein regions. The position of veins (lacking *net* mRNA) is indicated. (B) *net* expression is reduced in discs $ash2^{112411}/ash2^{112411}$. However, the central domain of the wing pouch shows expression of *net*. (C) Absence of *net* mRNA in $ash2^{I1}$ wing discs. (D) Wing of net^1 homozygous mutant with ectopic vein tissue. (E) net^1/net^1; $ash2^{I1}$ heterozygous flies show posterior wing blistering and enhanced ectopic vein connections, especially between L2-L3. (F) Wings of net^1; $ash2^{112411}$ homozygous flies exhibit a total fusion of L2-L3 and L4-L5 with the corresponding loss of intervein regions B and D. The size of the wing is also reduced and the posterior margin is partially lost. (G) High magnification of L2 and L3 fusion, which is mostly associated with L2 thickening, as L5 is for L4-L5 fusion. Note the ventral pattern of corrugation in L2 and the *sensillia campaniforme*, and dorsal corrugation in L3.

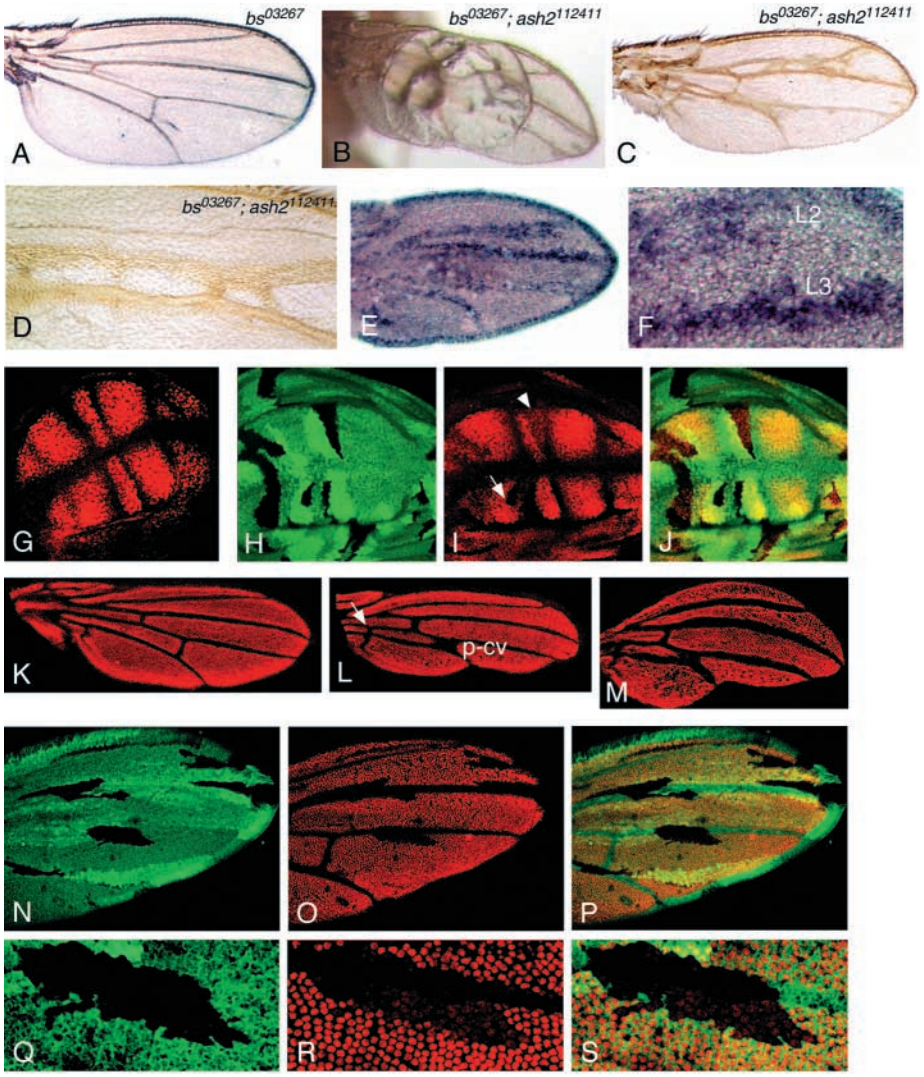

Fig. 5. *ash2* is required for activation of *bs*. (A) Wings of heterozygous bs^{03267} flies display a small ectopic vein in intervein region D. (B,C) $bs^{03267}/+$; $ash2^{112411}/ash2^{112411}$ wings show blisters with extra vein tissue (B) and reduction of interveins B and D alongside partial fusion of L2-L3 and L4-L5 (C). (D) Detail of extra crossveins connecting L2 and L3. (E) *rho* expression in a $bs^{03267}/+$; $ash2^{112411}/ash2^{112411}$ pupal wing. (F) High magnification of the same image. (G) Bs staining in a wild-type third instar wing disc. The vein territories and the DV boundary are devoid of antibody staining, which is only associated with intervein territories. (H) $ash2^{I1}$ clones in a wing imaginal disc (visualised as black spots by the absence of GFP). (I) Bs staining of the same disc. The reduction of Bs staining is more pronounced in the clone located between L2 and L3 (arrow) than in the one between L3 and L4 (arrowhead). (J) Merged green and red channels. (K-M) 24-30 hours APF wings stained with anti-Bs. (K) Wild type. (L) $ash2^{112411}/ash2^{112411}$ where extra vein tissue is restricted to wider p-cv and an extra proximal crossvein (arrow). (M) $ash2^{112411}/ash2^{112411}$ pupal wing of an individual with thicker vein tissues. In both L and M, some interveins are reduced and show some negatively stained cells within the interveins. (N) $ash2^{I1}$ mitotic clones in pupal wings. (O) Bs staining of the same wing. (P) Merged green and red channels. (Q-S) High magnification of an $ash2^{I1}$ clone.

salm (Fig. 6F-H). However, homozygous $ash2^{112411}$ discs express *salm* in a central domain (Fig. 6C), as in net^{1}/net^{1}; $ash2^{112411}/ash2^{112411}$ and $bs^{03267}/+$; $ash2^{112411}/ash2^{112411}$ discs (Fig. 6D,E). In all these cases, the expression pattern resembles wild type. We also tested for possible alterations of *brk*, an antagonist of the Dpp signalling pathway that is expressed in peripheral cells of the wing disc in a pattern complementary to the *sal-C* domain (Campbell and Tomlinson, 1999; Jazwinska et al., 1999). However, $ash2^{I1}$ clones in the wing did not show any perturbation of *brk* expression (Fig. 6I-L).

ash2 inhibits *kni* expression

We next examined whether the function of *ash2* is exclusive to vein/intervein differentiation or whether vein positioning genes are also targeted. As $ash2^{112411}$ shows a crooked L2 and the expression of *kni-C* is required to organise this vein, we studied a possible interaction between the two genes. Analysis with anti-Kni reveals *kni* expression in L2 and in some cells of the wing pouch margin in wild-type discs (Fig. 7A), while in $ash2^{I1}$ homozygous mutant discs its expression is expanded throughout the whole disc (Fig. 7B). Some expansion of *kni* expression is also found in $ash2^{112411}$ discs, which in addition show discrete Kni-staining of L2 (Fig. 7C). We also tested the effects in mitotic clones and found that *kni* is upregulated in $ash2^{I1}$ clones (Fig. 7D-L). This effect is cell-autonomous. Activation of *kni* was observed in twin and $Minute^{+}$ clones generated in early developing discs, as well as in small clones generated late in development, suggesting that the activation of *kni* by $ash2^{I1}$ can occur at any stage of larval development.

We observed that although *ash2* homozygous cells within the $Minute^{+}$ clone show a strong activation of *kni*, the heterozygous cells show residual activity in the whole wing disc (Fig. 7E). To test whether this residual activity is due to the lack of one copy of the *ash2* gene, we generated twin clones (Fig. 7G-I) and found that the residual *kni* expression seen in heterozygous cells was completely missing in cells homozygous for the wild-type allele (Fig. 7J-L). This result supports *kni* de-repression not only in *ash2* homozygous cells but also in *ash2* heterozygous cells. However, whereas Kni in the L2 vein is nuclear, in homozygous *ash2* clones, the localisation is both cytoplasmic and nuclear (Fig. 7M,N), suggesting that although there is an increase in Kni protein, it may not be fully functional. To gain insight into the interaction

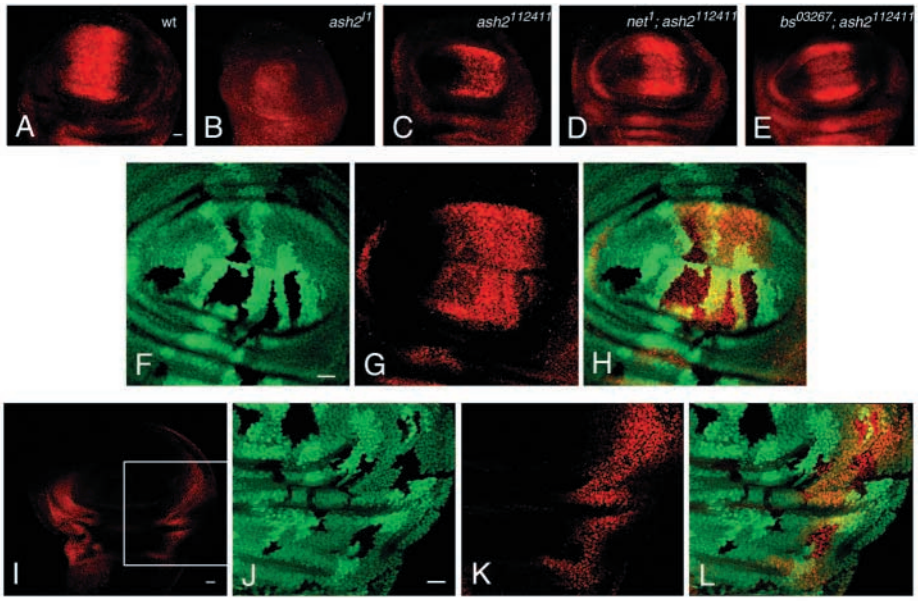

Fig. 6. Patterns of *sal* (A-H) and *brk* (I-L) expression in wing discs. (A) Wild type. (B) $ash2^{I1}/ash2^{I1}$ showing reduced sal expression. (C) $ash2^{112411}/ash2^{112411}$. (D) net^{1}/net^{1}; $ash2^{112411}/ash2^{112411}$. (E) $bs^{03267}/+$; $ash2^{112411}/ash2^{112411}$. (F-H) $ash2^{I1}$ mutant clones, marked by the absence of GFP. (G) *salm* expression is reduced in $ash2^{I1}$ clones. (H) Merged green and red channels. (I) *brk* expression is visualised by means of the *brk-lacZ* reporter *X47* in a wild-type wing disc. (J) High magnification of the boxed area indicated in I, $ash2^{I1}$ clones lack GFP. (K) There is no change in β-galactosidase expression in $ash2^{I1}$ mutant cells. (L) Merged images of J and K. Scale bars: 20 μm.

between *ash2* and *kni* we analysed mutant combinations of *ash2* and the hypomorphic allele kni^{ri-1}. In kni^{ri-1} wings, the L2 vein fails to develop and the rest of the veins develop normally (Fig. 7O), whereas in kni^{ri-1} $ash2^{112411}$ double homozygous mutants, the L2 vein is partially (25%, *n*=58) or almost completely restored (75%, *n*=58; Fig. 7P) and *rho* is expressed in the L2 primordium (Fig. 7Q,R). Further evidence in favour of a role for *ash2* as a repressor of *kni* was gained by semi-quantitative RT-PCR. We found that RNA from $ash2^{I1}$ homozygous larvae contains significantly increased levels of *kni*, compared with wild-type larvae (Fig. 7S).

ash2 regulates *kni* expression independently of *sal-C*

We also examined whether repression of *kni* by *ash2* is mediated by regulators of *kni* expression in the wing. A well-known regulator of *kni* in L2 is *sal-C*. Low levels of *sal-C* activate *kni* expression in the presumptive L2 region, whereas higher levels repress *kni-C* expression (de Celis and Barrio, 2000; Lunde et al., 1998). As we observed some perturbation of *salm* expression in $ash2^{I1}$ tissues, we investigated whether the low levels of *salm* could be responsible for the ectopic *kni* expression. To achieve this, we generated $ash2^{I1}$ clones in *UAS-salm* or *UAS-salr* backgrounds, using a *nubbin-Gal4* driver. Adult wings overexpressing *salm* or *salr* lose L2 and L5 and show severe size reduction (de Celis et al., 1996) (Fig. 8A). Surprisingly, we observed that *ash2* clones generated in those flies exhibit de-repression of *kni*, even when high levels of Salm are maintained in the clone (Fig. 8B-E). Therefore, we conclude that de-repression of *kni* induced by loss of function of *ash2 i*s independent of *sal-C*. This interpretation is strengthened by the observation that in mitotic clones and in homozygous $ash2^{I1}$ mutants the ectopic expression of *kni* is not exclusive to the *sal-C* domain (Fig. 7B,G). In regions outside the *sal-C* central domain, loss of *ash2* also activates *kni*, in anterior as well as posterior cells.

None of the other possible activators or inhibitors of the L2 enhancer, such as *sd*, *vg* or *en*, showed significant alteration of their expression in $ash2^{I1}$ clones (data not shown). Moreover, using a *lacZ* reporter construct that includes a minimal L2 enhancer element containing only the activator and repressor sites (*EX-lacZ*) (Lunde et al., 2003), we observed no β-galactosidase expression in $ash2^{I1}$ homozygous discs or in mitotic clones (data not shown). Taken together, these results indicate that the *ash2*-induced repression of *kni* is independent of *sal-C*, and suggest that *ash2* could be interacting with a *kni* enhancer other than L2.

Discussion

An important step towards understanding how cell determination is maintained is the identification of targets of the PcG and trxG genes. Although *ash2* has been considered to be a member of the trxG (Adamson and Shearn, 1996), its biological and molecular function is still unknown. In this work, we show evidence that *ash2* is required for normal vein/intervein patterning, and demonstrate that it plays a role in two major biological events – determination of intervein identity and maintenance of *kni* repression beyond L2.

Identifying intervein and vein target genes of *ash2*

Loss of *ash2* function causes differentiation of ectopic vein tissue, indicating that *ash2* is required for intervein development, where it functions as an activator of the intervein-promoting genes *net* and *bs*, restricting *rho* expression to vein regions. In addition, the loss-of-function phenotypes of *Egfr* alleles are rescued in *ash2* mutants, while the gain-of-function phenotypes are enhanced. Furthermore, *rho* mRNA exhibits an expanded expression pattern in *ash2* mutant tissues. Thus, *ash2* promotes the maintenance of intervein fate, either by activation of *net* and *bs* or by repression of the Egfr pathway. As *rho* and *bs*/*net* expression is mutually exclusive, we cannot determine whether the Ash2 complex interacts directly with one or all of them. However, as *bs* expression is inhibited by the loss-of-function of *ash2* during larval and pupal stages, we can propose that *ash2* acts as a long-term chromatin imprint of *bs* that is stable throughout development.

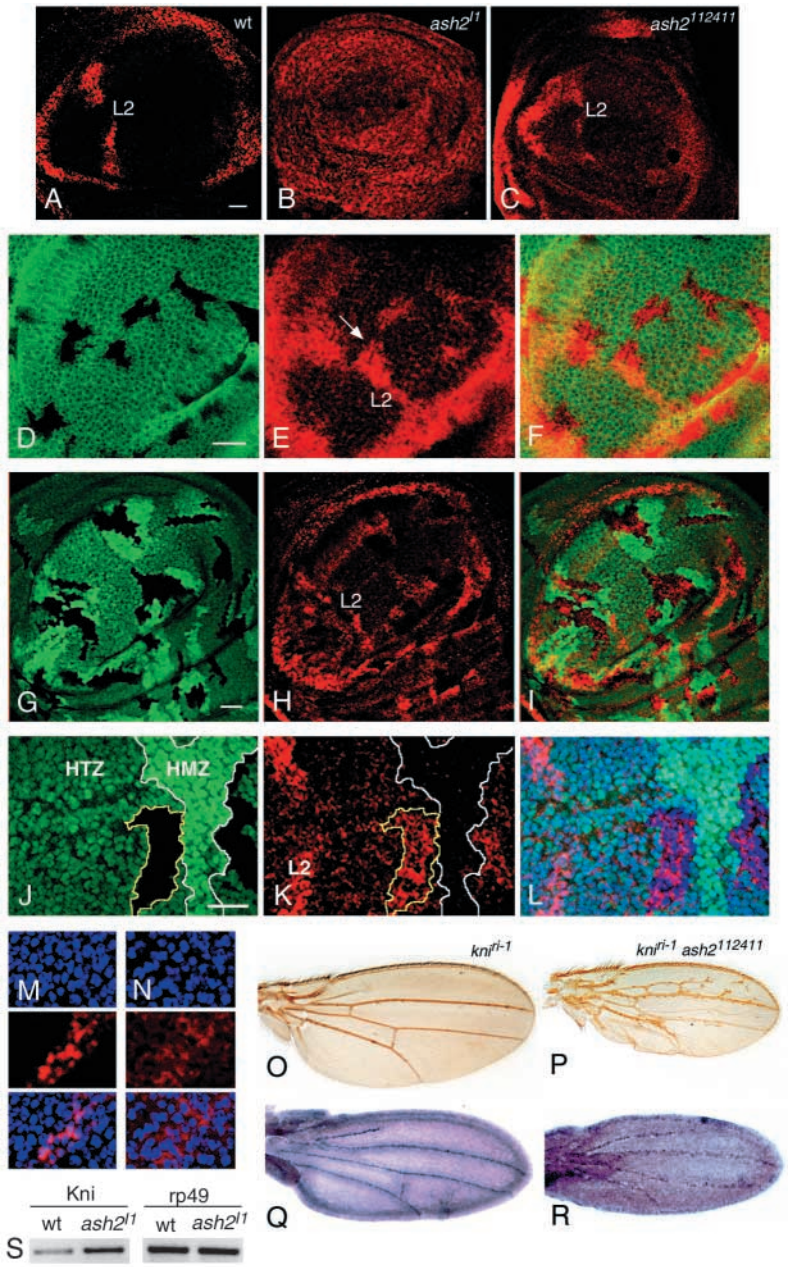

Fig. 7. *ash2* regulates *kni* expression. *kni* in wild-type (A), $ash2^{I1}/ash2^{I1}$ (B) and $ash2^{112411}/ash2^{112411}$ (C) wing discs. (D) *Minute*$^{+}$ clones homozygous for $ash2^{I1}$ induced at 110±4 hours AEL are marked by the absence of β-galactosidase. (E) Expression of *kni* is cell-autonomously upregulated by the loss of *ash2* function (arrow). There is residual *kni* expression in heterozygous tissue. (F) Merged images of C and D. (G) Clones homozygous for $ash2^{I1}$ induced at 52±4 hours AEL are marked as black spots by the absence of GFP and twin clones of wild-type cells appear as intense green spots. (H) *kni* expression is upregulated in homozygous $ash2^{I1}$ mutant cells and residual *kni* disappears in cells homozygous for GFP. (I) Merged green and red channels of F and G. (J) Detail of a clone. Homozygous GFP cells are outlined in white (HMZ) and homozygous $ash2^{I1}$ cells are outlined in yellow. HTZ. heterozygous tissue. (K) Anti-Kni staining of the same disc. One mutant copy of *ash2* is enough to de-repress *kni* expression. (L) Merged images of I and J, and an additional channel with nuclear labelling (propidium iodide, shown in blue). (M) Detail of endogenous Kni (red) showing nuclear localisation (blue) in the L2 domain (pink in lower panel corresponds to nuclear labelling). (N) Detail of an $ash2^{I1}$ clone; Kni is mainly cytoplasmic, although nuclei also contain some protein. (O) Adult kni^{ri-1}/kni^{ri-1} wing. (P) kni^{ri-1}/kni^{ri-1} $ash2^{112411}/ash2^{112411}$ wing showing rescue of L2 vein. (Q) *rho* expression in pupal kni^{ri-1}/kni^{ri-1} wing. (R) *rho* expression in pupal kni^{ri-1}/kni^{ri-1} $ash2^{112411}/ash2^{112411}$ wing. There is distal expression in L2. (S) RT-PCR showing increased *kni* mRNA in $ash2^{I1}$ homozygous larvae compared with wild type. Rp49 is shown as a control. Scale bars: 20 μm.

Our results in adult clones and from analysis of genetic interactions suggest that *ash2* acts principally by maintaining B and D intervein regions, as the C intervein remains unaltered in *ash2* mutants. This region is under the control of organising genes that respond to the Hh signal (Tabata and Kornberg, 1994; Zecca et al., 1995). One of these genes is *kn*, which prevents vein differentiation in the C intervein (Crozatier et al., 2002; Mohler et al., 2000) and is required for the expression of *bs* in this domain (Vervoort et al., 1999). *bs* expression is regulated by two enhancer elements: the boundary enhancer, which is dependent on *hh* and controls *bs* expression in the C intervein region through *kn*; and another enhancer dependent on Dpp activity, which controls *bs* expression in B and D intervein domains (Nussbaumer et al., 2000). Thus, the role of *ash2* as a positive regulator of *bs* is mainly restricted to regions beyond the *kn* domain where the Dpp dependent *bs* enhancer is active.

It has been found that some combinations of *dpp* alleles and mosaic clones of *sal-C* result in elimination of B and D intervein regions, along with fusion of their flanking veins (de Celis et al., 1996). Although the genetic interactions between *ash2* and either *bs* or *net* could be the result of a synergistic failure to activate genes downstream of Dpp, our results indicate that this may not be the case because *salm* is expressed in the central domain of the wing pouch of those mutant combinations.

It has been recently shown that another trxG complex, the Brm complex, is involved in regulating wing vein development (Marenda et al., 2004). The authors found that components of that complex interact genetically with *net* and *bs* at pupal stages to regulate the expression of *rho*, and that the complex is specifically required in cells within and bordering L5 to mediate proper signalling. There are some key differences

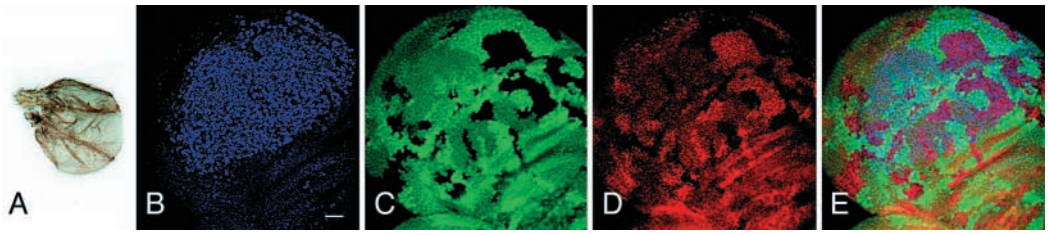

Fig. 8. *kni* upregulation by *ash2*^{I1} is independent of *sal-C* downregulation. (A) Overexpression of *sal-C* from *UAS-sal*^{64d} using a *nubbin-Gal4* driver results in loss of L2 and L5 and severe reduction of wing size. (B) Wing imaginal disc showing ectopic expression of *salm* throughout the wing pouch. (C) *ash2*^{I1} homozygous clones are devoid of GFP. (D) *kni* expression is also upregulated in *ash2*^{I1} mutant clones even, when *salm* is overexpressed. (E) Merged images. Scale bars: 20 μm.

between the Brm complex and Ash2: (1) Ash2 maintains *bs* expression from the third instar stage; (2) the Ash2 complex is mainly required for interveins B and D; and (3) the enhancement or suppression phenotypes of the genetic interactions with Egfr and intervein-promoting alleles are much stronger for *ash2* than for the Brm complex. Taken together, these results suggest that *ash2* plays a crucial role in intervein identity and that each trxG complex acts in a specific spatiotemporal program to maintain organ identity.

Ash2 complex maintains *kni* in an *off* state

The positioning of vein tissues depends on the *sal-C* patterning dictated by the Dpp signalling pathway (Sturtevant et al., 1997; Sturtevant and Bier, 1995). Low levels of *sal-C* in the anterior compartment are required for the expression of *kni-C*, which triggers the differentiation of L2 (de Celis and Barrio, 2000; Lunde et al., 1998). We have shown that lack of *ash2* activity results in downregulation of *salm* and upregulation of *kni*. Thus, it is possible that within the *sal-C* domain, the ectopic expression of *kni* is a result of low levels of *salm*. However, when high levels of *salm or salr* are maintained by ectopic activation, lack of *ash2* nevertheless results in de-repression of *kni*. Moreover, *kni* is also cell-autonomously de-repressed by loss-of-function of *ash2* in cells outside of the *sal-C* expression domain. Thus, the repression state in the whole wing must be maintained by factors other than *sal-C*. The *kni/knirl* L2-enhancer is subdivided into activation binding sites for Brk, En and the Sd/Vg complex, and repression binding sites for Sd/Vg, En, Salr and Brk (Lunde et al., 2003). We did not observe changes either in β-gal expression from the *EX-lacZ* enhancer or in *sd*, *vg*, *brk* or *en* expression in clones lacking *ash2*. Therefore the de-repression of *kni* in *ash2* mutant cells must be accounted for by a mechanism entirely different from that of the signal-dependent induction of L2, perhaps through another enhancer more global than that of L2.

The low levels of *salm* expression associated with *ash2*^{I1} clones may also be explained by de-repression of *kni*. In dorsal tracheal cells, *kni/knrl* activity represses *salm* transcription, and this repression is essential for branch formation. Similarly the establishment of the border between cells acquiring dorsal branch and dorsal trunk identity entails a direct interaction of Knirps with a *salm* cis-regulatory element (Chen et al., 1998). Also in the wing, *kni* and *knrl* are likely to refine the L2 position by positive auto-regulation of their own expression and by providing negative feedback to repress *salm* expression (Lunde et al., 1998).

It is possible that the de-repression of *kni*, intervein inhibition and appearance of extra vein tissues are linked events. The *kni-C* complex organises the development of the L2 vein by activating *rho* and inhibiting *bs* (Lunde et al., 1998; Montagne et al., 1996). Thus, *kni-C* participates in L2 morphogenesis by functioning downstream of *salm* and upstream of vein-intervein genes. The ectopic activation of *kni* by lack of *ash2* could trigger intervein repression and vein activation. Indeed, ectopic activation of UAS-*kni* results in broad expression of *rho* and elimination of Bs expression in pupal wings, leading to the production of solid vein material (Lunde et al., 1998). However, in adult clones not all *ash2* mutant cells develop vein tissue. This raises the possibility that de-repressed *kni* may not be fully functional, as ectopic *kni* is often localised to the cytoplasm rather than the nucleus. Alternatively, *ash2* could have independent functions in the wing, maintenance of the repressed state of *kni* alongside maintenance of the intervein condition, by acting on different targets.

The *ash2*^{112411} mutation can partially rescue the loss of L2 in *kni*$^{ri-1}$ mutants. This is in contrast to our observation that the L2 enhancer appears not to mediate the effect of *ash2*. The *kni*$^{ri-1}$ allele is a 252 bp deletion in the enhancer of L2 (Lunde et al., 2003) that results in lack of *kni* expression in L2 (Lunde et al., 1998). It has been shown, however, that it is possible to rescue the vein-loss phenotype of *kni*$^{ri-1}$ by expressing a *UAS-rho* transgene in L2 (Lunde et al., 2003). In addition, double mutant flies for *kni*$^{ri-1}$ and *net* partially rescue L2 (Diaz-Benjumea and Garcia-Bellido, 1990). It is therefore likely that the antagonistic effect of *ash2* on *rho* could account for the partial rescue of L2 in *kni*$^{ri-1}$ *ash2*^{112411} wings, as *rho* mRNA is expressed in the rescued L2.

Some PcG genes are known to be required for the maintenance of *kni* expression domains in the embryo (McKeon et al., 1994; Pelegri and Lehmann, 1994; Saget et al., 1998). It is also likely that some trxG genes or other complexes of trxG proteins, such as the Ash2 complex (Papoulas et al., 1998), may interact with repressor sequences necessary to keep *kni* expression in an off state beyond L2. Moreover, in a genome wide prediction screen it has been shown that *kni* contains PRE/TREs (Ringrose et al., 2003). Thus, we propose here that *ash2* acts as regulator of *kni* expression in the wing through an epigenetic mechanism of cellular memory similar to the trx-G regulation of homeotic genes, albeit that it remains to be seen whether *kni* is a direct or indirect target of *ash2*.

Cellular memory and morphogen gradients

A well-studied mechanism through which to induce and

preserve cell identities in wing imaginal discs is the response to gradients of the morphogen Dpp. This raises questions about the extent to which the response to Dpp occurs through concentration-dependent mechanisms or cellular memory. There is compelling evidence in favour of the existence of Dpp gradients that organise the pattern and growth of the wing imaginal disc (Podos and Ferguson, 1999; Strigini and Cohen, 1999). Dpp signalling causes a graded transcriptional regulation of *brk* by an interaction between the Dpp transducers and a *brk* morphogen-regulated silencer (Muller et al., 2003). Thus, *brk* appears to respond to direct morphogenetic signalling rather than remembering the inputs of previous developmental events. However, whereas activation of *salm* requires continuous signalling through the Dpp pathway (Lecuit et al., 1996; Nellen et al., 1996), other targets of Dpp, such as *omb*, remember exposure to the signal (Lecuit et al., 1996). We have shown here that stable regulation of other genes involved in wing development, such as *kni* repression, and *net* and *bs* activation, would also respond to the cellular memory conferred by epigenetic marks of the Ash2 complex. Thus, both mechanisms – morphogen-dependant, which will be required for growth and patterning, and epigenetic, which will keep specific genes in an *off* or *on* state – are likely to act simultaneously to maintain cellular identities within the wing.

Because many developmental regulators are only expressed transiently during development, the function of epigenetic complexes is likely to be very dynamic. The developmental events required for the construction of the wing, as with many other morphogenetic events, cannot only rely on an on or off state of gene expression. Instead, morphogenesis is rather malleable and epigenetic marks could act as a means to facilitate, rather that fix, the preservation of developmental fates. It may well be that the epigenetic marks of the Ash2 complex allow changes in chromatin structure to assist the access of proteins that activate or repress gene expression. From an epigenetic point of view, the ultimate refinement of morphogenesis and maintenance of cellular memory will depend upon the interaction of these chromatin remodelling complexes with the factors that trigger or inhibit transcription.

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