

Activation of *knot (kn)* specifies the 3-4 intervein region in the *Drosophila* wing

Jym Mohler^{1,*}, Maria Seecoomar¹, Shradha Agarwal¹, Ethan Bier² and Jennifer Hsai¹

¹Department of Biological Sciences, Barnard College, New York, NY 10027, USA

²Department of Biology, University of California San Diego, La Jolla, CA 92093, USA

*Author for correspondence (e-mail: jmohler@barnard.columbia.edu)

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SUMMARY

Hedgehog (Hh) plays an important role in *Drosophila* wing patterning by inducing expression of Dpp, which serves to organize the wing globally across the A-P axis. We show here how Hh signalling also plays a direct role in patterning the medial wing through the activation of the Hh-target gene, *knot (kn)*. *kn* is expressed in Hh-responsive cells near the A-P compartment boundary, where its expression is dependent on *fu*, a component of Hh signalling. *kn* is required for the proper positioning of veins 3 and 4 and to prevent ectopic venation between them. Furthermore, the expansion anteriorly of the normal *kn* expression domain causes an associated anterior shift in the position of vein 3 in the resultant wing. Ectopic expression of *kn* elsewhere in the wing imaginal disc results in the failure to properly activate the vein initiation genes, *rho* and *Dl*. Expression of the gene encoding the EGF-receptor (*EGFR*), which is

required for vein initiation and subsequent differentiation, is normally depressed in the 3-4 intervein region. This downregulation of *EGFR* in the medial portion of the imaginal disc is dependent on *kn* activity and ectopic expression of *kn* inactivates *EGFR* elsewhere in the wing primordium. We propose *kn* expression in Hh-responsive cells of the wing blade anlagen during the late third instar creates a zone of cells in the medial wing in which vein primordia cannot be induced. The primordia for veins 3 and 4 are laid down adjacent to the *kn*-imposed vein-free zone, presumably by a signalling factor (such as Vn) also synthesized in the medial region of the wing.

Key words: *knot (kn)*, Hh signalling, EGF-receptor, *Drosophila*, wing vein

INTRODUCTION

The organization of the *Drosophila* wing veins is a simple system in which to study developmental patterning. The venation pattern consists primarily of five longitudinal veins (thickened cuticular regions providing structural support and axonal/hemolymph conduits, numbered 1 to 5 from anterior to posterior) separated by thin diaphanous intervein cuticle. Furthermore, unlike many other developing organs where patterning and morphogenesis are reiterative events, in the developing wing, the overall pattern of the veins is set up at a distinct time (during late larval stages) prior to morphogenesis and differentiation of wing (which occurs during pupal stages).

The events that define the organization of the wing initiate with the division of the second thoracic segment (from which the wing imaginal disc derives) into two compartments in the early embryo, defined by the expression of the transcription factors, Ci and En, in the anterior and posterior compartments, respectively (see Lawrence, 1992, for a review). Throughout larval development, Hh is secreted from the posterior compartment and activates an Hh-response in the adjacent cells, in the anterior compartment along the compartment boundary, by antagonizing the basal inhibitory activity of the Hh-receptor, Patched (Ptc). This signalling response involves

the activation of the Ci transcription factor and results in the transcriptional activation of a number of target genes in the wing imaginal disc, most notably the upregulation of the Ptc receptor and induction of a secondary signal, Dpp (Tabata and Kornberg, 1994; Zecca et al., 1995). Dpp, once made by the Hh-responsive cells, disperses from the central region of the disc and during the third larval instar activates different target genes, notably *omb* and *sal*, at distinct threshold levels to create nested domains of transcription factor expression across the wing (Nellen et al., 1996; de Celis et al., 1996; Grimm and Pflugfelder, 1996).

These Hh- and Dpp-signalling processes and their activated target genes lead to the specification of vein primordia at specific locations along the anterior-posterior axis in the late third instar larval disc. These primordia can be detected by the expression of vein initiation marker genes, such as *rho* and *Dl*. *rho* is expressed in the primordia of all the longitudinal veins at the end of the third larval instar, as well as in two stripes flanking the presumptive wing margin (Sturtevant and Bier, 1995); *Dl* is expressed similarly, except it is absent from the primordia for vein 2 (Huppert et al., 1997). *rho* and *Dl* are essential components of EGF- and Notch-mediated signalling, respectively, both of which processes are required for resolution of vein and intervein cell-fate determination

(Sturtevant and Bier, 1995). The expression of *rho* and *Dl* in individual vein primordia is presaged by the expression of vein-specific vein-promoting genes, each of which is necessary for the specification of a particular vein. These vein-specific vein-promoting genes include *kni*, which is required for vein 2 (Sturtevant and Bier, 1995; Lunde et al., 1998), *ara/caup*, a pair of partially redundant genes of the *iroquois* complex that are required for the odd-numbered veins (veins 1, 3 and 5; Gomez-Skarmeta and Modolell, 1996; Gomez-Skarmeta et al., 1996) and *ab*, which is required for vein 5 (Sturtevant and Bier, 1995). In the case of vein 2, expression of *kni* is induced just outside the anterior boundary of the expression domain of *sal* (a Dpp target) by some unknown signal from *sal* expressing cells (Lunde et al., 1998). For the other three predominant longitudinal veins, it is not clear how the global patterning elicited by Hh- and Dpp signalling results in the positioning of the vein primordia within the wing disc.

One gene that appears to play a role in positioning veins 3 and 4 is *knot* (*kn*). The original viable *kn¹* allele causes veins 3 and 4 to form closer together and produces a corresponding shift in their primordia (as detected by *rho* expression) in the late third instar disc (Sturtevant and Bier, 1995). Mosaic analysis of strong, embryonic *kn* alleles by Nestoras et al. (1997) indicated that *kn* is important in suppressing vein formation between veins 3 and 4, but not in other regions of the wing. Because vein 4 runs just posterior to the A-P compartment boundary, the region affected by *kn* mutant clones corresponds approximately to the Hh-responsive cells along the A-P compartment boundary. *kn* is also required for suppressing vein formation in *ptc*- clones in the anterior compartment; these *ptc*- clones mimic Hh-responsive cells in which Hh has bound to the Ptc receptor. Nestoras et al. (1997) proposed that *kn* functions to separate veins 3 and 4 by imposing a vein-free region in response to Hh signalling. Mosaic analysis of strong, pupal lethal alleles of *fu*, a serine/threonine kinase required in Hh-responsive cells for a normal response, showed a similar requirement for *fu* in preventing ectopic vein induction between veins 3 and 4 (Alves et al., 1998), suggesting a direct role for Hh signalling in controlling the 3-4 intervein space.

In order to decipher how *kn* functions to control patterning in the medial wing, we adopted a positional cloning strategy (M. S., S. A., K. Vani, G. Yang and J. M., unpublished). We found that the *kn* gene is identical to the previously described *collier* (*col*) gene (Crozatier et al., 1996), which encodes a COE-family transcription factor. Strong *kn* alleles result in embryonic lethality, whereas other *kn* alleles, likely to affect regulatory sequences, affect only wing vein patterning. In this report, we show that *kn* is expressed in the wing imaginal disc precisely between the primordia for veins 3 and 4, within the Hh-responsive cells of the wing blade. The expression of *kn* in this region is dependent on the activity of *fu*, a known component of Hh-signal transduction. An artificially induced anterior expansion of the domain of *kn* expression causes an anterior displacement of vein 3. Global ectopic expression of *kn* in other regions of the wing imaginal disc suppresses the expression of vein-initiation markers, *rho* and *Dl*, and the final wing venation. Furthermore, the expression of *EGFR* (encoding the EGF-receptor, a critical component of EGF signalling that is required for proper vein initiation) is normally downregulated in the medial wing. This downregulation of *EGFR* is dependent on *kn*

function, both normally in the medial region of the wing and elsewhere in the wing when *kn* is ectopically expressed. Taken together, these results indicate that expression of *kn* in the medial wing in response to Hh signalling specifies a vein-free zone, outside which veins 3 and 4 form to either side.

MATERIALS AND METHODS

Generation of UAS-*kn*

The UAS-*kn* line was constructed as follows. The 5' portion of the *kn* coding region was amplified by PCR from the F1 cDNA (Crozatier et al., 1996) using a primer binding approximately 100 bp upstream from the initiation codon (with a single mutation to yield an *EcoRI* site: TCOL44SECO: AAGCTGAATTCCGAAGAGCCG) and a primer binding downstream to an internal *BglIII* site (RCOL8: AATTGAATTCCGCTGCCGGGCGACG). This PCR fragment was digested with *EcoRI* and *BglIII* and cloned into the same sites in pUAST. The 3' portion of the coding region was isolated as a 1.8 kb *BglIII* fragment from the F1 cDNA and ligated into the *BglIII* site of 5'-pUAST construct to reconstruct the entire coding region in pUAST. Three independent constructs were injected into flies and tested to verify that ectopic expression of each of the three has similar effects on wing venation. All experiments described here utilized the construct named '15+1' inserted onto the X chromosome.

In situ hybridization

In situ hybridizations using a single DIG-labelled RNA probe or in combination with antibody labelling, were done according to the methods of Sturtevant et al. (1993) and stained discs were mounted in Canada Balsam. Mouse monoclonals were used for all antibody staining: α -Dl was generated in the laboratory of M. Muskavitch; α - β gal was obtained from Promega (Madison, WI, USA). Expression patterns of *kn*, *sal*, *rho*, *EGFR*, *vn*, *caup* and *kni* were determined by in situ hybridization; expression patterns of *ptc*, *dpp* and *ara/caup* were determined using lacZ-reporters and β -gal immunohistochemistry.

Double in situ hybridizations were done using a modification of the single in situ protocol of Sturtevant et al. (1993). Larval carcasses were hybridized with a combination of two probes, one labelled with digoxigenin and one with fluorescein. The fluorescein-labelled probe was generated identically to the digoxigenin-labelled probe, except that fluorescein-11-UTP was substituted for digoxigenin-11-UTP in the labelling reaction. The fluorescein-labelled probe was first bound with AP-conjugated α -fluorescein Fab and stained with Fast Red. After the first staining, the larval carcasses were washed five times in PBT to remove excess stain and twice in 0.1 M glycine, pH 2.2, to remove the α -fluorescein Fab. The carcasses were then rinsed five times in PBT and bound to AP-conjugated α -digoxigenin Fab and stained with NBT/BCIP. Discs were dissected from the carcasses and mounted in glycerol. In all the double in situ hybridizations shown here, the fluorescein-labelled probe was a *kn* anti-sense transcript.

Clonal analysis

y w hsFLP; FRT42D / FRT42D pwn knKN4 larvae were heat-shocked at 37°C for 2 hours after 5-6 days growth at 21°C after egg lay to generate small wing clones induced in mid-third instar (pupariation was approximately 1 day later). Adult wings were mounted in Canada Balsam and observed for venation defects and *pwn* clones.

RESULTS

kn controls the spacing of veins 3 and 4 and prevents formation of ectopic veins between them

In the *Drosophila* wing, *kn* is specifically required for the

proper development of the intervein region between veins 3 and 4 (Nestoras et al., 1997). Clonal analysis of *kn* mutations in the wing indicates that *kn* has two related functions in the medial wing: *kn* is required primarily for the formation of the intervein region separating veins 3 and 4 and, secondarily, to prevent ectopic vein formation in that region. Flies mutant for wing-specific alleles of *kn* show a reduction in the 3-4 intervein region and fusions of veins 3 and 4, particularly proximal to posterior crossvein (Fig. 1B). Similarly, large clones of strong, embryonic-lethal alleles of *kn* that encompass the entire medial region of the wing cause the complete loss of the 3-4 intervein region and the complete fusion of veins 3 and 4 (Nestoras et al., 1997). This shift in the positions of veins 3 and 4 in the *kn* mutants relative to wild type and the associated reduction in the intervein space reflect changes in the initial patterning of vein primordia in the late third instar, as manifested by the expression of vein initiation genes such as *rho* (Fig. 1D,E; Sturtevant et al., 1993).

In contrast, smaller clones generated within the 3-4 intervein region have little or no effect on the position of veins 3 and 4, but are usually associated with ectopic vein material. This is most evident in clones generated in the early third instar larvae, typically less than five cells in size, which are associated with ectopic vein material only if situated between veins 3 and 4 (Fig. 1C). The ectopic vein material associated with these small, late clones often extends outside the clone proper (Fig. 1F); this result is consistent with the activation of vein primordia initiation with the *kn* clone followed by vein extension outside the clone (a process occurring later in vein differentiation that normally assures contiguity of each longitudinal vein; Garcia-Bellido, 1977; Sturtevant and Bier, 1995). The phenotypes associated with these clones indicate that *kn* has a role to prevent ectopic vein formation in the 3-4 intervein space, in addition to its role in positioning veins 3 and 4.

***kn* is expressed in the medial region of the wing imaginal disc**

To decipher the role of *kn* in patterning the medial *Drosophila* wing, we adopted a positional cloning strategy to clone the *kn* locus (M. S., S. A., K. Vani, G. Yang, J. M., unpublished). In brief, breakpoints of rearrangement mutations of *kn* were mapped within genomic clones obtained from the European and Berkeley *Drosophila* Genome Projects. This analysis indicated that *kn* was identical to the previously described gene *collier* (*col*; Crozatier et al., 1996), since the rearrangements associated with the *kn* mutations affected the integrity of the *col* transcription unit. A similar conclusion, equating the *kn* and *col* genes, was derived by genomic rescue recently by Vervoort et al. (1999).

In situ hybridization of the corresponding *kn* cDNA to wing imaginal discs indicates that strong *kn* expression is limited primarily to a

stripe in the middle of the wing blade region of the imaginal disc (Fig. 2A). Weaker *kn* expression can also be detected in more posterior regions of the wing pouch and in portions of the hinge region of the disc. Significantly, *kn* medial expression is reduced in mutants for *fu* (Fig. 2B, *fu*¹, a moderate adult viable allele), which encodes a ser-thr kinase that functions in many aspects of Hh signalling.

Colabelling of *kn* RNA expression with a *ptc-lacZ* reporter construct, reveals that the medial expression of *kn* is located within the *ptc*-expressing cells (the Hh-responsive cells, Fig. 2D). The posterior margin of *kn* expression matches the posterior margin of *ptc-lacZ* expression at the anterior-posterior compartment boundary, although *kn* expression does not extend quite as far anterior as the *ptc-lacZ* reporter. In contrast, most of the *kn* expression is posterior to the expression domain of a *dpp-lacZ* reporter construct (marking a region of strong *dpp* expression), overlapping this domain only slightly (Fig. 2C). Thus, *kn*, like *en* and *ptc* (Strigini and Cohen, 1997) is expressed close to A-P boundary, whereas

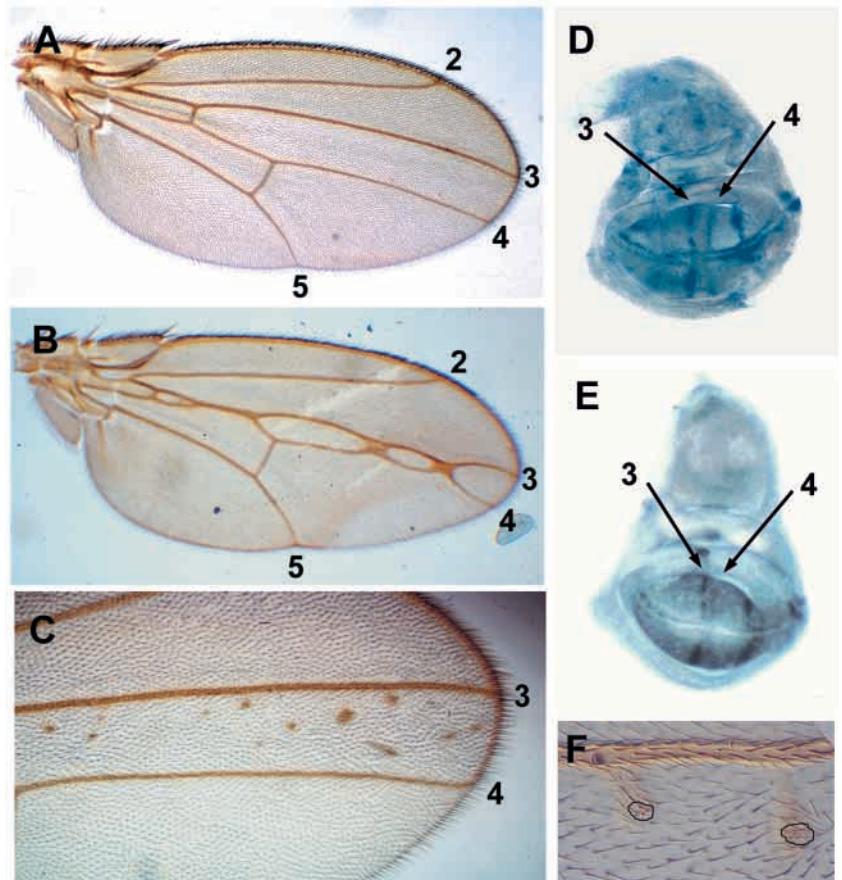


Fig. 1. Effects of *kn* mutations on wing venation. (A,B) Adult wings. (A) Wild-type wing. (B) *kn*¹/*kn*^{SA1} wing is lacking most of the intervein space between veins 3 and 4. (D,E) *rho* expression patterns in late third-instar wing imaginal discs. (D) Wild type. (E) *kn*¹/*kn*^{SA1}; the *rho* expression specific for veins 3 and 4 is shifted closer together. (C) Ectopic vein material induced by homozygous *kn*^{KN4} clones generated in the mid-third instar, marked with *pwn*. Only clones between veins 3 and 4 are associated with ectopic vein material. (F) Close-up of *pwn kn*^{KN4} clones showing domineering non-autonomy. Vein material extends outside the clone. (In this and all subsequent figures, wings are oriented anterior up and proximal left, whereas discs are oriented dorsal up, anterior left; veins are numbered 1-5.)

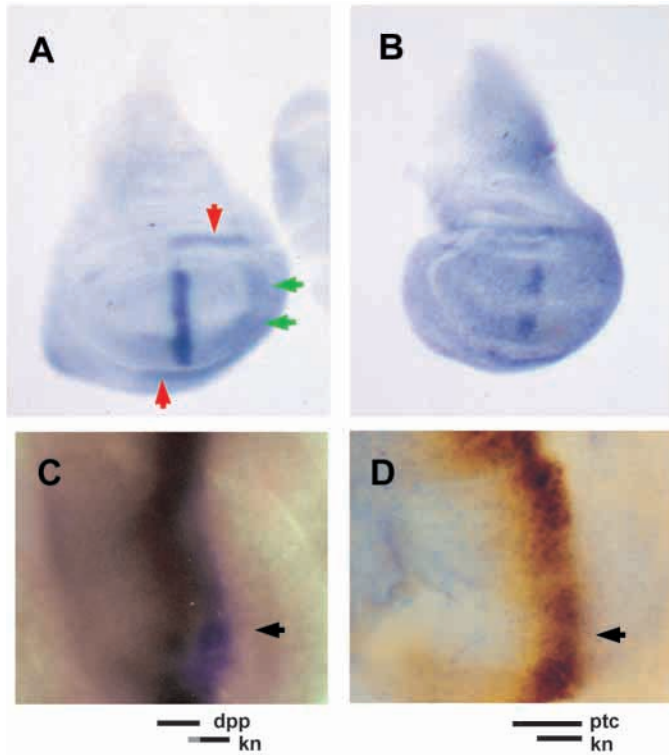


Fig. 2. Expression of *kn* compared to other target genes of Hh signalling. (A,B) *kn* RNA expression pattern in late third-instar wing imaginal discs of (A) wild-type and (B) *fu1* mutant. Low level expression in hinge region (red arrows) and posterior wing blade (green arrows) is marked in the wild-type disc. (C,D) *kn* RNA expression (blue) in the wing blade region compared with *lacZ* reporter gene expression (detected by HRP-labelled immunohistochemistry in brown or yellow) marking (C) *dpp* or (D) *ptc* expression. Beneath the stained discs are bars representing the width of the expression domains for the indicated gene at the point in the disc marked with an arrow; black bar, observed expression; grey bar, expected *kn* expression based on the normal width of the *kn* expression domain, but which cannot be observed due to the intensity of the *dpp* signal.

strong *dpp* expression is displaced slightly anteriorly with respect to *kn* and the A-P boundary. Similar results showing *kn* expression close to the A-P boundary, dependent on Hh signalling, have recently been reported by Vervoort et al. (1999).

Expression of the *rho* gene has been demonstrated to be a determinant for the vein primordia laid down in the late third instar wing disc (Sturtevant et al., 1993). Double-labelled in situ hybridizations reveal that *kn* is expressed precisely between the vein primordia for veins 3 and 4 (Fig. 3B). Thus, *kn* RNA expression matches the region determined by mosaic analysis for *kn* function: between veins 3 and 4 in the Hh-responsive cells of the wing blade.

***kn* expression inhibits expression of vein initiation genes**

Ectopic expression of *kn* under the control of the *MS1096-GAL4* driver reduces or eliminates the expression of two vein primordia markers, *rho* and *Dl*, as well as the resulting venation of the wing. In *MS1096-GAL4 UAS-kn* discs, *kn*

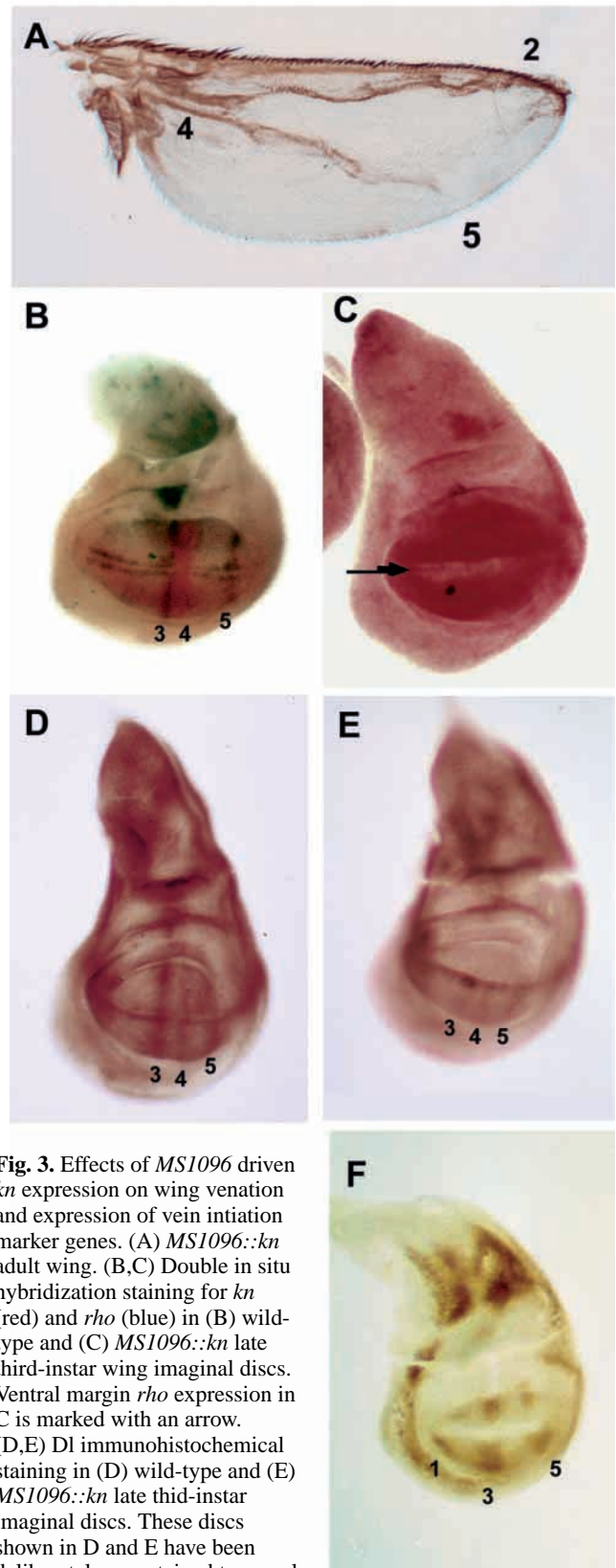


Fig. 3. Effects of *MS1096* driven *kn* expression on wing venation and expression of vein initiation marker genes. (A) *MS1096::kn* adult wing. (B,C) Double in situ hybridization staining for *kn* (red) and *rho* (blue) in (B) wild-type and (C) *MS1096::kn* late third-instar wing imaginal discs. Ventral margin *rho* expression in C is marked with an arrow. (D,E) DL immunohistochemical staining in (D) wild-type and (E) *MS1096::kn* late third-instar imaginal discs. These discs shown in D and E have been deliberately overstained to reveal the low-level expression in the ventral wing primordia in the *MS1096::kn* wing discs. (F) *iro* expression in *MS1096::kn* imaginal discs, as detected by immunohistochemical staining to β -galactosidase in the rF209 *lacZ*-reporter.

expression is driven at high levels in the dorsal wing blade of 3rd instar discs and more weakly in the proximal portion of the ventral region (Fig. 3C, in red). The only portion of the normal *rho* RNA expression domain still present in the *MS1096::kn* discs is the expression along the ventral margin (Fig. 3C, in blue marked with arrow) within the region of the wing blade where *kn* is not driven by *MS1096*. Similarly, *Dl* protein expression is approximately normal in the same ventral margin domain, greatly reduced in the ventral portions of the vein primordia for veins 3, 4 and 5, and completely absent from all of its normal domains in the dorsal portion (veins 3, 4, 5 and distal margin domain) of the wing blade in *MS1096::kn* discs (Fig. 3E). The relatively low expression of *Dl* in the ventral regions of all three vein primordia can only be observed following overstaining of the immunohistochemical detection (as is the case in Fig. 3E); because of the higher background levels of the *rho* in situ hybridizations it was not possible to detect similar low levels of *rho* expression that might also exist in the ventral regions. Taken together, these results suggest that the expression of the vein initiation genes on the dorsal side is completely eliminated where *kn* ectopic expression is high and at best poorly expressed in the vein primordia on the ventral side where *kn* ectopic expression is lower. Adult wings developing from such discs lack vein 3, the distal portion of vein 4 and the proximal portion of vein 5 (Fig. 3A). The veins most affected are normally dorsal veins that arise on the dorsal surface, where *rho* and *Dl* expression has been eliminated in the *MS1096::kn* discs. The ventral veins (veins 2, proximal 4 and distal 5) are diffuse, wide and plexate, suggesting that the compromised initiation of *Dl* and *rho* in the ventral vein primordia does not block vein formation but rather causes later problems in vein resolution during differentiation.

The expression of the vein initiation genes *rho* and *Dl* is anticipated by the prior expression of vein specific regulatory genes that serve to activate *rho* and *Dl* in individual stripes corresponding to each vein primordia (Sturtevant and Bier, 1995). *kn* ectopic expression driven by *MS1096* does not affect the expression of these vein-specific marker genes, i.e. *kni* (vein 2, Lunde et al., 1998) and *ara/caup* (odd-numbered veins, Gomez-Skarmeta and Modolell, 1996; Gomez-Skarmeta et al., 1996), nor does it affect the expression of *dpp* or *sal*, regulatory

genes functioning earlier in the vein patterning hierarchy (*ara/caup*, Fig. 3F; otherwise, data not shown). Thus, it appears that *kn* expression directly interferes with the activation of the vein initiation genes that mark the establishment of the vein primordia.

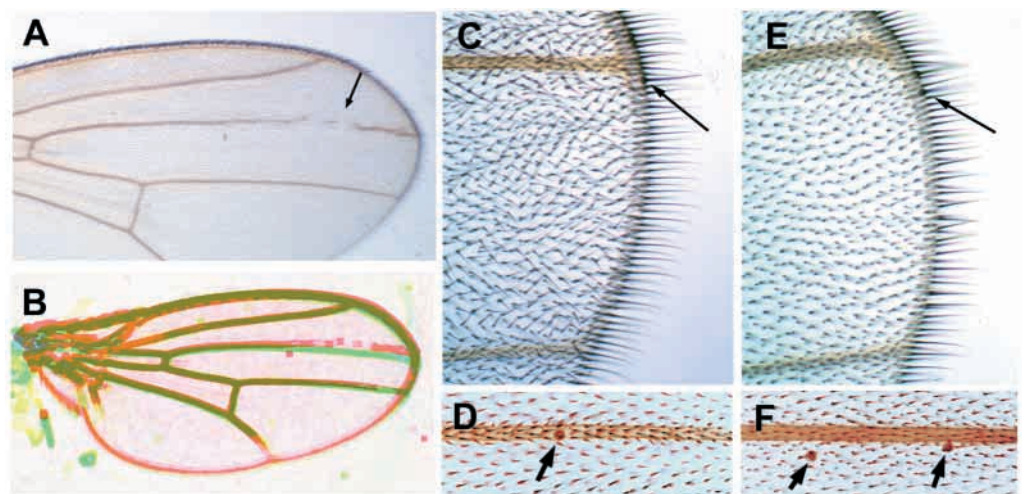
Anterior broadening of the normal *kn* expression domain shifts vein 3 anteriorly

As can be seen in Fig. 2C, the expression domain of *dpp* extends further anterior than that of *kn* and covers the primordium for vein 3 (Sturtevant et al., 1997). Because the primordium for vein 3 is laid down just anterior to the *kn* expression domain, *dpp-GALA* driven expression of *UAS-kn* should extend the *kn* expression domain anteriorly over the normal site for vein 3 formation. Vein 3 in *dpp::kn* wings is often disrupted in distal portions (Fig. 4A) and is shifted anteriorly (Fig. 4B). In normal wings, socketed ‘chemosensory’ bristles are found along the margin anterior to vein 3, with at most two such bristles posterior to vein 3 (Fig. 4C). In contrast, in *dpp::kn* wings vein 3 meets the margin considerably anterior to the posterior-most socketed bristle (by 7.7 ± 3.1 margin bristles, Fig. 4E). The suppression of the formation of socketed margin bristles posterior to vein 3 is believed to be due to *en* gene activity (Tabata and Kornberg, 1995; Hidalgo, 1994), and hence the displacement of the posterior-most socketed bristle from vein 4 is an indication of the range of *en* activation by Hh signalling. In *dpp::kn* wings, this distance is unchanged (38.4 ± 2.9 bristles in wild type versus 38.9 ± 1.8 bristles in *dpp::kn* wings). Thus, in the *dpp::kn* wings the range of Hh signalling appears to be unchanged while the 3-4 intervein space is increased by approximately 20% due to the anterior displacement of vein 3. In about 10-20% of the wings, this anterior shift in vein 3 is further confirmed by the presense of campaniform sensilla just posterior to vein 3, instead of in their normal location on this vein (Fig. 4D,F). Thus, the position of vein 3, which normally forms just anterior to the *kn* expression domain, is dependent on the width of the *kn* expression domain.

kn activity downregulates EGF-receptor expression

Vein induction is highly dependent on EGF signalling, as indicated by the activation of *rho*, a protein generally induced

Fig. 4. Effect of *dpp*-driven *kn* expression on the position of wing vein 3. (A) *dpp::kn* wing vein 3 exhibits gaps (arrow) in distal portions. (B) Overlay of wild-type (green) and *dpp::kn* (pink) wings. The veins in this figure have been artificially broadened for clarity. (C,E) Margin bristles of (C) wing-type and (E) *dpp::kn* wings. The posteriormost socketed bristle on the margin is marked with (arrow). (D,F) Close-up of vein 3 and associated campaniform sensilla on (D) wild-type and (F) *dpp::kn* wings.



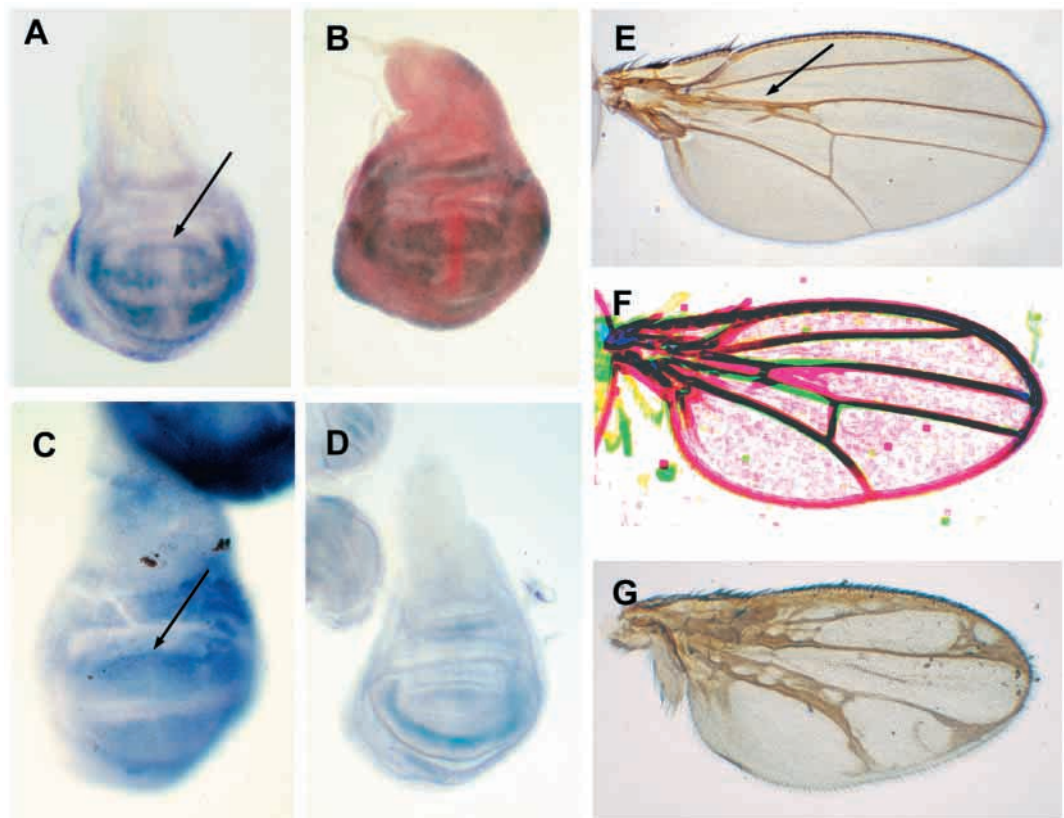
in regions of active EGF signalling that functions to amplify the EGF signalling process (Sturtevant et al., 1993; Guichard et al., 1999). Activation of EGF signalling, by ectopic expression in the developing wing blade of known *Drosophila* EGF-ligands (Vn and a secreted form of Spi), leads to the formation of ectopic venation in the wing (Schnepp et al., 1998). We therefore investigated the role of *kn* in the control of expression of genes involved in EGF signalling. Only one EGF-ligand, Vn, is known to be activated in the wing during the third larval instar at the time of vein initiation: *vn* mRNA is expressed in a stripe in the medial region of the wing just anterior to the A-P compartment (Simcox et al., 1996). However, no alteration of *vn* expression was noted either in *kn/kn^{SA1}* mutant discs or in discs in which *kn* had been ectopically expressed by the *MS1096* driver (data not shown), indicating that the expression of *vn* in this region is not controlled by *kn*.

Another gene involved in EGF signalling whose transcription is modulated at the time of vein initiation is the gene encoding the EGF-receptor, *EGFR*. Although transcription of *EGFR* is initially uniform throughout the wing blade, by the end of the third instar *EGFR* transcription has been repressed along the wing margin and in a medial stripe across the wing blade region (Guichard et al., 1999; Fig. 5A). Double in-situ hybridization reveals that the medial region of *EGFR* downregulation coincides with the region of *kn* gene expression (Fig. 5B). In *kn* mutants, downregulation of *EGFR* expression in the medial region is almost completely eliminated (Fig. 5C), indicating that the downregulation of *EGFR* expression is dependent on *kn*. The residual downregulated region of *EGFR* expression in *kn* mutants is

similar in width to the reduced 3-4 intervein space observed by *rho* expression (compare Figs 5C and 1G). Reciprocally, *EGFR* expression is greatly reduced in discs in which *kn* is ectopically expressed under the control of the *MS1096-Gal4* driver (Fig. 5D). *EGFR* transcripts are virtually completely absent in the dorsal portion of these discs and present only in the more distal portions of the ventral wing blade where *kn* is not ectopically expressed (compare *EGFR* staining in Fig. 5D with *kn* staining in Fig. 3C).

To determine whether downregulation of *EGFR* is necessary for the formation of the 3-4 intervein region we expressed *EGFR* ectopically, using *ptc-GAL4* to express *EGFR* in the region in which it is usually downregulated. Ectopic expression of *EGFR* in the medial wing driven by the *ptc-GAL4* driver (Fig. 5E) generates wings with fusion of veins 3 and 4 in the proximal portion of the wing. This phenotype is roughly similar to that observed in weak *fu* or *kn* mutations, in which vein fusions are limited to only the proximal region of the wing, suggesting that the downregulation in *EGFR* in the medial wing may play an important role in the spacing of veins 3 and 4. More distally, however, the spacing of veins 3 and 4 appears normal in the *ptc-GAL4* wings (Fig. 5F). Furthermore, ectopic expression of *EGFR* driven by the *MS1096* driver throughout the wing blade dorsally and weakly ventrally does not cause formation of ectopic veins between veins 3 and 4, although a significant amount of ectopic vein material is induced anterior to vein 3 and posterior to vein 4 (Fig. 5G). This suggests that the creation of a vein-free zone between veins 3 and 4 is not likely to be explained solely by downregulation of *EGFR* by *kn*.

Fig. 5. Effects of *kn* misexpression on *EGFR* expression in late third-instar wing discs. (A,C,D) *EGFR* RNA expression in (A) wild-type, (C) *kn¹/kn^{SA1}* and (D) *MS1096::kn* discs. Arrows in A and C denote the medial region of *EGFR* downregulation. (B) Double in situ RNA expression patterns for *kn* (red) and *EGFR* (blue) in late third-instar disc. (E-G) Venation associated with ectopic *EGFR* expression in the wing. (E) Wing from *ptc-GAL4, UAS-EGFR* fly. Arrow marks the region of 3-4 vein fusion in proximal wing. (F) Overlay of wild-type (green) and *ptc::EGFR* (pink) wings. (G) Wing from *MS1096, UAS-EGFR* fly. Ectopic venation is extensive in most of the wing, except between veins 3 and 4.



DISCUSSION

In this paper, we have demonstrated that the *kn* gene, encoding a COE-family transcription factor, is transcriptionally activated in Hh-responsive cells in the wing blade region of the third-instar imaginal disc adjacent to the anterior-posterior border (as has also been recently demonstrated by Vervoort et al., 1999). Expression of *kn* is dependent on the activity of *fu*, a component of Hh-mediated signalling, and further evidence by Vervoort et al. (1999) has demonstrated that *kn* expression is dependent on high levels of Hh signalling. The expression of *kn* in this region is required for the correct spatial activation of genes involved in the specification of the vein primordia, such as *rho* and *DI*, to properly position veins 3 and 4. Global expression of *kn* in the imaginal disc inhibits the activation of these vein initiation marker genes in their normal positions. Furthermore, widening the *kn* expression domain, in a manner independent of other elements of Hh signalling, widens the spacing between veins 3 and 4. We conclude that Hh signalling acts to directly specify the positioning of veins in the medial portion of the wing through its activation of *kn* expression. This activation of *kn* in the medial wing defines a vein-free zone, such that the primordia for veins 3 and 4 are laid down to either side.

While ectopic expression of *kn* throughout most of the wing imaginal disc results in the suppression of the *rho* and *DI* vein initiation markers (and of subsequent venation), this ectopic *kn* expression has no effect on the expression of upstream coordinate genes, such as *sal*, or the vein-specific regulatory genes. Most significantly, the expression of the functional redundant *ara* and *caup* homeobox genes of the *iro* (*iroquis*) complex, which are required for the proper initiation of all odd-numbered wing veins (including vein 3, whose position is controlled by *kn*; Gomez-Skarmata and Modolell, 1996; Gomez-Skarmata et al., 1996), are unaffected by *kn* ectopic expression. This indicates that *kn* functions to block the translation of the coordinate system of patterning genes into the activation of the various vein primordia. In this regard, it is significant that *kn* expression, either in its normal position in the medial wing or elsewhere in the wing when expressed ectopically, leads to the downregulation of the gene encoding the EGF-receptor, a required component for EGF signalling which is involved in the initiation of the all the longitudinal wing veins. This requirement for EGF signalling is evidenced by vein loss in wings with ectopically expressed antimorphic forms of *EGFR* or loss-of-function *EGFR* alleles (Diaz-Benjumea and Garcia-Bellido, 1990; Guichard et al., 1999) and by ectopic vein formation in wings with ectopically expressed activated *EGFR* (Queenan et al., 1997; Guichard et al., 1999) or the EGF ligands, Vn and secreted Spi (Schnepp et al., 1998).

Nestoras et al. (1997) noted that *kn* is required to prevent ectopic vein induction in the wing in two contexts: (1) normally in the medial wing (where Hh signalling normally occurs) and (2) in *ptc*-clones in the anterior compartment (in which an Hh response is activated because of the loss of *ptc* activity). Those results indicated that *kn* activity is under Hh signalling control, which is confirmed by the demonstration in this study and those of Vervoort et al. (1999) that *kn* transcription in the medial wing is limited to Hh-responsive cells and is dependent on components of Hh signalling, most notably on Fu kinase activity. Further, the mosaic analysis indicates that *kn*'s role in

the medial wing is to suppress vein formation. The ectopic veins that form in *kn* clones in the medial region or in *ptc kn* clones elsewhere in the anterior compartment can easily be explained by the failure of repression of vein initiation in those clones, which would be induced by a ligand synthesized within the clones or in adjacent medial region tissue. The existence of a vein-promoting factor activated by Hh signalling is based on the analysis of *ptc* mutant clones where a vein is invariably induced just outside the clone (Phillips et al., 1990).

Vn is an excellent candidate for a vein-promoting factor produced in the medial wing under Hh-control. Vn is expressed in the medial portion of the wing during the late third instar (Simcox et al., 1996), and hence is a candidate for such a signal. Ectopic expression of Vn (as well as the secreted form of the other somatic EGF-ligand, Spi) leads to the production of veins throughout the wing, with the exception of the medial wing where *kn* activity blocks vein initiation (Schnepp et al., 1998; J. M., unpublished results). The viable, *vn*¹ allele removes most of vein 4 with a small gap in the proximal region of vein 3 (Puro, 1982), indicating a dependence of veins 3 and 4 for *vn* function, and clones of strong (though hypomorphic) *vn* alleles located in the anterior compartment lead to the non-autonomous loss of vein 4 in the posterior compartment (Garcia-Bellido et al., 1994). Analysis of heteroallelic combinations of *vn*¹ with strong embryonic lethal alleles and its synergistic interactions with other wing patterning genes, however, suggest that *vn* also contributes to the formation of other veins (Diaz-Benjumea and Garcia-Bellido, 1990; Sturtevant and Bier, 1995; Simcox et al., 1996). Furthermore, the observation that *vn* transcription in the medial wing is independent of *kn* activity would suggest its underlying vein-promoting activity would remain in *kn*-mutant clones, thereby causing the formation of ectopic vein material in those clones.

Fig. 6 shows our working model for how the signalling events in the medial wing might define the position of the primordia for veins 3 and 4. In this model, Hh signalling across the anterior-posterior compartment boundary inactivates the Ptc receptor and activates the Ci transcription factor in a process requiring the participation of the ser/thr kinase, Fu. Activated Ci leads to the transcription of *kn*, whose product interferes with vein initiation. One way in which *kn* activity may interfere with vein initiation maybe by downregulation of *EGFR* transcription. Since the vein-promoting EGF ligand, Vn, is also synthesized in the medial wing (Simcox et al., 1996), it can potentially induce vein formation only where the EGF-receptor has been synthesized on either side of the zone of Hh-responsive cells in which *kn* is active. The activation of EGF signalling by Vn leads to the initiation of vein primordia and the expression of vein initiation genes, such as *rho* and *DI*. Subsequent events in vein differentiation, most importantly vein resolution involving Notch-dependent lateral inhibition, position veins 3 and 4 in the middle of their respective vein primordia a few cells away from the original margin of *kn* expression.

This proposed mechanism by which veins 3 and 4 appear to be positioned in the wing is remarkably similar to that proposed by Bier and colleagues (Sturtevant et al., 1997; Biehs et al., 1998; Lunde et al., 1998) for the positioning of vein 2. In both cases, the vein primordia are induced just outside a distinct region of gene expression: vein 2 is induced along the anterior border of the *sal* expression domain whereas veins 3

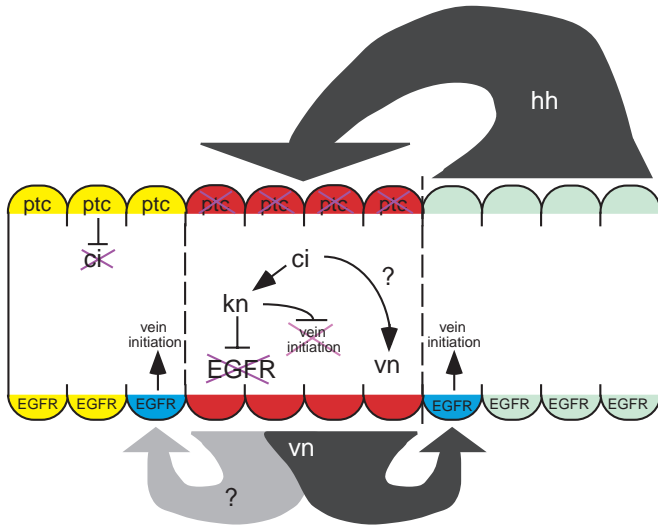


Fig. 6. Model for how Hh signalling in the medial wing may define the position of the veins 3 and 4 primordia. Hh morphogen is secreted from posterior compartment cells (green) to activate adjacent anterior compartment cells (red), causing the inactivation of the Ptc receptor (red). Inactivation of the Ptc receptor causes the activation of the transcription factor Ci. Activated Ci initiates the transcription of Hh-target genes, including *kn* and *vn* (as well as *ptc*, *dpp* and *en*, not shown). Kn activity creates a vein-incompetent region in the medial wing, in part by downregulating EGFR transcription (red). The secretion of the Vn ligand from these cells activates the EGF-receptor in adjacent cells, inducing the primordia for veins 3 and 4 and stimulating the transcription of the vein initiation genes *rho* and *Dl* (blue).

and 4 are induced to either side of the *kn* expression domain. In the case of vein 2, *sal* expression acts nonautonomously to induce vein initiation in adjacent tissue, suggesting that *sal* (itself a transcription factor) controls the expression of a vein-inducing factor (denoted as factor X by Sturtevant et al., 1997) and, secondarily, causes the cells expressing that factor to be non-responsive to that signal. Similarly, in the medial wing, the vein-inducing signal, Vn, is induced in response to Hh signalling, while induction of *kn* in these same cells makes them nonresponsive to vein induction. Thus, for at least these three of the five *Drosophila* longitudinal wing veins, each appears to be positioned adjacent to a distinct region of gene expression in the imaginal disc, which secretes a vein-inducing signal to the flanking tissue, but is itself non-responsive to that signal.

It should be noted that downregulation of *EGFR* is not the sole mechanism by which *kn* suppresses vein formation in the medial wing, especially in the distal portion of the wing blade. If *EGFR* downregulation were the principal mechanism, we would expect that driving expression of *EGFR* in the medial wing would result either in the fusion of veins 3 and 4 or, at least, the formation of ectopic veins between veins 3 and 4. Indeed, *ptc*-driven ectopic expression of *EGFR* does result in the loss of 3-4 intervein material proximal to the anterior crossvein (Fig. 5E). However, it does not significantly alter the spacing of veins 3 and 4 distal to the posterior crossvein nor does it induce ectopic venation between them. Similarly, ectopic expression of *EGFR* throughout most of the wing blade

(when driven by *MS1096*) results in ectopic vein material in most of the wing, except for the intervein space between vein 3 and 4. These results indicate that a second mechanism, in addition to downregulation of the EGF-receptor, must exist to position veins 3 and 4 and prevent vein formation between them, at least in the distal portion of the wing.

This secondary mechanism for blocking vein formation between veins 3 and 4 may reflect the fact that Vn is not the only vein-promoting factor synthesized in the medial wing: local Dpp signalling may also play a significant role in the activation of vein 3. Gomez-Skarmeta and coworkers (Gomez-Skarmeta and Modolell, 1996; Gomez-Skarmeta et al., 1996) have demonstrated that Dpp signalling in concert with Hh signalling is required for the activation in the medial wing of the *iro*-complex genes, *ara* and *caup*, which in turn are required for the formation of vein 3. The expression of the *iro* genes is not sufficient to activate vein 3 initiation, since *kn* overexpression in this region does not affect *iro* gene expression, but does eliminate expression of the vein initiation genes. We currently favor the hypothesis that, whereas vein 4 is activated primarily by Vn signalling onto the adjacent EGFR-active domain, vein 3 is combinatorily activated by Vn signalling and by Dpp signalling mediated through *iro* complex activation. While the Vn and Dpp-inducers may be expressed over a fairly broad domain in the medial wing, because of the block on vein initiation provided by *kn* activity, vein 3 must form just outside the *kn* expression domain. The gaps that form in vein 3 when the *kn* expression domain is expanded anteriorly may be caused by moving the border of the *kn* expression domain out of range of Vn signalling and either high-level Dpp signalling or the resultant *iro* expression domain. This process is most likely to produce gaps in the distal portion of vein 3, since the expression of both *vn* and *iro* is not as wide in the distal regions of the disc as in the proximal regions (Simcox et al., 1996; Gomez-Skarmeta and Modolell, 1996; Gomez-Skarmeta et al., 1996).

The expansion of the 3-4 intervein region in wings resulting from *dpp*-driven *kn* expression, without a concerted shift in margin bristle type suggests that these two features of the 3-4 intervein region (the position of vein 3 and transition to socketed bristles) are controlled independently by Hh signalling in this region. Marginal bristle character seems to be primarily under the control of *en*, which is also induced by Hh signalling in medial region of the wing blade. *en* clones induced in the 3-4 intervein region along the margin contain socketed bristles (Hidalgo, 1994; Tabata et al., 1995) and, while *ptc* clones induced on the margin in other regions of anterior compartment suppress the formation of socketed bristles within the clone, *ptc en* double mutant clones do not (Tabata and Kornberg, 1994). In contrast, *kn* mutant clones in the 3-4 intervein region and *ptc kn* double mutant clones elsewhere retain the ability to suppress socketed bristles along the margin, although allowing ectopic vein formation within the clone (Nestoras et al., 1997). These results suggest that *kn* and *en* have relatively independent roles in the 3-4 intervein region: *kn* to specify the positions of veins 3 and 4, *en* to specify the 'identity' of the region, at least with respect to marginal bristle character. However, some link between these two patterning events must exist, since in wings of hypomorphic, viable *kn* alleles (*kn¹* and *kn^{SA2}*), the transition to socketed bristles still occurs on vein 3 despite its shift

posteriorly closer to the A-P compartment boundary (Nestoras et al., 1997). Presumably, *kn* must also act to coordinate these two characters of medial wing pattern.

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