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Connecting Hh, Dpp and EGF signalling in patterning of the *Drosophila* wing; the pivotal role of *collier/knot* in the AP organiser

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SUMMARY

Hedgehog (Hh) signalling from posterior (P) to anterior (A) cells is the primary determinant of AP polarity in the limb field in insects and vertebrates. Hh acts in part by inducing expression of Decapentaplegic (Dpp), but how Hh and Dpp together pattern the central region of the *Drosophila* wing remains largely unknown. We have re-examined the role played by Collier (Col), a dose-dependent Hh target activated in cells along the AP boundary, the AP organiser in the imaginal wing disc. We found that *col* mutant wings are smaller than wild type and lack L4 vein, in addition to missing the L3-L4 intervein and mis-positioning of the anterior L3 vein. We link these phenotypes to *col* requirement for the local upregulation of both *emc* and N, two genes involved in the control of cell proliferation, the EGFR ligand Vein and the intervein determination gene *blistered*. We further show that attenuation of Dpp signalling in the AP organiser is also *col* dependent and, in conjunction with Vein upregulation, required for formation of L4 vein. A model recapitulating the molecular interplay between the Hh, Dpp and EGF signalling pathways in the wing AP organiser is presented.

Key words: Limb patterning, Hh, Dpp, EGF, Signalling, *collier*, *Drosophila melanogaster*, Proliferation

INTRODUCTION

The secreted proteins of the Hedgehog (Hh) family play a crucial role in the patterning of many structures in vertebrates and invertebrates, by providing differentiating cells and tissues with positional information (Ingham and McMahon, 2001). Hh is a central patterning signal in the *Drosophila* wing, which, like all adult appendages in the fly, derives from a monolayer epithelium known as an imaginal disc, which is subdivided by a lineage restriction into anterior (A) and posterior (P) compartments. Hh synthesised by P cells diffuses into the A compartment forming a short-range activity gradient that activates the localised expression of a number of target genes, including *patched* (*ptc*) and *decapentaplegic* (*dpp*, a TGFβ family member). By sequestering extracellular Hh, Ptc limits the diffusion and range of activity of Hh (Chen and Struhl, 1996). Dpp, a long-range morphogen, diffuses into both the A and P compartments, and acts to relay positional information from the AP organiser, controlling growth and patterning of the distal regions of the wing (Brook et al., 1996; Lawrence and Struhl, 1996). A similar relay mechanism has been proposed for anteroposterior patterning of the vertebrate limb. In this case, Sonic Hedgehog (Shh) initially acts at long range to prime the region of the limb competent to form digits, and later acts at short range to induce expression of bone morphogenic proteins (Bmps/TGFβ), the morphogenetic action of which specifies digit identity (Drossopoulou et al., 2000). In both the *Drosophila* wing and vertebrate limb, however, it remains unclear how Hh (Shh) exerts its direct patterning activity.

One morphological read-out of the AP patterning information in the *Drosophila* wing is the positioning of intervein and longitudinal L2 to L5 provein domains in larval imaginal discs (Sturtevant and Bier, 1995). Position of veins is prefigured by expression of *rhomboid* (*rho*) and activation of the EGF pathway in the central provein cells, which will differentiate as the adult vein cells (De Celis, 1998; Guichard et al., 1999; Sturtevant et al., 1993). Activation of *blistered/Drosophila Serum Response Factor* (*bs*/*D-SRF*) in complementary regions endows cells with an intervein fate (Montagne et al., 1996; Price et al., 1989; Roch et al., 1998; Sturtevant et al., 1993).

Hh short-range activity is responsible for patterning the central region of the *Drosophila* wing: specification of the L3-L4 intervein region, the anterior L3 vein and the posterior L4 vein (Mullor et al., 1997; Strigini and Cohen, 1997). We have previously shown that this activity is mediated by activation of the transcription factor Collier/Knot (Col/Kn), in a narrow stripe of cells along the AP boundary that we designate below as the AP organiser. The restricted domain of *col* expression, compared with that of *dpp*, established that *dpp* and *col* are induced by different levels of Hh activity, providing a paradigm for Hh morphogen properties (Vervoort et al., 1999). Col activates and/or maintains high levels of expression of BS and downregulates expression of EGFR in the AP organiser (Mohler et al., 2000; Vervoort et al., 1999), therefore preventing these cells from adopting a provein fate. Formation of posterior L4 vein requires activity of *vein* (*vn*), which encodes a diffusible neuregulin-like protein, one of the

known EGFR ligands and activates the EGF pathway (Schnepp et al., 1996; Simcox et al., 1996). Like *col*, *vn* is transcribed in anterior cells along the AP border, consistent with mosaic analyses indicating that *vn* activity is provided by anterior cells (Garcia-Bellido et al., 1994). Together, these data led to the proposal that Hh patterns the central part of the wing via activation of *col* and *vn* in cells along the AP boundary (Mohler et al., 2000; Vervoort et al., 1999). *col* requirement for L4 vein formation has not been previously investigated, however, and the links between *col* and *vn* activity remain elusive. Moreover, the molecular mechanisms involved in positioning of the L3 vein remain to be firmly established.

In this report, we re-examine in depth the role of *col* activity in establishing the vein pattern and size of the wing. First, detailed morphological comparison of the hypomorphic and null *col* mutant wings allowed to precise the null mutant phenotype: a reduced size of the wing contributed by both reduction of the posterior compartment and lack of L3-L4 intervein, the loss of L4 vein, and a posterior shift in the position of L3 vein. At the molecular level, we show that, in addition to BS, *col* upregulates transcription of *vn* in the AP organiser, providing a first molecular interpretation for Col requirement for L4 vein formation. Col is also required for local upregulation of *extramacrochaete* (*emc*) and Notch (N), two genes involved in the control of cell proliferation, thus linking Hh signalling to cell division in the L3-L4 intervein primordium. Furthermore, we show that the recently reported downregulation of Dpp signalling in cells receiving high doses of Hh (Tanimoto et al., 2000) is mediated by *col* activity. By manipulating the levels of *vn* expression and Thickvein (Tkv, a type 1 Dpp receptor) activity, we provide evidence that modulation of Dpp signalling in the AP organiser is essential both for positioning L3 vein and formation of L4 vein. The integrative role of Col thus revealed a complex and previously underemphasised interplay between the Hh, Dpp and EGF pathways in determining the size and vein pattern of the *Drosophila* wing.

MATERIALS AND METHODS

Mutant and transgenic *Drosophila* strains

col^1 is classified as a null allele and kn^1 as an hypomorphic allele of *col* (Crozatier et al., 1999; Vervoort et al., 1999). The *P[col5-cDNA]* transgene allows to rescue the embryonic lethality (Crozatier et al., 1999), but not the adult wing phenotype of col^1 mutants. vn^{M2} /*TM3, Sb* was provided by A. Garcia-Bellido.

The transgenic lines *UAS-Vn*, *UAS-TkvDN* (TkvDN corresponds to Tkv1DGSK, which lacks the GS box and kinase domain), *dpp-Gal4* and *ptc-Gal4* have previously been described (Haerry et al., 1998; Morimura et al., 1996; Simcox et al., 1996). For ectopic expression of *vn* and *tkvDN* in the col^1 genetic background, the *ptc-Gal4, UAS-TkvDN* and *UAS-Vn* transgenes were introduced into the col^1, *P(col5-cDNA)* chromosome by recombination.

Clonal analysis

Clones of col^1 mutant cells were generated by Flp-mediated mitotic recombination (Xu and Rubin, 1993) by heat-shock treatment at 37°C for 2 hours of first and second instar larvae of the following genotype: *y, w, Hs-Flp/+; FRT-42D, ubi-GFP/ FRT-42D,* sha^{VAIS1}, col^1. The *FRT-42D,* sha^{VAIS1}, col^1 chromosome was constructed by recombination. The cell marker was *shavenoid* (*sha*).

In situ hybridisation and antibody staining

In situ hybridisation of wings of third instar larvae and pupae were carried out as described elsewhere (Sturtevant et al., 1993). Double labelling with two probes simultaneously was described elsewhere (Crozatier et al., 1996), using the alkaline phosphatase substrate kit 1 provided by Vector. RNA probes were synthesised from cDNA plasmids except for the *col* probe, which contains intronic sequences (Crozatier and Vincent, 1999). The following primary antibodies were used: rabbit anti-Col, anti-P-Mad (Persson et al., 1998) (provided by T. Tabata), anti-phospho histone H3 (from Upstate Biotechnology), rat anti-Ci (which recognises both Ci155 and Ci75, a gift of R. A. Holmgren), mouse monoclonal N (C17.9C6) from Hybridoma bank (a gift of Véronique Van de Bor) and mouse monoclonal anti-BS produced by Michael Gilmam at Cold Spring Harbor Laboratory.

RESULTS

Col/Kn specifies the central part of the wing and controls wing size

We have previously reported that in col^1 mutant flies [col^1 is a null mutation; col^1 adults are obtained upon rescue of the embryonic lethality by a *col* transgene (Crozatier et al., 1999)], the wings completely lacked the intervein normally separating L3 and L4 veins. In col^1/kn^1 mutant wings (kn^1 is a hypomorphic viable mutation) (Diaz-Benjumea and Garcia-Bellido, 1990; Nestoras et al., 1997) the L3-L4 intervein was only reduced in size, with an occasional partial apposition of L3 and L4 veins (Vervoort et al., 1999) (Fig. 1A-C). Detailed inspection indicated that, in both hypomorphic and null *col* mutants, there is a 20% reduction in the overall wing size compared with wild type (Fig. 1D). To investigate this phenotype, we compared the surface area of each intervein in col^1, col^1/kn^1 and wild-type wings. Average values were obtained from measurements of 10 individual wings of each genotype (data not shown). In col^1/kn^1 wings, the size of the L3-L4 intervein was 66±6% smaller that of wild type, whereas the size of the other A interveins (anterior margin to L2 and L2-L3) was unchanged, indicating that the reduction in number of cells is specific to this intervein. Surprisingly, however, in col^1 mutant wings in which the L3-L4 intervein (and L4 vein, see below) were missing, the L2-L3 domain was 12±5% larger than in either wild-type or col^1/kn^1 wings. Finally, in both col^1 and col^1/kn^1 wings, the L4-L5 and L5 to posterior margin intervein sectors were smaller than in wild type, by 17±5% and 27± 4%, respectively. Taken together, our data indicated that the reduced size of *co1* mutant wings was due to the reduced size (or absence) of both the L3-L4 intervein and posterior interveins (Fig. 1D). As *col* expression is restricted to the L3-L4 intervein (Vervoort et al., 1999), its activity on the regulation of cell proliferation in the P compartment must be cell non-autonomous. The larger size of the L2-L3 intervein in col^1 wings raised both questions of the identity of the single central thick vein observed in these wings and its position relative to the AP boundary. This vein showed the presence of campaniform organs and dorsal corrugations, which are specific for L3 vein (reviewed by Campuzano and Modolell, 1992; Milan et al., 1997). The *col* mutant phenotype therefore does not correspond to apposition of L3 and L4 veins, as first proposed (Vervoort et al., 1999; Mohler et al., 2000) but to loss of L4 vein and repositioning of L3 vein.

The central vein in *col*^{1} wings is of L3-type and is displaced closer to the AP boundary

We have previously shown that BS, an intervein cell marker, is not expressed in the presumptive L3-L4 intervein in *col*^{1} wing discs of third instar larvae (Vervoort et al., 1999). We asked whether this central area devoid of BS staining corresponded to presumptive L3 or L4 vein, or was composite and examined BS expression in 24 hours APF (after puparium formation) pupae; BS expression enables each longitudinal wild type provein to be visualised as a three- to four-cell wide stripe devoid of staining (Fig. 2A). In *col*^{1} mutant wings, instead of L3 and L4 proveins, there was a single central stripe of cells, wider than the wild type L3, which did not express BS (Fig. 2B). A truncated stripe of BS-negative cells located near the presumptive hinge (arrow in Fig. 2B) correlated with the residual L4 vein observed in a proximal position in *col*^{1} adult wings (arrow in Fig. 1C). This indicated that L4 vein was lacking and suggested that the central vein in *col*^{1} mutant wings was a widened L3 vein. In order to confirm this, wings of wild-type and *col*^{1} pupae at 28-30 hours APF were stained for Ci, which is expressed only by A cells (Blair, 1992) (Fig. 2C,D). At that stage, resolution of provein into vein has occurred and the *col*^{1} central vein is morphologically distinguishable (arrowhead in Fig. 2D). All cells of this vein expressed Ci, indicating that it was entirely located in the anterior compartment, confirming its L3-like identity (L3m – m for mutant). However, although the posterior limit of Ci expression bisected the wild type L3-L4 intervein (Fig. 2C), it coincided with the posterior limit of *col*^{1} L3m vein. This shows that the position of L3m vein is shifted towards the AP boundary, correlating with the increased size of the L2-L3m intervein of *col*^{1} adult wings (see above). Taken together, morphological observations and size measurements of adult wings and immunostaining of pupal discs revealed three types of defects in *col*^{1} mutant wings: a reduced number of cells, the lack of L4 vein and a posteriorwards shift in the position of a wider L3 vein.

Reduced proliferation of A cells close to the AP boundary; *col* requirement for *emc* and N expression

The observation that *col*^{1}/*kn*^{1} wings displayed both an intervein L2-L3 of normal size and a reduced L3-L4 intervein suggested that the loss of the L3-L4 intervein and the displacement of L3 vein observed in *col*^{1} mutant were not necessarily linked. The specific loss or reduction of the L3-L4 intervein territory in *col* adult wings could be due to local programmed cell death and/or reduced cell proliferation. To distinguish between these possibilities, we stained wild-type and *col*^{1} pupal wing discs with a probes for *reaper* (*rp*), a marker for apoptotic cells (Abrams et al., 1993), and an antibody against a phosphorylated form of histone H3 (H3P), which reveals mitosis. No difference in *rp* expression was detected between wild-type and *col*^{1} mutant wings, which excluded a major contribution from apoptosis and suggested instead defective proliferation. In wild-type pupal wings, a wave of mitosis takes place in each intervein primordium between 15 and 21 hours APF (Schubiger and Palka, 1987). H3P staining of wild-type pupal wings 18-20 hours APF allowed the visualisation the proliferation of intervein cells along the proveins (Fig. 3A). Staining was specifically absent from the central region of either *col*^{1} or *col*^{1}/*kn*^{1} mutant wings (Fig. 3B and data not shown), correlating with changes from intervein to provein fate. However, the reduced size of the medial region of the wing already detectable at this early pupal stage probably reflects an earlier proliferation defect. Double staining with BS and propidium iodide (PI; not shown), which labels all nuclei, enabled us to count the number of rows of cells that separate the L2-L3 and L4-L5 intervein primordia in wild-type and *col*^{1} larval discs. Ten independent measurements gave average numbers of 10.5 and 7.5 rows, respectively, indicating a significant reduction in *col*^{1} mutants (not shown). We then looked at the expression of *extramacrochaetae* (*emc*), which encodes a helix-loop-helix (HLH) protein lacking a basic motif, and Notch (N), as both genes have previously been shown to be involved in the control of cell proliferation in the wing (De Celis and Garcia-Bellido, 1994; De Celis et al., 1995; Ellis et al., 1990; Garcia-Bellido et al., 1976; Garrell and Modolell, 1990). In third instar larvae, *emc* is expressed at a low level throughout the wing disc and at a higher level in two stripes of cells corresponding to the prospective A margin and the AP organiser (Baonza and Garcia-Bellido, 1999; de Celis et al., 1995) (Fig. 3C). Unmodified at the A margin, *emc* expression was completely lost from the AP organiser cells in either *col*^{1} or *col*^{1}/*kn*^{1} mutant discs (Fig. 3D and data not shown), showing that Col is required for *emc* transcription in the L3-L4 intervein primordium. Levels of N protein are high in intervein regions and low in presumptive vein territories in late third instar (De Celis et al., 1997). In *col*^{1} mutants, N is downregulated in the L3m provein domain (data not shown). *col* requirement for *emc* and N upregulation in the AP organiser cells is consistent with the reduced cell number in the central region of *col*^{1} mutant discs. This could not, however, account for the reduced number of cells in P interveins also observed in *col* mutant wings.

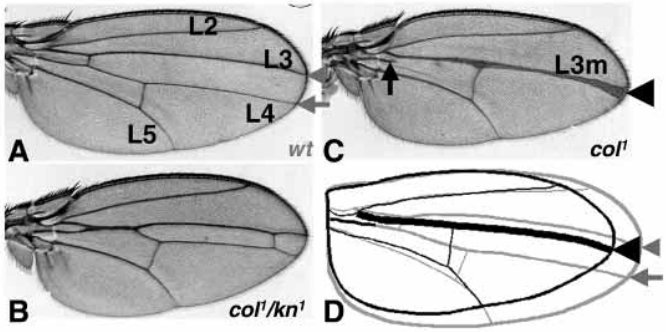

Fig. 1. *col/kn* mutant wings: abnormal vein pattern and reduced size. (A) Wild-type wing with the longitudinal L2 to L5 veins indicated. The small arrowhead and arrow point to veins L3 and L4, respectively. (B) *col*^{1} /*kn*^{1} mutant wing; veins L3 and L4 are closer to each other than in wild-type wing and partly apposed proximally. (C) *col*^{1}/*col*^{1} wing (abbreviated *col*^{1} in subsequent figures). L2 and L5 veins are normal, but a central larger L3-type vein is present (L3m vein, large arrowhead), whereas only a small, proximal region of L4 vein forms (vertical arrow). *col* wings are smaller than wild type, owing to (partial) loss of the region between L3 and L4 veins and a reduced size of the posterior compartment. In addition, *col*^{1} wings display a wider L2-L3 intervein than do wild type or *col*^{1} /*kn*^{1}. (D) Schematic superimposition of wild type (grey) and *col*^{1} (black) wings adjusted so as to align the proximal L4 vein. All wings are shown at the same magnification.

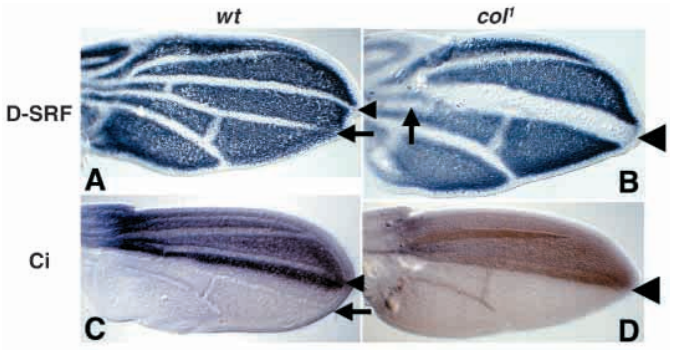

Fig. 2. Loss of L4 vein and posteriorwards shift of L3 vein in *col*^{1} mutant wings. (A,B) BS (D-SRF in figure) expression in 24 hours APF pupae. The central BS labelling which prefigures the L3-L4 intervein in wild type wings (A) is missing in *col*^{1} pupae (B), confirming the presence of a single central provein in *col*^{1} mutants. (C,D) Ci expression in pupal wings 28 hours APF. The posterior limit of Ci expression bisects the L3-L4 intervein in wild-type wings (C) but coincides with the posterior limit of L3m vein in *col*^{1} wings, indicating a posterior shift in its position (D). Positions of the L3, (residual) L4 and L3m veins are indicated as in Fig. 1.

rho expression in *col*^{1} mutant discs prefigures the posterior shift of L3m vein and the absence of L4 vein

That Hh short-range patterning activity in the wing (Biehs et al., 1998; Mullor et al., 1997; Strigini and Cohen, 1997) is mediated by *col* was first revealed by *hh* overexpression experiments in which the anterior displacement of L3 vein observed in these conditions could be reverted by reducing the *col* dose (Vervoort et al., 1999). This was confirmed by the posterior shift of L3 vein in *col*^{1} mutant wings (Fig. 2C,D). However, the molecular mechanisms involved remained unknown. We first examined the position of vein primordia in *col*^{1} mutant discs, using *rho* in situ hybridisation, which labels the vein primordia (Sturtevant et al., 1993). In wild-type third instar wing discs, single rows of *rho*-positive cells marked the positions of L3 and L4 veins on either side of the AP boundary (Fig. 4A). In *col*^{1} mutant discs, we observed two types of changes: first, whereas *rho* was only expressed in a few cells in the dorsal presumptive L4 vein, it was expressed in two to four rows of cells, rather than a single one, in the presumptive L3 vein (Fig. 4B). Second, the distance separating the L3 and residual L4 veins was reduced (Fig. 4B), suggesting a posterior shift, in addition to widening, of the presumptive L3m vein, compared with L3. We next directly compared *rho* and *col* expression, as *col* transcription, which is not modified in *col* mutant discs, marks the cells receiving high doses of Hh, thus enabling the AP boundary to be positioned. In wild-type third instar imaginal discs, *rho*-expressing cells flanked the *col* expression domain on both sides, with one to two rows of intercalary cells expressing neither gene (Fig. 4C). In *col*^{1} mutant discs, the L3m *rho* and *col* expression domains partially overlapped, confirming that the position of L3m vein has been shifted posteriorwards by several cell diameters relative to wild type (Fig. 4C,D), as previously deduced from Ci labelling of 24 hours APF pupae (Fig. 2D). However, *rho* labelling did not abut the AP boundary, indicating that *rho* transcription remained repressed in the anterior cells, which express En (Blair, 1992) (Fig. 4I). The changes in position and number of *rho*-expressing cells in L3m and the lack of *rho* expression in L4 vein show that the abnormal vein pattern of *col*^{1} adult wings reflects patterning defects occurring in third instar larvae (Fig. 4I).

Positioning of L3 vein involves Col-dependent down-regulation of Dpp signalling in the AP organiser

The displacement of L3m *rho*-expressing cells in *col*^{1} discs confirmed that Col mediates Hh activity in positioning the L3 vein. However, specification of L3 vein per se does not depend on *col*, but on activity of the homeobox-containing genes *araucan* (*ara*) and *caupolican* (*cau*) from the *iroquois* complex (*iro-C*) (Gomez-Skarmeta et al., 1996; Gomez-Skarmeta and Modolell, 1996). In order to investigate how the processes of vein specification and vein positioning were connected, we examined the expression domain of *ara* and *col* in wild type and *col*^{1} third instar imaginal discs. In wild-type discs, *ara* and *col* expression partly overlapped, with *ara* expression extending anteriorly by about three rows of cells into the region corresponding to presumptive L3 vein (Fig. 4I). In *col*^{1} mutant discs, the stripe of *ara* expression was both weaker and narrower. The posterior border of *ara* expression which is situated within the *col* expression domain and defined by En repression (Gomez-Skarmeta and Modolell, 1996) was not modified, but its anterior border was shifted by two to three rows of cells closer to the AP boundary (Fig. 4F,I). It has previously been reported that positioning of the anterior border of *iro-C* expression involved transcriptional repression by *sal/salr*, which are themselves targets of Dpp signalling (De Celis and Barrio, 2000). This regulation by *sal/salr* raised the possibility that the posterior shift of L3m vein position in *col*^{1} wing discs could be due to a modified range of Dpp signalling. In that respect, the recent report that Dpp signalling was

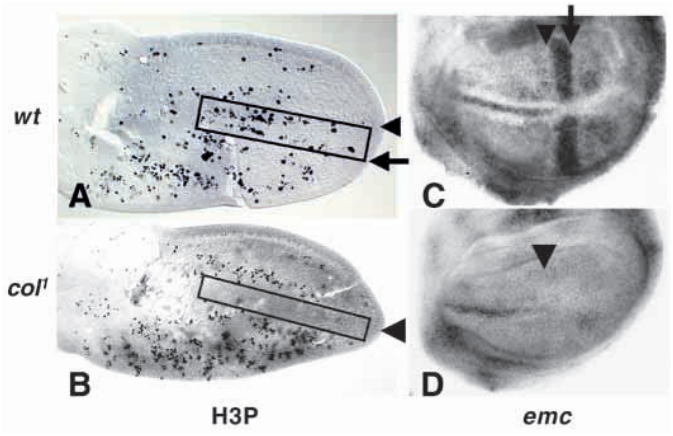

Fig. 3. *col* requirement for *emc* upregulation and cell proliferation in the L3-L4 intervein primordium. Cell proliferation in wild-type (A) and *col*^{1} (B) pupal wings, 18-20 hours APF, as visualised by the phosphorylation pattern of Histone H3 (H3P). (A) In wild type, a wave of cell proliferation occurs along veins in each intervein region at this stage, including the L3-L4 presumptive intervein (black frame) No such wave occurs in the medial region of a *col*^{1} wing (B). (C,D) *emc* expression in third instar wing discs. Upregulation in the AP organiser of wild-type discs (C) is specifically lost in *col*^{1} mutant discs (D). The small arrowhead and arrow indicate proveins L3 and L4, respectively.

attenuated in the AP organiser because of downregulation of expression of Tkv (Funakoshi et al., 2001; Tanimoto et al., 2000) was particularly intriguing. Dpp signalling can be monitored in situ, using antibodies recognising the phosphorylated form of the Mothers against Dpp (p-Mad) protein (Persson et al., 1998). In wild-type discs, levels of p-Mad are low in the AP organiser and high in both A and P flanking cells (Tanimoto et al., 2000) (Fig. 4G). In *col*^{1} mutant discs, a uniform staining of p-Mad was observed in the centre of the disc (Fig. 4H), indicating that downregulation of Dpp signalling in the AP organiser requires *col* activity.

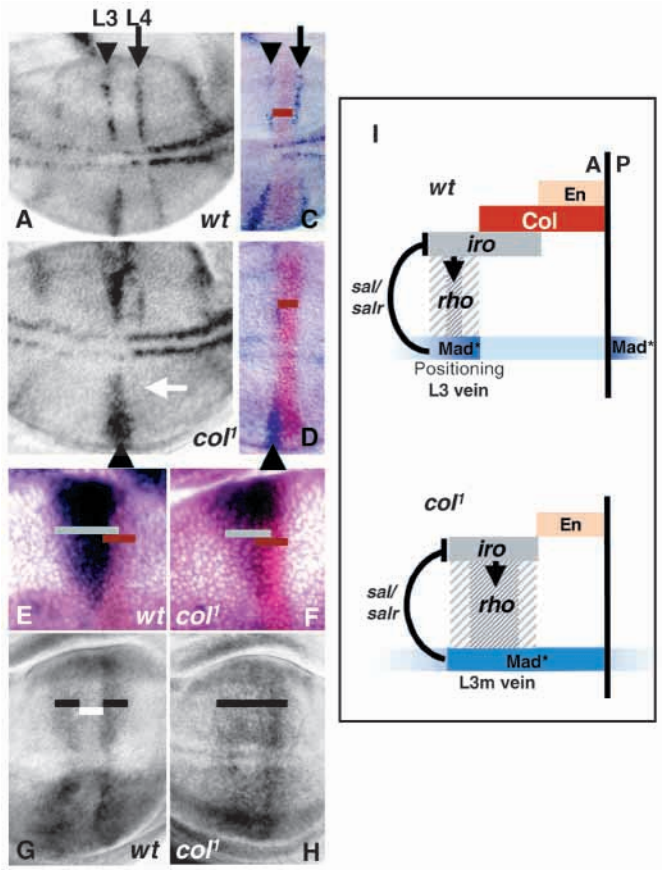

Fig. 4. Changes in *rho* and *iro* expression in larval discs prefigure the abnormal vein pattern of *col*^{1} wings. (A,B) *rho* expression in wild-type and *col*^{1} third instar wing discs. In *col*^{1} discs (B), *rho* expression in the presumptive L3 vein is wider than in wild type (A), while it is strongly downregulated dorsally or absent ventrally (white arrow) in the L4 vein primordium. The L3 and residual L4 primordia are closer to each other than in wild type. (C,D) Double in situ hybridisation for *rho* (blue) and *col* (pink, red bar) transcripts. In wild-type discs, there is no overlap between *rho* and *col* expression (C). The overlap observed in *col*^{1} discs (D) indicates a posterior shift of L3 vein. (E,F) Double in situ hybridisation for *ara* (blue, grey bar) and *col* (pink, red bar) transcripts. In *col*^{1} discs (F), the anterior border of *ara* expression is shifted closer to the AP boundary. (G,H) Dpp signalling in wing discs, revealed by the distribution of phosphorylated P-Mad. In wild-type discs (G), Dpp signalling is strongly downregulated in the AP organiser (white bar) and peaks on either side (black bars). In *col*^{1} discs (H), a uniform labelling is observed in the centre of the disc (black bar). (I) Schematic diagram of the expression domains of En (orange box), Col (red box), *iro* (grey box), *rho* (heavy grey hatching) and high Dpp signalling (Mad*, blue) in wild-type (top) and *col*^{1} (bottom) wing discs, based on data presented in A-H (see Blair, 1992; Gomez-Skarmeta et al., 1996; de Celis and Barrio, 2000). The L3 provein is indicated in light grey hatching and the AP border as a vertical black bar. This scheme postulates that the anterior shift of *iro* expression in *col*^{1} mutants is due to changes in *sal/salr* activity in response to changes in Dpp signalling, while increased *rho* expression reflects the loss of repression by Col in posterior-most *iro*-expressing cells.

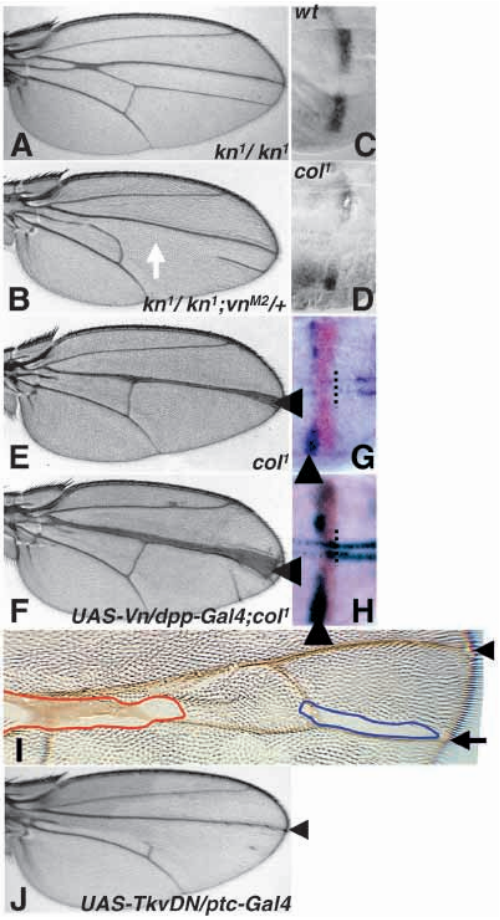

Fig. 5. Col-mediated upregulation of *vn* transcription in the AP organiser is required but not sufficient for L4 vein formation. (A) *kn*^{1}*/kn*^{1} and (B) *kn*^{1}*/kn*^{1}*; vn*^{M2}*/+* adult wings. In *kn*^{1}*/kn*^{1}*; vn*^{M2}*/+* wings, a large region of L4 vein is missing (white arrow). (C,D) *vn* transcription is strongly downregulated in the L3-L4 intervein primordium in *col*^{1} (D) relative to wild type (C) third instar wing discs, except in cells close to the presumptive hinge; (E-H) *col*^{1} and *UAS-Vn/dpp-Gal4; col*^{1} adult wings (E,F) and corresponding larval discs (G,H). In the absence of *col*, *vn* expression in the AP organiser does not rescue formation of L4 vein, while vein L3m is widened (F). Double in situ hybridisation for *rho* (blue) and *col* (pink) transcripts in wing discs shows that *rho* expression is not activated in posterior L4 vein cells but is upregulated in cells corresponding to L3m vein or the posterior margin (H). The broken black line indicates the position of the AP boundary. (I) *col*^{1} mutant clones in the wing, marked by *shavenoid (sha)*. L4 vein forms posterior to mutant clone spanning three to four rows of cells along the AP border (blue line); L4 vein is missing and L3 is wider and shifted posteriorly when the *col*^{1} clone fills the entire L3-L4 region (red line). (J) *UAS-TkvDN/ptc-Gal4* adult wings showing that sequestering Dpp by overexpression of TkvDN in the AP organiser results in wings smaller than wild type and specific loss of the L4 vein.

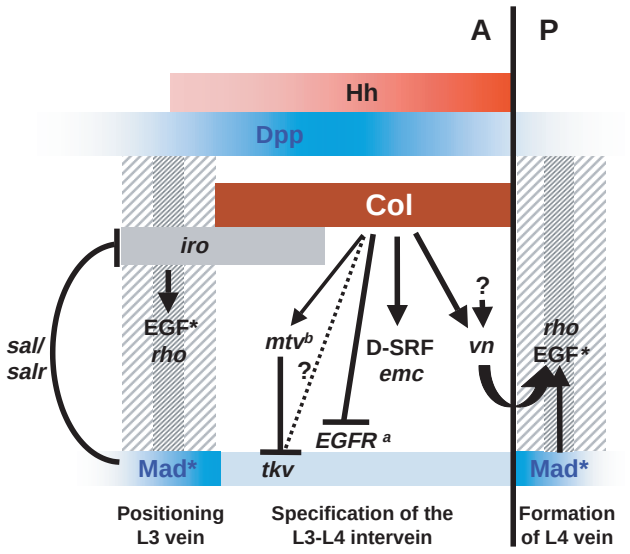

Fig. 6. Proposed model for the interplay between the Hh, Dpp and EGF signalling pathways in the AP organiser and patterning of the medial region of the wing. Anterior cells that receive high doses of Hh (the AP wing organiser) activate Col, which, in turn, upregulates expression of BS, *vn*, *emc* and *mtv*, and represses expression of *EGFR* ([a]Mohler et al., 2000). BS (D-SRF) is required for commitment of Col-expressing cells to an intervein fate (L3-L4 intervein) and Emc for their proliferation. Repression of *tkv* by Mtv leads to low levels of Dpp signalling in the AP organiser cells and high levels in both A and P flanking cells ([b]Funakoshi et al., 2001). Vn diffuses and activates EGF signalling in posterior cells (visualised by *rho* expression; heavy grey hatching). Col-mediated upregulation of Vn expression and modulation of Dpp signalling are both required for activation of EGF signalling in posterior cells and formation of L4 vein. *rho* repression in posterior-most *iro*-expressing cells positions the L3 vein competent domain anterior to the AP organiser (see Fig. 4I for the role of *sal/salr* in positioning *iro* expression). The signals triggering EGF activation in L3 vein and positioning *rho* expression at the centre of L3 and L4 provein domains (Biehs et al., 1998) remain unknown.

Lack of L4 vein in the absence of Col activity; impaired *vn* transcription in the AP organiser

Differentiation of posterior L4 vein specifically requires the activity of Vein (Garcia-Bellido et al., 1994). Genetic mosaic experiments indicated that this activity is provided by anterior cells close to the AP boundary where the *vn* transcripts accumulated in third instar larval discs (Simcox et al., 1996) (Fig. 5C). The absence of L4 vein in col^1 wings suggested that *col* and *vn* might genetically interact. To test for this possibility, we first looked for dominant genetic interactions between the two genes. Flies either homozygous for a weak *col/kn* allele, kn^1, or heterozygous for a strong allele of *vn*, vn^{M2}, formed a normal L4 vein. However, in $kn^1/kn^1; vn^{M2}/+$ flies, L4 vein was missing, except for its proximal- and distal-most parts, showing that *vn* and *col* cooperate to promote L4 vein formation (Fig. 5A,B). We then looked at *vn* expression in col^1 mutant discs. In the absence of *col* activity, a strong downregulation of *vn* transcription was observed in the AP organiser, except in the cells located near the presumptive hinge. *vn* transcription outside the wing pouch was not affected (Fig. 5C,D and data not shown). Together, these data indicate that *col* acts upstream of *vn* transcription in the AP organiser cells.

vn expression in the AP organiser is not sufficient to rescue formation of L4 vein in *col* mutant discs

To ensure that *col* requirement for L4 vein formation was only through the upregulation of *vn* transcription in the AP organiser, we asked whether re-introducing *vn* expression would be sufficient to rescue of L4 vein in the absence of *col*. As expression of *UAS-col* under the control of a *dpp-Gal4* driver in *col* mutant wings restores formation of L4 vein (data not shown), we expressed *vn* under control of the same driver. In contrast to *col*, *vn* expression did not restore formation of L4 vein, however. Instead, it led to the formation of a wider L3m vein (Fig. 5E,F). Accordingly, *rho* expression in larval discs was not restored in the L4 vein primordium, while strongly upregulated in the anterior L3m provein and over a distance of several cell diameters in the posterior wing margin (Fig. 5G,H). One possible reason for the failure of presumptive L4 vein cells to respond could be a limited range of diffusion of Vn as the P compartment is separated by three to four cell diameters from the source of Vn when expressed under control of the *dpp-Gal4* driver, due to the transcriptional repression of *dpp* by anterior En (Alves et al., 1998; Strigini and Cohen, 1997). To rule out this possibility, we examined L4 vein formation in col^1 mutant clones located in the A compartment, at varying distances from the AP boundary. We observed a loss of vein L4 when col^1 mutant clones encompassed most, if not all, dorsal and ventral cells in the L3-L4 region. By contrast, L4 vein formed in cases where dorsal and ventral col^1 cells spanned three to four rows of cells along the AP boundary (Fig. 5I). These data indicated that Vn is able to induce L4 vein formation, even when expressed at a distance of three to four cell diameters. The inability of Vn to activate *rho* expression in the L4 vein primordium and restore L4 vein formation in col^1 mutants indicated that another signal regulated by *col* in the AP organiser is required, in addition to *vn*. We therefore analysed the possible role of Col-mediated downregulation of Dpp signalling (Fig. 4H,I).

Modulation of Dpp signalling by the AP organiser cells is required for formation of L4 vein

In order to determine whether Col-dependent downregulation of Dpp signalling in the AP organiser was involved for L4 vein formation, we expressed a dominant-negative form of Tkv, TkvDN (*UAS-Tkv1ΔGSK*) (Haerry et al., 1998) in the AP organiser, using a *ptc-Gal4* driver. The TkvDN protein has a dual effect when overexpressed: blocking Dpp signalling and trapping Dpp itself, thus limiting its diffusion (Haerry et al., 1998). Expressing TkvDN in the AP organiser resulted in smaller wings with a loss of L4 vein and anterior crossvein (Fig. 5J). The decrease in wing size has been attributed to the titration of Dpp molecules when high levels of TkvDN (or Tkv) are expressed, leading to reduced growth of the posterior wing compartment (Haerry et al., 1998) (see Discussion). More striking is the loss of the L4 vein, suggesting that this is the first vein affected when Dpp levels are reduced. That the loss of L4 vein is an early patterning defect was confirmed by *rho* in situ hybridisation on larval wing discs. In *UAS-TkvDN/ptc-Gal4* discs, *rho* was not expressed in presumptive L4 vein (data not shown). Taken together, our data suggested that increased

sequestering of Dpp in the AP organiser in either *col* mutant or *UAS-TkvDN/ptc-Gal4* discs could modify the range of Dpp signalling and lead to both a reduced size of the posterior compartment and preferential loss of the L4 vein. Activation of Col expression by high doses of Hh thus appears to be an essential component of the interplay between Hh and Dpp signalling that specifies the provein fate of cells located on either side of the AP organiser.

DISCUSSION

The *Drosophila* wing primordium is subdivided into anterior (A) and posterior (P) compartments, with the AP boundary reflecting a true lineage restriction (Garcia-Bellido, 1975). How this discontinuity serves as a source of positional information, which patterns the imaginal disc and adult appendage, has been the subject of intensive investigation for over 25 years (Crick and Lawrence, 1975). The current model is a signalling-relay model where Hh secreted from the P cells diffuses into the A cells along the AP boundary and exerts a patterning function both independently and through the induction of *dpp* expression; Dpp in turn diffuses in both directions and acts at long-range to organise the pattern of more distal regions (Basler and Struhl, 1994; Chen and Struhl, 1996; Tabata and Kornberg, 1994; Mullor et al., 1997; Neumann and Cohen, 1997; Strigini and Cohen, 1997). We show here that activity of the Hh, Dpp and EGF signalling pathways in patterning of the *Drosophila* wing are coordinated by Col, a Hh target specific to the wing AP organiser (Fig. 6).

Col activity is required for downregulation of Dpp signalling in the AP organiser

An unanticipated intricacy of the independent, versus Dpp-mediated, Hh patterning activity in the wing arose from comparison of the gradient of Dpp activity with *dpp* expression. It revealed that Dpp signalling is downregulated in the AP organiser and upregulated in both A and P flanking cells (Tanimoto et al., 2000). This downregulation of Dpp activity was shown to result from the localised transcriptional repression of *tkv*, which is itself due to activation of the transcriptional repressor Master of thick vein (Mtv) in response to Hh signalling (Funakoshi et al., 2001). We show here that Col activity is required for the downregulation of Dpp signalling in the AP organiser and link this regulation to Col requirement for positioning L3 vein and formation of L4 vein. While the observation that *mtv* transcription is downregulated in *col* mutant discs (data not shown) and tkv is upregulated in clones of *col* mutant cells (T. Tabata, personal communication) suggests that *col* may act upstream of *mtv* in the regulatory cascade, attenuating Dpp signalling in the AP organiser, the relative functions of these two genes remain to be examined in detail.

Positioning L3 vein: crosstalk between Hh and Dpp signalling via *col* and *iro* regulation

It has previously been proposed that Hh does directly control the position of L3 vein, although the molecular mechanisms of this control were not firmly established (Mullor et al., 1997; Stigini and Cohen, 1997). In both *col* (Nestoras et al., 1997) (this paper) and *mtv* mutant clones (Tanimoto et al., 2000), the position of L3 vein is shifted posteriorwards. That both *col* and *mtv* control the position of L3 vein suggested that this position is defined by Hh signalling through the modulation of Dpp signalling. It has previously been established that *iro* was required for *rho* activation in the L3 primordium and formation of L3 vein (Gomez-Skarmeta et al., 1996; Gomez-Skarmeta and Modolell, 1996). *iro* is activated by both Dpp and Hh signalling and its anterior border of expression is under control of *sal/salr*, a target of Dpp (De Celis and Barrio, 2000). We show that the patterns of *col*, *iro* and *rho* expression are intimately connected. We observed both an increased number of cells expressing *rho* and a posterior shift of the anterior border of *iro* expression in col^1 mutant discs. We interpret this posterior shift as reflecting a modified range of Dpp signalling relayed, at least in part, by *sal/salr* activity. The increased number of *rho*-expressing cells, for its part, indicated that Col is able to antagonise *rho* activation by *iro* in cells, which express both *iro* and Col. This correlates well with the wing phenotype – anteriorwards shift of the L3 vein, together with gaps in its distal region – which results from anterior extension of Col expression, in *UAS-Col/dpp-Gal4* wing discs (Mohler et al., 2000) (M. C., unpublished). The distal gaps could reflect the complete absence of *rho* expression close to the DV border, because of the complete overlap between *col* and *iro* expression where *iro* expression is narrower. From *col* loss- and gain-of function experiments, we therefore conclude that the primordium of L3 vein corresponds to cells that express *iro* but not *col* (Fig. 4I). Col thus appears to play a dual role in defining the position and width of L3 vein: activating BS and repressing EGFR in the wing AP organiser cells, endows these cells with an intervein fate, while attenuating Dpp signalling indirectly positions the anterior limit of *iro* expression domain, and L3 vein competence anterior to the AP organiser (Fig. 6).

Formation of L4 vein depends upon appropriate regulation of both Dpp and EGF signalling in the AP organiser

We have shown that Col regulates *vn* transcription in the AP organiser. This expression of *vn* is required for formation of L4 vein (Garcia-Bellido et al., 1994; Schnepp et al., 1996; Simcox et al., 1996). The loss of this vein in col^1 mutants could not, however, be rescued by expressing high level of Vn in the AP organiser, suggesting that a second signal dependent upon Col was also required. The specific loss of L4 vein was previously observed in conditions of reduced levels of Dpp signalling caused by ubiquitous expression of either Tkv or TkvDN (Haerry et al., 1998). Together with this, the col^1 wing phenotype and Col requirement for downregulation of Dpp signalling in the AP organiser suggested a role of Dpp signalling in formation of L4 vein. Indeed, expressing a dominant-negative form of Tkv (TkvDN) in the AP organiser resulted in L4 loss. At first sight, it may appear contradictory that either upregulation (in *col* mutants) or downregulation (by expressing TkvDN) of Dpp signalling in the AP organiser leads to the preferential loss of posterior L4 vein. In both cases, however, there is increased sequestering of Dpp in the AP organiser, which limits its range of diffusion and signalling in posterior cells (Haerry et al., 1998; Lecuit and Cohen, 1998). Therefore attenuation of Dpp signalling in the AP organiser and increased signalling in posterior flanking cells (Tanimoto et al., 2000) appears to be required in addition to Vn activity for formation of L4 vein. By modulating Dpp signalling and

vn transcription in cells receiving high doses of Hh, Col thus links Hh short-range activity to both positioning of the anterior L3 vein and formation of the posterior L4 vein.

Dual Col function in the control of cell proliferation in the wing disc

In both null and hypomorphic *col* mutants, the wing blades are 20% smaller than wild type, owing to a reduced number of cells, both in the L3-L4 intervein and the posterior compartment. Reduced proliferation in the posterior compartment is a cell non-autonomous effect that is probably linked to the decreased range of Dpp diffusion that results from the upregulation of Tkv in the AP organiser cells (Lecuit and Cohen, 1998; Tanimoto et al., 2000). By contrast, the reduced size or the lack of L3-L4 intervein in col^1/kn^1 and col^1 mutants, respectively, is an independent defect as Dpp signalling is up- rather than downregulated in this region. Defective cell proliferation in the central region of *col* wings in pupae could be linked to Col requirement for upregulation of *emc* and N, previously shown to be required for cell proliferation (Garcia-Bellido et al., 1976; De Celis and Garcia-Bellido, 1994; Baonza and Garcia-Bellido, 1999). Thus, the size reduction of col^1 mutant wings therefore reflects the cumulative effect of changes in Dpp signalling and decreased expression of *emc* and/or N in the AP organiser.

The wing AP organiser: a tissue-specific signalling centre

Col was initially characterised for its function in the segmentation of the embryonic head, acting upstream of Hh in this process (Crozatier et al., 1996; Crozatier et al., 1999). Its key role in mediating short-range Hh activity in the wing was therefore unanticipated. Col role in the wing illustrates the pivotal importance of tissue-specific mediators of Hh and Hh→Dpp signalling pathways in patterning different appendages. The concentration of Shh is the primary determinant of AP polarity in the vertebrate limb (Lewis et al., 2001; Yang et al., 1997). In this case, as in *Drosophila* imaginal discs, a signalling relay involving Bmps is implicated (Drossopoulou et al., 2000; Duprez et al., 1996). Col/Kn is the single *Drosophila* member of the family of COE transcription activators that includes products of the vertebrate *ebf*/*olf-1*/*coe* genes and *C. elegans unc-3* (for a review, see Dubois and Vincent, 2001). Whether Col function in mediating tissue-specific interpretation of Hh signalling in *Drosophila* has some equivalent in vertebrates or corresponds to the co-option of Col in a highly derived patterning process remains to be investigated.

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