brinker and *optomotor-blind* act coordinately to initiate development of the L5 wing vein primordium in *Drosophila*

Orna Cook[1], Brian Biehs[2] and Ethan Bier[1,*]

[1]Section of Cell and Developmental Biology, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0349, USA
[2]University of California, San Francisco, Third and Parnassus, San Francisco, CA 94143-0448, USA
*Author for correspondence (e-mail: bier@biomail.ucsd.edu)

Accepted 28 January 2004

Development 131, 2113-2124
Published by The Company of Biologists 2004
doi:10.1242/dev.01100

Summary

The stereotyped pattern of *Drosophila* wing veins is determined by the action of two morphogens, Hedgehog (Hh) and Decapentaplegic (Dpp), which act sequentially to organize growth and patterning along the anterior-posterior axis of the wing primordium. An important unresolved question is how positional information established by these morphogen gradients is translated into localized development of morphological structures such as wing veins in precise locations. In the current study, we examine the mechanism by which two broadly expressed Dpp signaling target genes, *optomotor-blind* (*omb*) and *brinker* (*brk*), collaborate to initiate formation of the fifth longitudinal (L5) wing vein. *omb* is broadly expressed at the center of the wing disc in a pattern complementary to that of *brk*, which is expressed in the lateral regions of the disc and represses *omb* expression. We show that a border between *omb* and *brk* expression domains is necessary and sufficient for inducing L5 development in the posterior regions. Mosaic analysis indicates that *brk*-expressing cells produce a short-range signal that can induce vein formation in adjacent *omb*-expressing cells. This induction of the L5 primordium is mediated by *abrupt*, which is expressed in a narrow stripe of cells along the *brk*/*omb* border and plays a key role in organizing gene expression in the L5 primordium. Similarly, in the anterior region of the wing, *brk* helps define the position of the L2 vein in combination with another Dpp target gene, *spalt*. The similar mechanisms responsible for the induction of L5 and L2 development reveal how boundaries set by dosage-sensitive responses to a long-range morphogen specify distinct vein fates at precise locations.

Key words: *optomotor-blind*, *omb*, *brinker*, *brk*, *abrupt*, *ab*, L5 vein, Wing disc, *Drosophila*, Patterning, Morphogenesis

Introduction

Cells must determine their positions in order to develop into specific tissues or organs in a complex multi-cellular organism. One source of positional information is concentration gradients of diffusible secreted morphogens. Cells respond to these gradients in a threshold-dependent fashion by activating distinct patterns of gene expression. In the *Drosophila* wing imaginal disc, the Hedgehog (Hh) and Decapentaplegic (Dpp) morphogens act sequentially to specify central (Hh) and lateral (Dpp) positions along the anterior-posterior axis (reviewed by Klein, 2001; Lawrence and Struhl, 1996; Strigini and Cohen, 1999). Hh, which is expressed in posterior compartment cells, is prevented from eliciting a response in these cells by the selector gene *engrailed*. Hh diffuses over a distance of six to eight cells into the anterior compartment where it activates expression of various target genes including *dpp* (Cadigan, 2002; Sanson, 2001; Vervoort, 2000). The borders of this Hh-responsive central organizer determine the positions of the centrally located L3 and L4 wing veins (Biehs et al., 1998; Crozatier et al., 2002; Mohler et al., 2000; Strigini and Cohen, 1997; Vervoort et al., 1999). As summarized below, Dpp produced in the central organizer then acts over a longer range to specify the positions of the more lateral L2 and L5 wing veins.

The Bone Morphogenetic Protein (BMP)-related ligand Dpp functions as a morphogen during several stages of *Drosophila* development, including patterning the dorsal/ventral (DV) axis of the embryo (Bier, 1997; Rusch and Levine, 1996) and establishing the anterior/posterior (AP) axis of the wing disc (reviewed by Affolter et al., 2001; Klein, 2001; Lawrence and Struhl, 1996; Strigini and Cohen, 1999). In the wing disc, Dpp is produced in a stripe just anterior to the AP border, and diffuses in both anterior and posterior directions to form a concentration gradient and a corresponding BMP activity gradient (Entchev et al., 2000; Fujise et al., 2003; Klein, 2001; Lawrence and Struhl, 1996; Strigini and Cohen, 1999; Teleman and Cohen, 2000). This BMP activity gradient, which is established by the synergistic action of the ligands Dpp and Glass Bottom Boat (Gbb) (Haerry et al., 1998; Wharton et al., 1999), functions in a dosage-sensitive fashion to control the nested expression of a series of BMP target genes. The BMP target genes *spalt-major* (*salm*) and *spalt-related* (*salr*) (these related and neighboring genes will be referred to as *sal* hereafter), *optomotor-blind* (*omb*; *bifid*, *bi* – FlyBase), and *vestigial* (*vg*) are expressed in progressively broader domains due to their increasing sensitivity to BMP signaling (Kirkpatrick et al., 2001; Lecuit et al., 1996; Nellen et al., 1996).

A crucial Dpp target gene is *brinker* (*brk*), which is repressed in a graded fashion by Dpp signaling in the central region of the wing disc (Marty et al., 2000; Muller et al., 2003; Torres-Vazquez et al., 2000). Brk encodes a transcriptional repressor (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Minami et al., 1999) that acts in a dosage-dependent manner to establish the centrally nested expression of the transcription factors encoded by *omb*, *sal* and *vg* (Jazwinska et al., 1999b; Sivasankaran, 2000; Kirkpatrick et al., 2001; Muller et al., 2003). Thus, the opposing and complementary activities of Dpp and Brk along the AP axis of the wing disc lead to differential activation of target genes such that the more responsive a gene is to BMP signaling and the less sensitive it is to repression by Brk, the broader its expression domain will be.

Although much is understood regarding the formation of the Dpp gradient and how the resulting graded activation of BMP signaling elicits different patterns of gene expression, little is known about how these target genes direct differentiation of defined tissues in specific locations. A mechanism that links broad patterns of gene expression to specification of particular cell types is the creation of sharp borders between different domains. As described above, graded BMP signaling subdivides the wing disc into nested domains expressing the target genes *sal*, *omb* and *vg*. These expression domains create boundaries that then can act as local organizers along the AP axis (Lawrence and Struhl, 1996) to induce formation of specific morphological structures such as longitudinal wing veins (Bier, 2000; Sturtevant et al., 1997; Sturtevant and Bier, 1995).

A well-studied example of vein induction at a boundary is formation of the L2 primordium along the anterior border of the *sal* expression domain. Cells expressing high levels of *sal* induce expression of *knirps* (*kni*) and *knirps related* (*knrl*; *knirps-like* – FlyBase) genes in a narrow stripe of neighboring anterior cells, which express low levels of *sal* (de Celis and Barrio, 2000; Lunde et al., 1998). Analysis of an L2 vein-specific enhancer element of the *kni* locus revealed that it consists of an activation domain containing functionally important Scalloped (Sd)-binding sites, as well as a repressor domain containing consensus binding sequences for Sal and Brk (Lunde et al., 2003). Kni/Knrl organize development of the L2 primordium by activating expression of the vein promoting gene *rhomboid* (*rho*), as well as by repressing expression of the intervein gene *blistered* (*bs*) in the vein primordial cells (Lunde et al., 1998). In addition, because Kni/Knrl expression must be confined to a narrow stripe to promote vein development (Lunde et al., 1998), it may also control expression of a lateral inhibitory factor that represses vein development in adjacent intervein cells. Therefore, Kni/Knrl play a key role in translating positional information at the anterior border of the *sal* expression domain into a coherent gene expression program in the L2 primordium.

The position of the L5 primordium, like that of L2, is determined by a threshold response to the Dpp gradient (Sturtevant et al., 1997). However, the border(s) of gene expression domains responsible for inducing formation of L5 are unknown. In this study we examine the initiation of L5 development and show that it is dependent on the two abutting Dpp target genes, *omb* and *brk*. The L5 primordium forms within the *omb* domain adjacent to cells expressing high levels of *brk*. We show that *omb* is required for responding to a for-export-only signal produced by *brk*-expressing cells. This combination of constraints results in the activation of *abrupt* (*ab*), which plays a key role in organizing gene expression in a sharp line within the posterior extreme of the *omb* expression domain.

Materials and methods

Fly stocks

w^{1118}, ab^{1}, $omb^{1,}$ *w[1118] P{Ubi-GFP(S65T)nls}X P{neoFRT}18A* and *MKRS, P{hsFLP}86E/TM6B, Tb[1]* stocks were obtained from the Bloomington stock center. The *y w* brk^{m68} f^{36a} *FRT18a/FM7a* stock was kindly provided by C. Rushlow (Jazwinska et al., 1999a), the *yw hsFLP* f^{36a}; *ab*>f^{+}>*GAL4-lacZ*/CyO stock was kindly provided by K. Basler (Moreno et al., 2002), and the omb^{D4} *w*/*FM6* stock was kindly provided by G. Pflugfelder. Flies used for expression pattern markers included: *X47* (Campbell and Tomlinson, 1999) for *brk-lacZ* expression; bi^{x35} for *omb-lacZ* expression (Sun et al., 1995); and *P{ry*$^{+t7.2}$*=PZ}salm*^{03602} *cn*^{1}*/CyO; ry*^{506} (*Drosophila* genome project) for *sal-lacZ* expression. Lines for ectopic expression using the GAL4/UAS system (Brand and Perrimon, 1993) included: *MS1096*-GAL4, *C765*-GAL4 (kindly provided by Gomez-Skarmeta) and Vg^{B} GAL4 (kindly provided by S. Carroll), UAS-*brk* (C. Rushlow and E. Moreno) and UAS-*omb* (Grimm and Pflugfelder, 1996).

Clonal analysis

Homozygous loss-of-function clones were generated by hs*FLP-FRT* recombination (Xu and Rubin, 1993). *y w* brk^{m68} f^{36a} *FRT18a/FM7a* and omb^{D4} *w*/*FM6* stocks were recombined to generate the *w* omb^{D4} f^{36a} *FRT18a/FM0* and *w* omb^{D4} brk^{m68} f^{36a} *FRT18a/FM0* stocks. Each of these stocks was crossed with *w[1118] P{Ubi-GFP(S65T)nls}X P{neoFRT}18A; MKRS, P{hsFLP}86E/TM6B, Tb[1]* and larvae were heat shocked 24-72 hours after egg-laying at 37°C for 1-2 hours. Wing discs were dissected and analyzed after 24-72 hours, or vials were kept at 25°C until flies hatched and wings were analyzed. Mutant clones in the wing disc were detected by lack of GFP expression, and in the adult wing by f^{36a} phenotype.

Flip-out clones ectopically expressing *ab* were generated in larvae of the genotype yw *hsFLP* f^{36a}; *ab*>f^{+}>*GAL4-lacZ*/*UAS-ab* following recombination between FRT elements (>), initiated by heat induction of the HS-FLP recombinase transgene for 30 minutes at 34°C. These clones were marked by gain of *lacZ* expression in the disc, and by the cell-autonomous f^{36a} trichome phenotype in adult wings. A similar set of crosses was used to generate flip-out clones misexpressing high levels of *omb*.

Generation of an anti-Abrupt antibody

An Abrupt-GST fusion protein consisting of the 88 C-terminal amino acids of Ab fused to GST was purified from soluble whole bacterial protein extracts, using a glutathione column, and injected into rabbits. The antiserum was partially purified by ammonium-sulfate precipitation (25% cut) and preabsorbed 1:10 against fixed embryos. Titration of this antibody revealed that a final 1:1000 dilution gave a strong signal with low background.

Immunostaining

Immunohistochemical staining was performed using the following antibodies: Guinea pig anti-Kni (kindly provided by D. Kosman), mouse anti-Delta (kindly provided by M. Muskavitch), mouse anti-DSRF (kindly provided by M. Affolter), mouse anti-β-Gal (Promega), and rabbit β-Gal (Cappel), as previously described in (Sturtevant et al., 1993). Fluorescent detection using secondary Alexa Fluor 488, 555, 594 or 647 conjugated antibodies (Molecular Probes) was visualized using a Leica scanning confocal microscope.

In situ hybridization to whole-mount larval wing discs

In situ hybridization using digoxigenin-labeled antisense RNA probes was performed either alone (O'Neill and Bier, 1994) or in combination with antibody labeling, as previously described (Sturtevant et al., 1993).

Mounting fly wings

Wings from adult flies were dissected in ethanol and mounted in 50% Canada Balsam (Aldrich #28,292-8), 50% methylsalicilate, as described by Ashburner (Ashburner, 1989).

Results

ab organizes gene expression in the L5 primordium

The *ab* gene, which encodes a zinc finger protein containing a BTB/POZ domain, is required for L5 development as revealed by viable alleles such as ab^1, which bypass the early embryonic requirement for this gene in motor neuron axon guidance and result in distal truncation of the L5 vein (Fig. 1A,B) (Hu et al., 1995). We have also recovered four additional viable *ab* alleles in a genome-wide screen for new wing vein mutants, one of which results in a somewhat stronger phenotype in which the L5 vein is consistently truncated proximal to the posterior cross-vein (data not shown). We examined expression of *ab* in the wing disc and found that it is expressed as a single stripe in the posterior compartment (Fig. 1C). The viable ab^1 allele is likely to be a regulatory mutation, as *ab* expression is greatly reduced in ab^1 mutant wing discs (Fig. 1D). *ab* expression is similarly reduced or undetectable in the other four independently isolated viable *ab* alleles (data not shown). Double-label experiments with the vein marker Delta (Dl), which is expressed in L1 and L3-L5 (Biehs et al., 1998), revealed that *ab* is co-expressed with Dl in the L5 primordium (Fig. 1E).

Extension of our previous analysis of *ab* in initiating L5 development (Biehs et al., 1998; Sturtevant and Bier, 1995) showed that *ab* functions early in L5 specification. Activation of all known vein genes, including *rho* (Fig. 1F,G), *Dl* (Fig. 1I,J), the *caupolican* and *araucan* genes of the Iroquois Complex (IroC), and *argos* (data not shown), and repression of the intervein genes *bs* (also known as *DSRF*; Fig. 1L,M) and *net* (data not shown), is lost in cells corresponding to the L5 primordium in ab^1 mutant wing discs. We also determined whether it is critical that *ab* expression is confined to a narrow stripe for regulating expression of vein or intervein genes. We ubiquitously misexpressed *ab* in the wing disc using the *MS1096*-GAL4 driver and found that such global activation of *ab* suppressed expression of vein genes, such as *rho* and *Dl*. This *ab* misexpression also caused vein-specific downregulation the intervein gene *bs*, in the wing disc (Fig. 1H,K,N), but did not repress expression of other genes, including *hh*, *ptc* and *dpp* (data not shown). This phenotype may result from unregulated production of a lateral inhibitory signal normally produced by vein cells to suppress vein development in adjacent intervein cells.

We also investigated whether restricted expression of *ab* in small clones was sufficient to induce vein development. We used the flip-out misexpression system (Struhl and Basler, 1993) to generate clones of cells ectopically expressing *ab* in the wing disc, and found that these cells (identified by Ab or β-Gal expression) ectopically expressed the vein marker Dl (Fig. 1O,P) and downregulated expression of the intervein marker Bs (Fig. 1Q,R) in a cell-autonomous fashion when located anywhere within the wing pouch. Adult wings containing small *ab*-expressing clones marked with *forked* also produced ectopic vein material cell autonomously (Fig. 1S). These results, in conjunction with those described above, demonstrate that *ab* is necessary to control known gene expression in the L5 primordium, and is sufficient to induce vein development when expressed in a restricted number of cells. These data are consistent with *ab* acting in a vein-organizing capacity to direct L5 development.

ab is expressed along the border of *omb* and *brk* expression domains

As previously shown, the L2 primordium forms along the anterior boundary of the *sal* expression domain, in cells expressing low levels of *sal* and facing those expressing high levels of *sal* (de Celis and Barrio, 2000; Lunde et al., 1998; Sturtevant et al., 1997). The symmetrical disposition of the L2 and L5 veins, and the positioning of both of these veins by Dpp rather than Hh signaling, suggested that the L5 vein might form along the posterior border of the *sal* expression domain in much the same way that L2 is induced along its anterior border. However, two lines of evidence indicate that *sal* is not likely to be directly involved in determining the position of L5. First, the posterior border of the *sal* expression domain is located several cells anterior to the L5 primordium (Sturtevant et al., 1997). Second, although $salm^-$ clones do occasionally result in the formation of ectopic posterior veins, they do so non-autonomously at a distance of several cell diameters from the clone border (Sturtevant et al., 1997). This phenotype is entirely different from the ectopic L2 veins that form at high penetrance immediately within the borders of anterior sal^- clones, located between the L2 and L3 veins (Sturtevant et al., 1997). Clones of a deficiency removing both *salm* and the related *salr* gene also result in the production of an ectopic vein (de Celis and Barrio, 2000), but this vein forms within the interior of such clones between L4 and L5, in a position corresponding to a cryptic vein, or paravein, which has a latent tendency to form along the posterior border of the *sal* domain (Sturtevant et al., 1997).

As the L5 primordium forms approximately four to six cell diameters posterior to the *sal* expression domain (Fig. 2A-C) (Sturtevant et al., 1997), we examined the expression of other BMP target genes, *omb* and *brk*, relative to the L5 primordium. The borders of these gene expression domains are known to form posterior to that of the *sal* domain (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Lecuit et al., 1996; Minami et al., 1999; Nellen et al., 1996). Previous studies revealed that the domains of cells expressing high levels of *omb* and *brk* (Campbell, 2002; Jazwinska et al., 1999a) are largely reciprocal, although these genes are co-expressed at lower levels in cells along the border. We therefore determined the relative positions of the border of high level *omb*/*brk* expression with respect to vein primordia marked by Dl (L1, and L3-L5) and Kni (L2). These experiments revealed that the L5 stripe of Dl expression forms inside and along the posterior border of the domain expressing high levels of *omb*, whereas the anterior border of the *omb* domain extends well beyond the L2 primordium (Fig. 2D-F). A complementary pattern was observed in wing discs of *brk-lacZ* flies double stained for β-

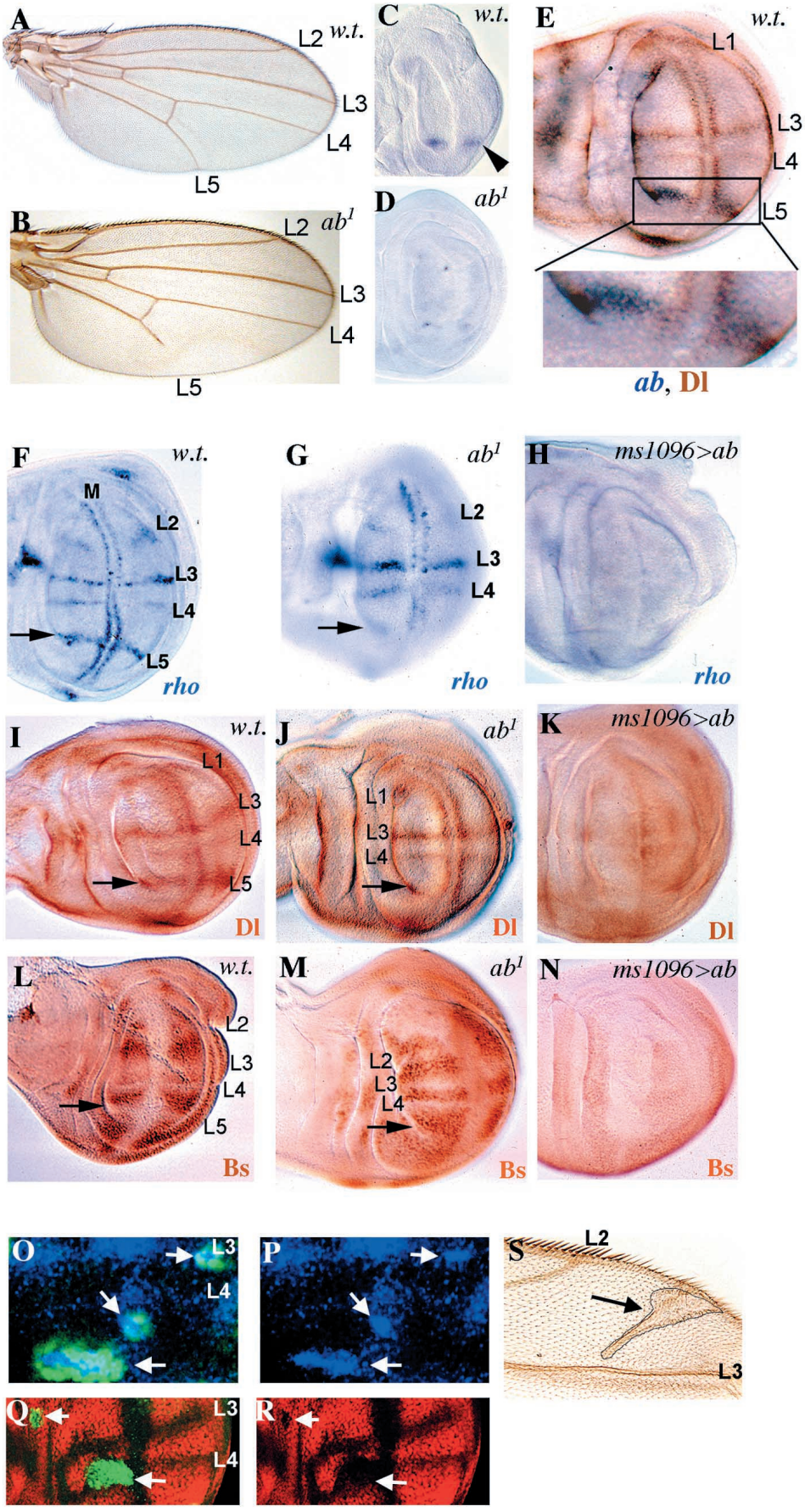

Fig. 1. *ab* is the L5 organizing gene. (A) A wild-type (wt) wing. The L2-L5 longitudinal veins are indicated. (B) An adult ab^1/ab^1 mutant wing. (C) *ab* expression in a wild-type third instar wing imaginal disc, visualized with an antisense *ab* probe (arrowhead indicates the *ab* stripe). (D) *ab* expression is greatly reduced in an ab^1/ab^1 mutant wing disc. (E) Double labeling of *ab* RNA (blue) and Dl protein (brown), shows that these genes are co-expressed in cells corresponding to the L5 primordium. Inset shows a higher magnification of the L5 primordium. The L1, and L3-L5, vein primordia are indicated. (F) *rho* is expressed in all longitudinal vein primordia in a wild-type third instar larval wing disc, detected with an antisense *rho* probe. Arrows indicate the approximate location of the L5 primordium in this and subsequent panels. (G) *rho* expression is lost in the L5 primordium of ab^1/ab^1 mutant wing discs. Wing discs are oriented with anterior at the top and dorsal to the left in this and subsequent panels. (H) Ubiquitous expression of *ab* with *MS1096*-GAL4 eliminates *rho* expression in all vein primordia. (I) Dl is expressed in the L1 and L3-L5 wing vein primordia. (J) Dl expression is lost in the L5 primordium of ab^1/ab^1 mutant wing discs. (K) Dl expression in all vein primordia is greatly reduced in discs ubiquitously expressing *ab* in *MS1096*-GAL4; UAS-*ab* wing discs. Weak Dl expression is visible in the ventral compartment of the disc, consistent with the lower levels of *MS1096*-GAL4 expression in ventral versus dorsal cells. (L) Blistered (Bs) protein is expressed at high levels in intervein cells, but is strongly downregulated in the L2-L5 vein primordia. (M) Bs downregulation in the L5 primordium is lost in ab^1/ab^1 mutant wing discs. (N) Bs expression is greatly reduced in all cells of *MS1096*-GAL4; UAS-*ab* wing discs. (O) A third instar wing imaginal disc misexpressing *ab* in flip-out clones (arrows) stained for β-Gal (green) and Dl (blue). Dl is expressed in a cell-autonomous fashion within a subset of β-Gal-expressing cells. Additional double-label experiments reveal that all cells in flip-out clones expressing β-Gal also express Ab at high levels (O.C., unpublished). (P) Dl channel only for the disc shown in O. (Q) A third instar wing imaginal disc misexpressing *ab* in a flip-out clone (arrows) stained for β-Gal (green) and the intervein marker Bs (red). Bs is downregulated in a cell-autonomous fashion within all cells of the clone. (R) Bs channel only for the disc shown in Q. (S) Ectopic veins form in a cell-autonomous fashion within small *ab*-expressing flip-out clones marked by being f^{36a} (outlined).

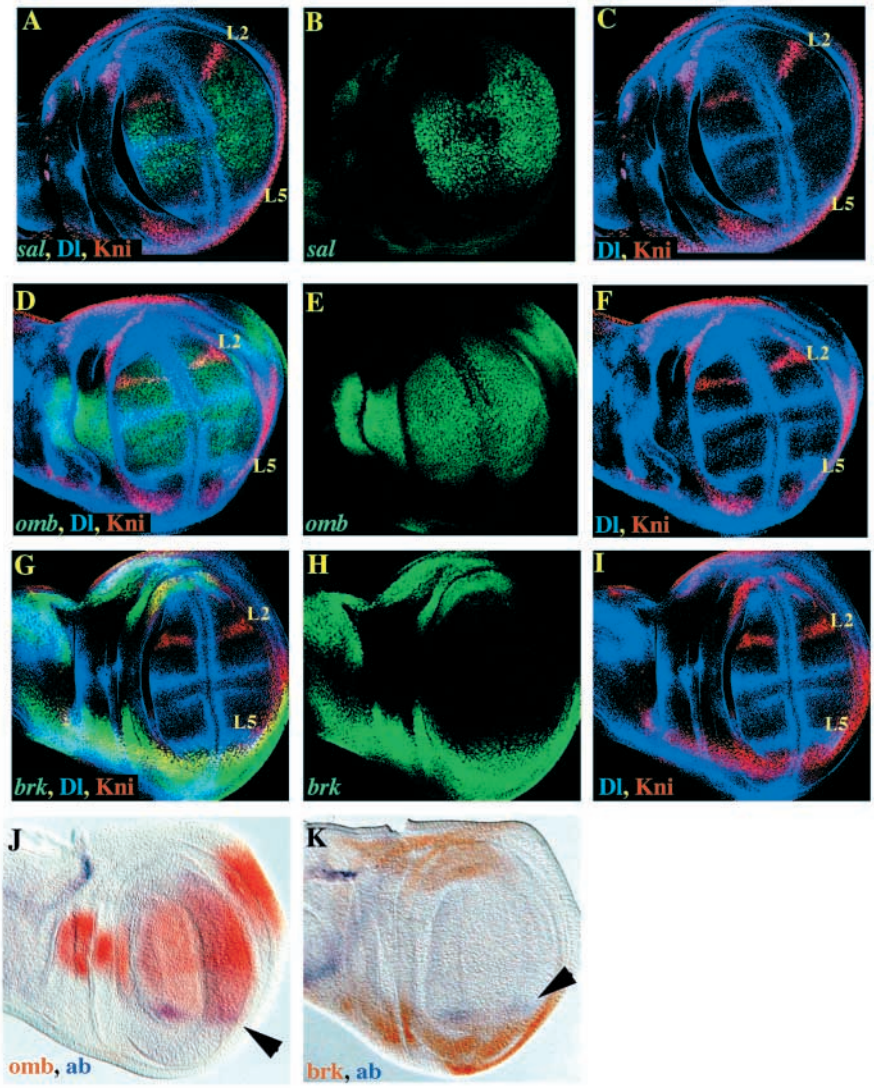

Fig. 2. The L5 primordium forms within the *omb* domain adjacent to *brk*-expressing cells. (A) A third instar wing imaginal disc triple labeled for Kni protein (red), Dl protein (blue) and *sal-lacZ* expression (green, β-Gal). (B) *sal-lacZ* (β-Gal) channel alone for the disc shown in A. (C) Kni and Dl channels for the disc shown in A. Kni is expressed anterior to cells expressing high levels of *sal*, but there is a significant gap between the posterior edge of the *sal* expression domain and the L5 primordium. (D) A third instar wing imaginal disc triple labeled for Kni protein (red), Dl protein (blue) and *omb-lacZ* expression (green, β-Gal). (E) *omb-lacZ* (β-Gal) channel alone for the disc shown in D. (F) Kni and Dl channels for disc shown in D. *omb* expression extends well past the Kni L2 stripe anteriorly, but just includes the L5 primordium posteriorly. (G) A third instar wing imaginal disc triple labeled for Kni protein (red), Dl protein (blue) and *brk-lacZ* expression (green, β-Gal). (H) *brk-lacZ* (β-Gal) channel alone for the disc shown in G. (I) Kni and Dl channels for disc shown in G. The L5 primordium lies along the outside border of the high level *brk* expression domain. (J) A third instar wing imaginal disc of *omb-lacZ* flies, double labeled for β-Gal protein (brown) and *ab* RNA (blue, arrowhead). The *ab* stripe runs just within the domain of strong *omb* expression. (K) A third instar wing imaginal disc of *brk-lacZ* flies, double labeled for β-Gal protein (brown) and *ab* RNA (blue, arrowhead). The *ab* stripe runs just adjacent to the domain of strong *brk* expression.

Gal and Dl, in which the L5 Dl stripe runs outside and along the border of the high level *brk* expression domain (Fig. 2G-I). We obtained similar results using *ab* as a marker for the L5 primordium, in which we found that the stripe of *ab*-expressing cells lies within the *omb* domain (Fig. 2J), adjacent to high level *brk*-expressing cells (Fig. 2K). These expression studies reveal that *omb* and *brk* are expressed in the right location to play a role in positioning the L5 primordium.

ab and *omb* interact genetically in promoting L5 formation

As a first step in determining whether *omb* or *brk* play a role in L5 development, we tested for genetic interactions between these genes and *ab*. Several viable or lethal *ab* alleles were crossed to stocks carrying the brk^{m68} allele or a deficiency of *brk*, and trans-heterozygous brk^{-}/+; ab^{-}/+ F1 flies were examined for L5 phenotypes. None of the combinations of *brk* and *ab* alleles tested resulted in any dominant vein-loss phenotype in trans-heterozygotes (e.g. brk^{m68}/+; ab^{1}/+ flies, data not shown). In addition, we did not observe any enhancement of the homozygous ab^{1}/ab^{1} L5 truncation phenotype in brk^{-}/+; ab^{1}/ab^{1} flies (Fig. 3A,E). By contrast, when we tested for trans-heterozygous interactions between *ab* and *omb* alleles we observed consistent genetic interactions. For example, omb^{1}/+; ab^{1}/+ flies exhibit truncations in the distal portion of L5 (with 3% penetrance, Fig. 3F), whereas neither ab^{1}/+ nor omb^{1}/+ heterozygotes ever show any L5 phenotype (Fig. 3B,C). Moreover, the omb^{1} allele, which causes notching of the wing margin when homozygous but has no associated L5 phenotype (Fig. 3D), strongly enhances the ab^{1}/ab^{1} L5 truncation phenotype. This interaction is evident in omb^{1}/+; ab^{1}/ab^{1} females (Fig. 3G), and is very pronounced in omb^{1}/omb^{1}; ab^{1}/ab^{1} double homozygous females (data not shown) or hemizygous omb^{1}/Y; ab^{1}/ab^{1} males (Fig. 3H). These results suggest that *omb* and *ab* function in concert to promote L5 formation.

Misexpression of *omb* and *brk* shifts or eliminates the L5 and L2 veins

As a next step in analyzing the potential role of *brk* and/or *omb* in L5 formation we assessed the requirement for sharp borders of *omb* or *brk* expression. We addressed this by misexpressing

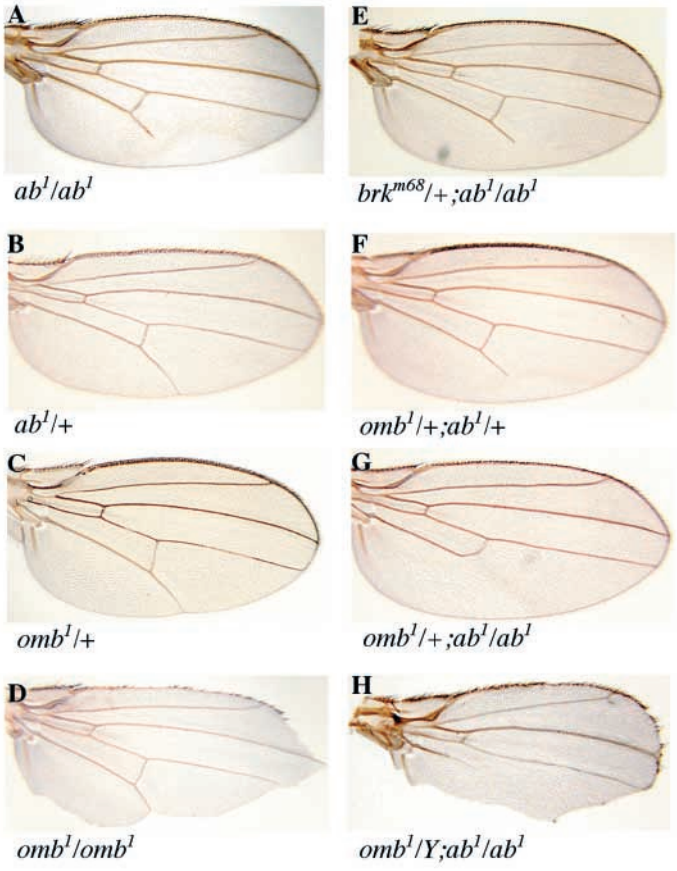

Fig. 3. *omb* interacts genetically with *ab*. (A) A homozygous ab^1/ab^1 mutant wing lacks the distal section of L5. (B) Heterozygous $ab^1/+$ flies have a fully penetrant wild-type wing phenotype. (C) Heterozygous $omb^1/+$ flies also have a fully penetrant wild-type wing phenotype. (D) An omb^1/omb^1 mutant female wing has a notched wing margin, but no L5 truncation. (E) A $brk^{m68}/+$; ab^1/ab^1 wing has a phenotype similar to that of ab^1/ab^1 homozygotes. (F) A trans-heterozygous $omb^1/+$; $ab^1/+$ female wing with a distal L5 truncation. This is a reproducible phenotype that occurs with low penetrance (3%). (G) An $omb^1/+$; ab^1/ab^1 female wing has an enhanced L5 vein truncation relative to that observed in ab^1/ab^1 controls (compare with A). (H) An omb^1/Y; ab^1/ab^1 double mutant male wing has a greatly enhanced L5 truncation phenotype relative to that observed in ab^1/ab^1 flies (compare with A).

omb or *brk* to erase or shift gene expression borders, and by performing clonal analysis (see below) to create new borders between these domains. For the misexpression experiments, we employed the GAL4/UAS system (Brand and Perrimon, 1993) to drive expression of *omb* or *brk* in different patterns and levels within the developing wing disc, and then examined the consequences of these manipulations in adult wings. As *brk* is a repressor of *omb*, misexpression of *brk* should eliminate *omb* expression in the regions where *brk* is ectopically expressed. However, the effect of misexpressing *omb* on *brk* expression has not been previously reported (see below).

To eliminate or blur the borders between *brk* and *omb* expressing cells we misexpressed *omb* or *brk* with weak or strong ubiquitous wing drivers, as well as the Vg^B-GAL4 driver, which activates localized gene expression along the wing margin (Fig. 4C) (Williams et al., 1994). Ubiquitous misexpression of either *omb* or *brk* in the wing using the strong *MS1096*-GAL4 driver resulted in small wings with a range of venation phenotypes, in which all or some veins were shifted, truncated or missing entirely (e.g. Fig. 4A). In these experiments the L2 and L5 veins were particularly sensitive to the effects of ubiquitous *brk* or *omb* expression, although other veins were also disrupted by high expression levels of these genes (data not shown). The global effects on wing patterning associated with strong ubiquitous expression of *omb* or *brk* may result from disrupting more general functions of these primary BMP response genes in defining regional identities within their broad domains of expression.

Ubiquitous misexpression of *brk* with the weaker driver, *C765*-GAL4, resulted in a range of venation phenotypes, including selective loss or displacement of the L2 and L5 veins (Fig. 4B), formation of a single central vein (L3) or the complete loss of veins (data not shown). In all cases, the L2 vein was either missing or reduced to a small posteriorly displaced remnant, whereas the L5 vein was shifted anteriorly (Fig. 4B). As *brk* is expressed in a reciprocal gradient to that of Dpp in the wing discs, ubiquitous misexpression of *brk*, added to its endogenous graded expression, should result in graded but higher than normal Brk levels in the peripheral regions of the disc. This increase in the basal level of *brk* expression would be expected to shift the borders between *brk* and *omb* or *sal* domains towards the center of the wing disc, consistent with the observed convergent displacement of L2 and L5 veins in *C765*-GAL4; UAS-*brk* flies (Fig. 4B). A similar centrally compressed vein phenotype (Fig. 4D) was observed when *brk* was misexpressed in a broad zone along the wing margin using the Vg^B-GAL4 driver (Fig. 4C), which substantially increases *brk* levels in peripheral regions of the wing when flies are raised at 25°C.

Misexpression of *omb* at modest levels also caused specific venation defects. For example, ectopic expression of *omb* along the posterior wing margin driven by Vg^B-GAL4 (Fig. 4C) causes distal truncation of the L5 primordium near its intersection with the margin (Fig. 4E, arrow). This loss of the endogenous L5 primordium may be a consequence of reduced *brk* expression in these cells since ectopic *omb* expression in peripheral regions of the wing disc results in downregulation of *brk* expression (O.C., unpublished). Another consequence of misexpressing *omb* with the Vg^B-GAL4 driver is the creation of a new *brk*/*omb* border posterior to L5. This border forms between the narrow strip of Vg^B-GAL4 expressing cells and the posterior edge of the endogenous *brk* expression domain, as can be observed in *brk-lacZ*; Vg^B-GAL4; UAS-GFP wing discs (Fig. 4C, arrow). In a fraction of Vg^B-GAL4; UAS-*omb* flies, we observed ectopic veins forming posterior to L5 (Fig. 4E, arrowhead), in addition to the posterior truncation of the endogenous L5 vein. This ectopic vein forms in the expected location of the new *brk*/*omb* border created by Vg^B-GAL4>*omb* expression. These observations suggest that having a sharp posterior *omb*/*brk* boundary is important for L5 formation.

omb is required cell autonomously for L5 development

In order to determine whether the boundary between *brk* and *omb* expression domains was necessary for inducing L5

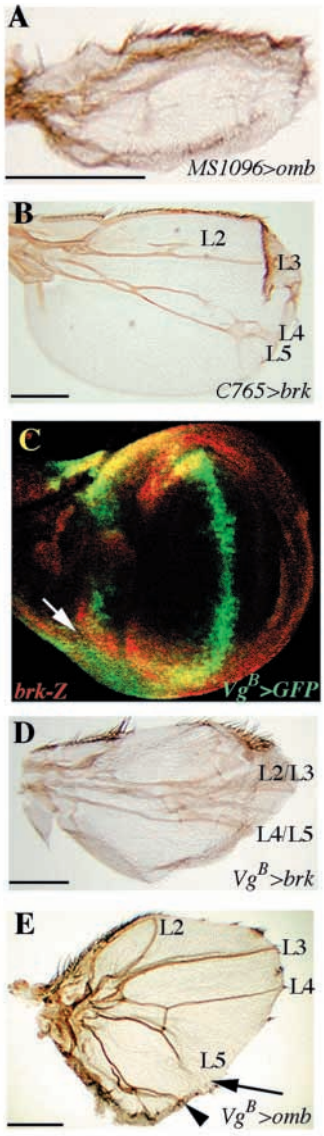

Fig. 4. Misexpression of *omb* or *brk* preferentially eliminates or displaces the L2 and L5 veins. (A) A wing from an *MS1096*-GAL4; UAS-*omb* fly is severely reduced in size and lacks posterior veins. (B) A wing from a *C765*-GAL4; UAS-*brk* fly displays a central shift of the L2 and L5 veins resulting in L2 approaching or fusing with L3 (i.e. 0-4 cells apart compared with 10-12 cells apart in wild type), and L5 approaching or fusing with L4 (i.e. 0-9 cells apart compared with 18-20 cells apart in wild type). By contrast, the space between the central L3 and L4 veins remained relatively unaltered (14-16 cells apart compared with 16-17 cells in wild type). All distance measurements between veins were made in the central region of the wing. (C) A third instar larval wing imaginal disc (anterior at the top) from a *brk-lacZ*; Vg^B-GAL4; UAS-*GFP* individual, grown at 22°C, stained for β-Gal expression (red) and examined for GFP fluorescence (green). Note the posterior domain in which green Vg^B>*GFP* expression abuts the posterior edge of the *brk* expression domain (arrow), and note that expression of GFP continues along the margin. Stronger and wider GFP expression was observed in discs of the same genotype raised at 25°C (data not shown). (D) A wing from a Vg^B-GAL4; UAS-*brk* fly grown at 25°C displays a central shift of the L2 and L5 veins resulting in fusion of L2 with L3, and of L4 with L5. (E) A wing from a Vg^B-GAL4; UAS-*omb* fly grown at 22°C, which has a notched margin, truncated L5 vein (arrow) and a long ectopic vein posterior to L5 (arrowhead). Scale bars: 0.5 mm.

development, we generated somatic *brk* or *omb* mutant clones or double-mutant clones lacking both *brk* and *omb* function (see Materials and methods for details). We first examined the requirement for *omb* by generating *omb*$^-$ null clones in different regions of the wing. Such *omb*$^-$ clones did not result in any vein phenotype when they were located in central regions of the wing (Fig. 5C, arrowhead), although these cells normally express high levels of *omb* in wing discs. This result indicates that simply having a border between *omb* expressing and non-expressing cells is not sufficient to induce vein formation. Moreover, although *omb* expression also extends into the L2 primordium (Fig. 2D-F), *omb*$^-$ clones located in this region of the wing did not disrupt formation of the L2 vein or expression of the L2 organizer gene *kni* in wing discs (Fig. 5A,B). By contrast, *omb*$^-$ clones in posterior regions of the wing that overlapped part of the L5 vein resulted in vein loss within the clone (Fig. 5C, arrow). Consistent with the L5 vein-loss adult phenotype, cells within *omb*$^-$ clones crossing the L5 primordium in third instar wing discs failed to express the vein marker Dl (Fig. 5D), whereas *omb*$^-$ clones located in central regions of the wing disc had no effect on Dl expression in the L3 or L4 primordia (data not shown). These data indicate that *omb* is required specifically for the formation of the normal L5 primordium, although a border of *omb* expression domain is not sufficient on its own to induce vein formation.

brk is required for the production of an L5 inductive signal

Having established that Omb is required cell autonomously for initiation of L5 development, we examined the role of *brk* in this process by generating *brk*$^-$ mutant clones. These *brk*$^-$ mutant cells also misexpress *omb* as a result of relieving repression by Brk (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Minami et al., 1999). Such *brk*$^-$ clones create an ectopic border between *brk* and *omb* if located within *brk*-expressing peripheral regions of the wing disc. When *brk*$^-$ clones were generated in the center of the wing they did not result in any phenotype (data not shown), as expected from the absence of endogenous *brk* expression in these cells. By contrast, *brk*$^-$ clones located in positions posterior to L5 (Fig. 5E), or anterior to L2 (Fig. 5G), induced ectopic veins that formed along the clone borders. In posterior *brk*$^-$ clones, ectopic veins formed strictly within the clone abutting wild-type cells outside the clone (Fig. 5E). Similarly, *brk*$^-$ clones examined in third instar wing discs ectopically expressed the vein marker Dl in a narrow ring of cells encircling the inside of the clone border (Fig. 5F). This arrangement of cells mimics the normal situation for L5 initiation, in that the induced vein forms within a domain of *omb* expression adjacent to *brk*-expressing cells.

Anterior *brk*$^-$ clones located several cell diameters away from the endogenous L2 vein also induced ectopic veins running within and along the clone border (Fig. 5G, red arrowhead). In third instar wing discs, comparably located clones ectopically expressed the L2 vein organizing gene *kni* within the clone (Fig. 5H). However, *brk*$^-$ clones located in the immediate vicinity of the endogenous L2 vein induced ectopic veins along the outside border of the clone (Fig. 5G, black arrowhead). The potential basis for the different behaviors of anterior *brk*$^-$ clones as a function of distance from the L2 vein is discussed below.

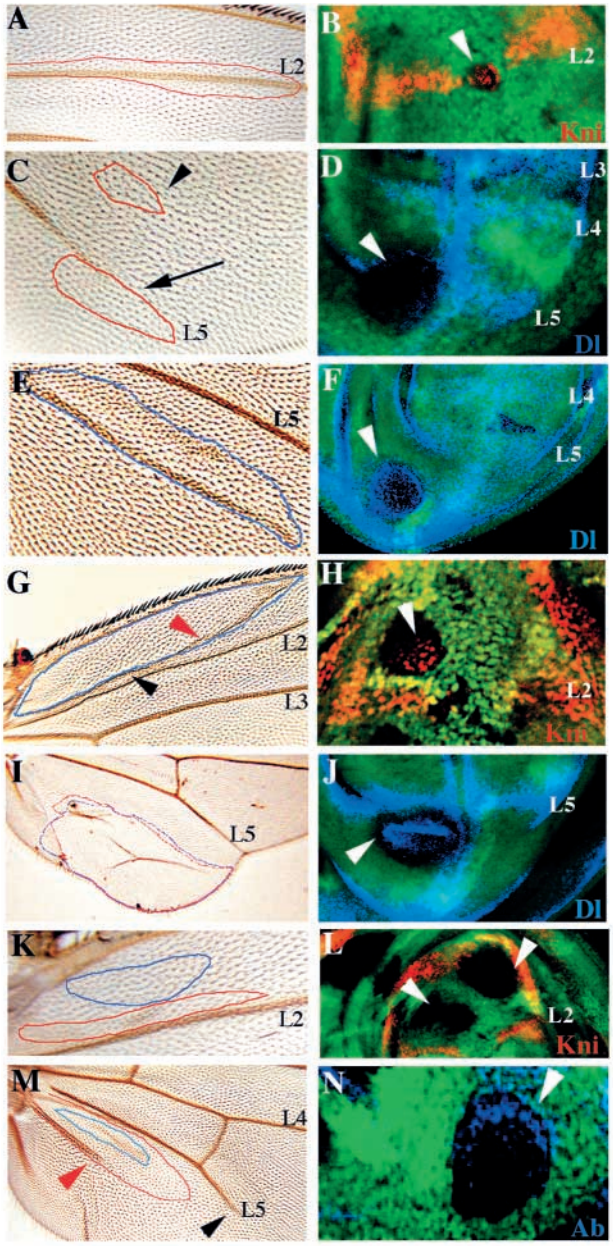

Fig. 5. *brk* induces L5 formation in adjacent *omb*-expressing cells. Clonal analysis of *omb* and *brk* function in L5 development. Adult wings and wing imaginal discs are oriented with the anterior at the top. In adult wings, ventral clones are outlined in blue and dorsal clones are outlined in red. Clones in wing discs are marked by the absence of GFP expression. (A) Wing with an anterior dorsal *omb*$^{-}$ clone. The L2 vein extends uninterrupted through the clone. (B) *kni* expression (red labeling) is normal inside an anterior *omb*$^{-}$ mutant clone (arrowhead) in a third instar larval wing imaginal disc. (C) A wing with posterior dorsal *omb*$^{-}$ clones. The clone anterior to L5 (arrowhead) does not cause any vein phenotype, whereas the clone that crosses L5 results in vein truncation (arrow). (D) Dl expression (blue labeling) is lost within a posterior *omb*$^{-}$ mutant clone (arrowhead) in a third instar larval wing imaginal disc. (E) A wing with a posterior ventral *brk*$^{-}$ clone. An ectopic vein runs strictly inside and along the clone border. (F) Dl is ectopically expressed (blue labeling) within and encircling a posterior *brk*$^{-}$ clone (arrowhead) in a third instar larval wing imaginal disc. (G) A wing with an anterior ventral *brk*$^{-}$ clone. An ectopic vein lies along the border, within the clone (red arrowhead), except near the branch-point with L2 (black arrowhead) where it runs just outside of the clone. (H) *kni* is ectopically expressed (red labeling) within an anterior *brk*$^{-}$ clone (arrowhead) in a third instar larval wing imaginal disc. (I) A wing with overlapping dorsal and ventral posterior *omb*$^{-}$ *brk*$^{-}$ double mutant clones. Ectopic disorganized veins form within the clone interior. (J) Dl (blue labeling) is expressed within a posterior *omb*$^{-}$ *brk*$^{-}$ double mutant clone (arrowhead) in a third instar larval wing imaginal disc. Dl is expressed in unorganized pattern, within the interior of the clone. (K) A wing with anterior ventral and dorsal *omb*$^{-}$ *brk*$^{-}$ double mutant clones. A short segment of vein runs within the interior of the dorsal clone. (L) *kni* is not misexpressed (red labeling) within anterior *omb*$^{-}$ *brk*$^{-}$ double mutant clones (arrowheads) in a third instar larval wing imaginal disc. (M) Posterior ventral and dorsal *brk*$^{-}$ clones in ab^{1}/ab^{1} mutant flies. The vein running along the posterior clone border is truncated (red arrowhead) in a similar location as the endogenous L5 vein (black arrowhead). In addition, a segment of vein forms within the center of the ventral clone. The phenotypes for the *brk*$^{-}$ clones shown in this panel and in E are representative clones scored posterior to or overlapping the L5 vein. Among a total of 44 such *brk*$^{-}$ clones generated in a wild-type background, 31 (70%) had veins extending for more than half of the proximal-distal length of the clone. These veins all formed inside and along the clone borders. The remaining 13 *brk*$^{-}$ clones had shorter segments of vein, which also ran along and within the clone borders. Among 15 comparably situated *brk*$^{-}$ clones generated in an *ab*$^{-}$ background, 3 (20%) had veins that extended along more than half the length of the clone border. The remaining clones had only short segments of vein and only 3 (20%) had disorganized vein material forming within the interior of the clone. No such internal disorganized veins were observed in any of the *brk*$^{-}$ clones generated in a wild-type background. (N) Ab expression (blue) along the border of a *brk*$^{-}$ clone located posterior to L5. No such ectopic Ab expression was observed in *brk*$^{-}$ *omb*$^{-}$ double mutant clones (O.C., unpublished).

The above analysis of the *brk*$^{-}$ and *omb*$^{-}$ single mutant clones suggests that *brk*-expressing cells induce L5 development in adjacent *omb*-overexpressing cells. This condition is met when *brk*$^{-}$ clones are generated in posterior regions of the wing, as loss of *brk* activity in these cells results in de-repression of *omb* expression (Sivasankaran et al., 2000). To test whether *omb* expression is required for the induction of ectopic veins within *brk*$^{-}$ clones, we generated *omb*$^{-}$ *brk*$^{-}$ double mutant clones. In contrast to *brk*$^{-}$ single mutant clones, we did not observe consistent induction of veins running along the edges of *omb*$^{-}$ *brk*$^{-}$ clone borders. In many cases, double-mutant *omb*$^{-}$ *brk*$^{-}$ clones contained no veins at all. In other cases, patches of vein material were observed that tended to be either short fragments of vein, which did not follow the clone boundary, or diffuse random veins meandering within the clone (Fig. 5I). Consistent with this adult wing-vein phenotype, posteriorly located double-mutant *omb*$^{-}$ *brk*$^{-}$ clones in third instar wing discs did not induce expression of the vein marker *Dl* along clones borders. In some of these *omb*$^{-}$ *brk*$^{-}$ clones, we observed diffuse expression of Dl or fragments of internal Dl expression (Fig. 5J), and in other cases we observed no Dl expression at all (data not shown). One unexpected result was that although *omb* is not required for formation of the endogenous L2 vein, it is essential for formation of ectopic veins observed in anteriorly located *brk*$^{-}$ clones. Thus, in contrast to the ectopic veins which formed along the inside borders of anterior *brk*$^{-}$ single clones, similarly positioned *omb*$^{-}$ *brk*$^{-}$ double mutant clones generally did not form any ectopic veins (Fig. 5K), nor did they induce ectopic Kni expression within the clone boundary (Fig. 5L). This finding suggests that the ectopic veins in anterior *brk*$^{-}$ clones may not have a simple L2 identity (see Discussion below).

Cumulatively, this clonal analysis reveals that induction of the L5 primordium depends on two conditions being met: (1) cell-autonomous Omb activity; and (2) non-autonomous induction by Brk acting across a sharp border with adjacent *omb*-expressing cells.

ab acts downstream of *brk* in L5 development

As *ab* functions at an early stage in L5 development (i.e. as a vein organizing gene), we investigated whether Ab was also misexpressed along the border of *brk*⁻ clones. We found that, as in the case of Dl, a ring of ectopic Ab expression circumnavigated the interior border of *brk*⁻ clones located in the vicinity of the endogenous L5 primordium (Fig. 5N). By contrast, no such Ab expression was observed in *omb*⁻ *brk*⁻ double mutant clones (data not shown). These results are consistent with activation of *ab* expression being downstream of a *brk*-induced signaling event.

We also determined whether *ab* is required to mediate the formation of ectopic veins observed in *brk*⁻ clones. We addressed this question by generating *brk*⁻ clones in an ab^1/ab^1 mutant background and scoring adult-vein phenotypes in various regions of the wing primordium. This analysis revealed that the frequency of ectopic veins within clones located in the vicinity of L5 was significantly reduced in *brk*⁻ clones produced in ab^1/ab^1 versus wild-type backgrounds. Some clones that formed posterior to L5 resembled *omb*⁻ *brk*⁻ double mutant clones, in that they either lacked veins entirely or had veins running diffusely within the clone region but not along the boundary (Fig. 5M, blue outlined clone). In larger clones, veins followed the clone border in proximal regions of the wing for a short distance, and then ended as the clone entered the distal regions (Fig. 5M, red arrowhead; compare with Fig. 5E), where the endogenous L5 vein is truncated in ab^1/ab^1 mutants (Fig. 5M, black arrowhead; see legend for quantification). These results suggest that *ab* is an essential mediator of *brk*- and *omb*-dependent induction of the L5 primordium.

Discussion

Morphogens play a central role in controlling growth and differentiation in both invertebrate and vertebrate development. A well-studied example of morphogen-dependent patterning is long-range diffusion of Dpp from its source in the center of the wing disc to establish AP positional information (Affolter et al., 2001; Cadigan, 2002). This graded positional information is translated into threshold-dependent expression of a set of transcription factors in broad domains along the AP axis. Several transcription factors, such as *sal*, *omb* and *vg*, are expressed in a nested series of central domains in direct correlation to Dpp protein levels and BMP pathway activation (Lecuit et al., 1996; Nellen et al., 1996). A key transcriptional mediator of BMP signaling is the repressor Brk, which is expressed in a reciprocal pattern to that of Dpp as a result of repression by the BMP signaling pathway (Muller et al., 2003). Each of these transcription factors specifies sub-populations of cells along the AP axis, which can send, and/or respond to, various secondary local signals. These local interactions between adjacent domains of cells create fine-scale positional information for organizing specific structures such as wing veins in precise locations.

Induction of L5 formation along the *brk/omb* border

In a previous model for establishing the position of the L5 primordium, it was proposed that *sal/salr* was the only Dpp target gene responsible for wing vein patterning, which determined the anterior position of the L5 primordium by repressing expression of IroC genes (de Celis and Barrio, 2000). It was also suggested that IroC gene expression was directly dependent on BMP signaling and that fading of the BMP activity gradient determined the posterior limit of IroC gene expression (de Celis and Barrio, 2000).

In the current study, we examined the role of two other Dpp target genes, which are expressed in domains abutting (*brk*) or just including (*omb*) the L5 primordium, in establishing the position of this vein. Our results, suggest an alternative model for how the BMP activity gradient induces formation of the L5 primordium in the posterior compartment of the wing (Fig. 6). According to this model, L5 development is initiated within

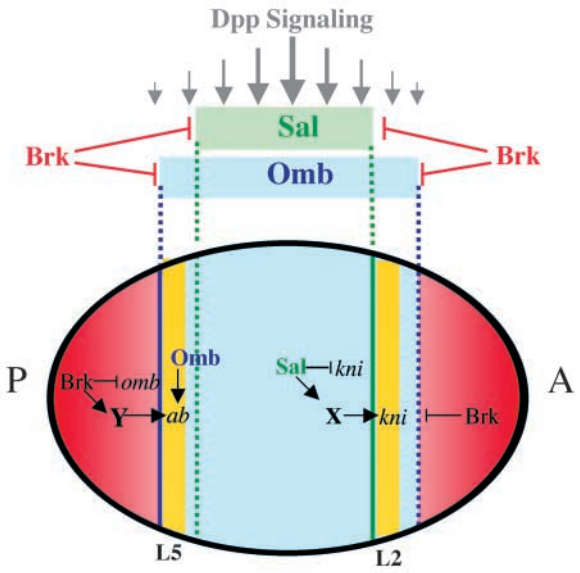

Fig. 6. A model for induction of the L5 primordium along the *omb*/*brk* border. The expression domain of the two Dpp signaling target genes *omb* (blue) and *sal* (green) is defined by their sensitivity to activation by Dpp and repression by Brk (red domains), in extreme anterior (A) and posterior (P) regions of the wing disc. L5 forms along the posterior border between *omb* and *brk* domains (solid blue line) following activation of the L5 organizer gene *ab*. *brk*-expressing cells on one side of the border produce a short range signal (Y) to which they cannot respond. This signal diffuses to the neighboring cells and, in combination with Omb, activates expression of *ab* along the border (yellow stripe). The green dotted line, which marks the posterior extent of strong *sal* expression, corresponds to a cryptic paravein border located midway between L4 and L5 (Sturtevant et al., 1997). This model is similar to that suggested for L2 formation along the border of the high level *sal* expression domain (Lunde et al., 1998). In the case of L2, *sal*-expressing cells produce a short range signal (X), to which they cannot respond due to repression by Sal, and this signal induces the expression of the L2 organizer gene *kni* along the border of cells just anterior to the *sal* domain (yellow stripe). The blue dotted line, which is indicated within the domain of strong *omb* expression along its anterior border and adjacent to cells expressing high levels of *brk*, may correspond to a cryptic paravein border located between L2 and the wing margin.

the posterior region of the wing where *brk* and *omb* are expressed in adjacent domains with a sharp border between them. As *brk*$^{-}$ clones induce vein development within the clone along the border with *brk*$^{+}$-neighboring cells, we suggest that *brk*-expressing cells produce a short-range vein-inductive signal, Y, to which they cannot respond. This signal acts on neighboring *omb*-expressing cells to initiate vein development. The additional cell-autonomous requirement for Omb activity to respond to this Brk-derived signal suggests that the intracellular effector of the vein inductive signal Y must act in combination with Omb to induce vein formation. Because Brk is a repressor of *omb* expression, the combined requirement for the short-range Brk-derived vein-inductive signal and Omb activity within responding cells constrains L5 initiation to *omb*-expressing cells adjacent to *brk*-expressing cells. In this scheme, Brk plays at least two distinct roles in L5 induction. First, as a repressor of *omb*, Brk defines the border between the *brk* and *omb* expression domains, and, second, *brk*-expressing cells are the source of a vein-inductive signal required to initiate L5 development within adjacent *omb*-expressing cells.

ab functions as the L5 vein organizing gene

A key mediator of L5 induction is the Ab transcription factor, which is expressed in a narrow stripe along the *brk*/*omb* border, just within the *omb* expression domain. *ab* is required for expression of all known vein genes and for downregulation of intervein genes in the L5 primordium (Fig. 1) (Biehs et al., 1998). Similarly, the ability of *brk*$^{-}$ clones to induce an ectopic posterior vein depends on *ab* function. In addition, localized misexpression of *ab* in small flip-out clones leads to induction of vein markers in wing imaginal discs and to the formation of ectopic patches of vein material. The vein-organizing activity of *ab* depends on its being expressed in a localized pattern, as ubiquitous expression of *ab* suppresses vein development throughout the wing disc. This effect of ubiquitous *ab* misexpression is similar to that observed previously for ubiquitous expression of *kni* or *knrl*, in which all distinctions between vein and intervein regions were lost although expression of other genes in the wing disc was not perturbed (Lunde et al., 1998). One explanation for this vein-erasing phenotype is that *kni*/*knrl* and *ab* control the expression of a lateral inhibitory signal. Consistent with this possibility, small *ab* flip-out clones autonomously express the lateral inhibitory signal Dl. According to the model in Fig. 6, establishment of the L5 primordium requires input from both *omb* (cell autonomous) and *brk* (cell non-autonomous), which collaborate to initiate *ab* expression in a narrow stripe along their borders.

A curious phenotype associated with some *brk*$^{-}$ clones generated in an *ab*^{1}/*ab*^{1} background is the formation of diffuse wandering veins within the interior of the clone. A similar disorganized ectopic vein phenotype is also observed in a fraction of *omb*$^{-}$ *brk*$^{-}$ double mutant clones. This phenotype may reflect the lack of a lateral inhibitory factor (e.g. Dl) produced by *ab*-expressing cells to suppress vein formation in neighboring cells. The observation that ubiquitous expression of *ab* suppresses vein formation throughout the wing disc is consistent with this possibility. It is also possible that *omb* plays a role in promoting intervein development as well as in activating *ab* expression. Additional analysis will be needed to address this question.

Brk plays a role in positioning the L2 primordium

Previous analysis of L2 initiation lead to a model in which *sal*-expressing cells produce a short-range vein-inductive signal (X) to which they cannot respond (Fig. 6) (Sturtevant et al., 1997). In response to signal X, neighboring cells outside of the *sal* domain express the L2 vein-organizing genes *kni* and *knrl* (Bier, 2000; Lunde et al., 1998). In addition, analysis of an L2-specific cis-regulatory element of the *kni*/*knrl* locus provided indirect evidence for negative regulation by a repressor, possibly Brk, expressed in peripheral/lateral regions of the wing disc (Lunde et al., 2003).

In the current study, we find that anterior *brk*$^{-}$ clones result in two different phenotypes, depending on their distance from the L2 primordium. First, as suspected from analysis of the L2-enhancer element, Brk acts in a cell-autonomous fashion to repress *kni*/*knrl* expression. This effect of Brk is observed in clones located several cell diameters anterior to the L2 primordium. The cell-autonomous induction of veins within the borders of these *brk*$^{-}$ clones can be explained by a mechanism similar to that operating within the posterior compartment, where *brk*-expressing cells induce vein development in adjacent cells. In such clones, loss-of-*brk* function does not result in significant levels of ectopic *sal* expression (Campbell and Tomlinson, 1999) (O.C., unpublished). The absence of a vein outside of these clones could result from a combination of three effects. First, the low levels of *sal* in such clones is not likely to be sufficient to activate expression of appreciable levels of signal X. Second, Brk levels outside of the clones are higher than in the L2 region, which presumably represses *kni* expression effectively in cells surrounding the *brk*$^{-}$ clones. Finally, there is evidence that low levels of *salr* and/or *salm* are required for L2 development (de Celis and Barrio, 2000), and detectable endogenous expression of *sal* extends only a short distance beyond the L2 primordium.

The second phenotype associated with *brk*$^{-}$ clones, which is restricted to clones located immediately anterior to L2, is a cell non-autonomous effect in which short segments of vein form along the clone border just outside of the clone. This non-autonomous effect of *brk*$^{-}$ clones located at branch points with L2 may be explained by the de-repression of Sal within such clones. As *sal* expression also requires a positive input from the BMP pathway, relieving repression by Brk induces high levels of Sal for only a short distance anterior of the L2 primordium (Campbell and Tomlinson, 1999) (O.C., unpublished). These *sal*-expressing cells should produce the L2 inductive signal X, which acts in a cell non-autonomous fashion, as proposed in the model for L2 formation (Fig. 6).

An interesting question regarding veins forming within more anteriorly located *brk*$^{-}$ clones is do they have an L2- or an L5-like identity? On the one hand, these veins express *kni*, but not Dl, suggesting that they have an L2-like identity. On the other hand, the ectopic veins induced anteriorly by *brk*$^{-}$ clones require *omb* function, as do L5-like veins generated in the posterior compartment of the wing. This latter observation suggests that the *brk*$^{-}$ border in anterior regions acts as it does in posterior regions of the wing disc, but that its effect may be mediated by the L2 organizing *kni*/*knrl* locus rather than the L5 organizing gene *ab*. This hypothesis might provide an explanation for why ectopic veins that form in various mutant backgrounds tend to form along a line running between the L2

vein and the margin (which we refer to as the P2 paravein) (Sturtevant et al., 1997). This sub-threshold vein promoting position may be defined by the anterior border of *brk* and *omb* expression. Further analysis of the identity of these ectopic veins will be required to resolve this question.

Similarities and differences between induction of the L2 and L5 vein primordia

As the L2 and L5 veins form at similar lateral positions within the anterior and posterior compartments of the wing, respectively, it is informative to compare the mechanisms by which positional information is converted into vein initiation programs in these two cases. The positions of these two veins are determined by precise dosage-sensitive responses to BMP signaling emanating from the center of the wing, which are mediated by the borders of the broadly expressed, Dpp signaling target genes *sal* and *omb.* Brk also plays a role in initiating both L2 and L5 development. In the posterior compartment, Brk leads to the production of a hypothetical vein-promoting signal Y, which has a similar function and range as the putative L2 vein-inducing signal X, produced by *sal*-expressing cells. It is not clear whether the signals X and Y are the same or different; however, an important difference between L2 and L5 initiation is that only L5 has an additional requirement for *omb* function. This dual requirement for *omb* function within the L5 vein primordium and a short-range inductive signal in neighboring *brk*-expressing cells provides a stringent constraint on where the L5 primordium forms. Brk may also directly repress expression of the vein-organizer gene *ab* in cells posterior to the L5 primordium, in analogy to its proposed role as a repressor of *kni*/*knrl* anterior to L2. One possible rationale for induction of the L5 vein depending on inputs from both *omb* and *brk* is that these genes are expressed in partially overlapping patterns and neither pattern may carry sufficiently detailed information to specify the position of the L5 primordium alone. Although the *omb* and *brk* expression levels fall off relatively steeply (i.e. over a distance of six to eight cells), these borders are not as sharp as the anterior *sal* border (two to three cells wide), which alone is sufficient to induce the L2 primordium.

A final similarity between the initiation of L2 and L5 formation is that induction of both veins is mediated by a vein-organizing gene that regulates vein and intervein gene expression in the vein primordium. Although *kni* and *ab* are members of different subfamilies of Zn-finger transcription factors, they are both expressed in a narrow stripe of cells along their respective inductive borders, and ubiquitous misexpression of either gene [see Fig. 1 for *ab*, and see Lunde et al. (Lunde et al., 1998) for *kni*] results in elimination of vein pattern in the wing disc. Thus, the L2 and L5 veins are induced by remarkably similar mechanisms and principles of organization. Further comparison of the mechanisms of these developmental programs should provide insights into the degree to which general and specific vein processes define the L2 versus the L5 vein identity.

Boundaries translate graded positional information into sharp linear responses

Induction of *Drosophila* wing veins at borders between adjacent gene expression domains provides a simple model system for studying how information provided by morphogen gradients is converted into the stereotyped pattern of wing vein morphogenesis. Each of the four major longitudinal veins (L2-L5) is induced by a for-export-only mechanism in which cells in one region of the wing produce a diffusible signal to which they cannot respond. In the case of L3 and L4, an EGF-related signal (Vein) is produced between these veins in the central organizer where expression of the EGF receptor is locally downregulated (Crozatier et al., 2002; Mohler et al., 2000; Vervoort et al., 1999). With respect to L2, response to the vein-inductive signal X is repressed in Sal-expressing cells that produce the hypothetical signal X (Lunde et al., 1998; Sturtevant et al., 1997). Finally, the L5 vein-inductive signal produced by *brk*-expressing cells depends on *omb*, the expression of which is repressed by Brk.

For-export-only mechanisms also underlie the induction of boundary cell fates in many other developmental settings. In the well-studied *Drosophila* wing, the earliest and most rigorously defined boundaries are the AP and DV borders, which are determined by Hh and Notch signaling, respectively. These compartmental borders define domains of non-intermixing groups of cells, and function as organizing centers by activating expression of the long-range morphogens Dpp and Wingless (Wg), respectively (reviewed by Sanson, 2001). In both cases, cells in one compartment produce a signal to which they cannot respond. This signal is constrained to act only on neighboring cells in the adjacent compartment. Other well-studied examples of for-export-only signaling include: induction of the mesectoderm in blastoderm stage *Drosophila* embryos by a likely cell-tethered Notch ligand expressed in the mesoderm (Cowden and Levine, 2002; Lecourtois and Schweisguth, 1995; Lunde et al., 1998; Morel et al., 2003; Morel and Schweisguth, 2000); induction of parasegmental expression of *stripe* via Wg, Hh and Spi signaling in gastrulating *Drosophila* embryos (Hatini and DiNardo, 2001); induction of mesoderm in *Xenopus* embryos by factors produced in the endoderm under the control of VegT (reviewed by Shivdasani, 2002); and formation of the DV border of leaves in plants controlled by the *PHANTASTICA* gene (Waites et al., 1998). The similar but distinct mechanisms for inducing the L2 and L5 vein primordia offers a well-defined system for examining these relatively simple cases in depth. These inductive events take place at the same developmental stage but within separate compartments of a single imaginal disc, and should provide general insights into the great variety of mechanisms that can be co-opted to accomplish for-export-only signaling.

We thank Jennifer Trimble for critical comments on the manuscript, and Dave Kosman and Mieko Mizutani for help with confocal analysis. We thank Chris Rushlow, Steve Cohen, Konrad Basler, Gert Pflugfelder, José-Luis Gomez-Skarmeta and Sean Carroll for providing fly stocks; and thank David Kosman, Marc Muskavitch, Markus Affolter, Gerard Campbell and Adi Salzberg for providing antibodies. We also thank the reviewers for their helpful comments. This work was supported by NIH grant R01 GM60585.

References

Affolter, M., Marty, T., Vigano, M. A. and Jazwinska, A. (2001). Nuclear interpretation of Dpp signaling in *Drosophila*. *Embo J.* **20**, 3298-3305.

Ashburner, M. (1989). Drosophila: *A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Biehs, B., Sturtevant, M. A. and Bier, E. (1998). Boundaries in the

Drosophila wing imaginal disc organize vein-specific genetic programs. *Development* **125**, 4245-4257.
Bier, E. (1997). Anti-neural-inhibition: a conserved mechanism for neural induction. *Cell* **89**, 681-684.
Bier, E. (2000). Drawing lines in the *Drosophila* wing: initiation of wing vein development. *Curr. Opin. Genet. Dev.* **10**, 393-398.
Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.
Cadigan, K. M. (2002). Regulating morphogen gradients in the Drosophila wing. *Semin. Cell Dev. Biol.* **13**, 83-90.
Campbell, G. (2002). Distalization of the *Drosophila* leg by graded EGF-receptor activity. *Nature* **418**, 781-785.
Campbell, G. and Tomlinson, A. (1999). Transducing the Dpp morphogen gradient in the wing of *Drosophila*: regulation of Dpp targets by *brinker*. *Cell* **96**, 553-562.
Cowden, J. and Levine, M. (2002). The Snail repressor positions Notch signaling in the *Drosophila* embryo. *Development* **129**, 1785-1793.
Crozatier, M., Glise, B. and Vincent, A. (2002). Connecting Hh, Dpp and EGF signalling in patterning of the *Drosophila* wing; the pivotal role of *collier*/*knot* in the AP organiser. *Development* **129**, 4261-4269.
de Celis, J. F. and Barrio, R. (2000). Function of the *spalt*/*spalt-related* gene complex in positioning the veins in the *Drosophila* wing. *Mech. Dev.* **91**, 31-41.
Entchev, E. V., Schwabedissen, A. and Gonzalez-Gaitan, M. (2000). Gradient formation of the TGF-beta homolog Dpp. *Cell* **103**, 981-991.
Fujise, M., Takeo, S., Kamimura, K., Matsuo, T., Aigaki, T., Izumi, S. and Nakato, H. (2003). Dally regulates Dpp morphogen gradient formation in the *Drosophila* wing. *Development* **130**, 1515-1522.
Grimm, S. and Pflugfelder, G. O. (1996). Control of the gene optomotor-blind in Drosophila wing development by decapentaplegic and wingless. *Science* **271**, 1601-1604.
Haerry, T. E., Khalsa, O., O'Connor, M. B. and Wharton, K. A. (1998). Synergistic signaling by two BMP ligands through the SAX and TKV receptors controls wing growth and patterning in *Drosophila*. *Development* **125**, 3977-3987.
Hatini, V. and DiNardo, S. (2001). Distinct signals generate repeating striped pattern in the embryonic parasegment. *Mol. Cell* **7**, 151-160.
Hu, S., Fambrough, D., Atashi, J. R., Goodman, C. S. and Crews, S. T. (1995). The *Drosophila abrupt* gene encodes a BTB-zinc finger regulatory protein that controls the specificity of neuromuscular connections. *Genes Dev.* **9**, 2936-2948.
Jazwinska, A., Kirov, N., Wieschaus, E., Roth, S. and Rushlow, C. (1999a). The *Drosophila* gene *brinker* reveals a novel mechanism of Dpp target gene regulation. *Cell* **96**, 563-573.
Jazwinska, A., Rushlow, C. and Roth, S. (1999b). The role of *brinker* in mediating the graded response to Dpp in early *Drosophila* embryos. *Development* **126**, 3323-3334.
Kirkpatrick, H., Johnson, K. and Laughon, A. (2001). Repression of *dpp* targets by binding of Brinker to Mad sites. *J. Biol. Chem.* **276**, 18216-18222.
Klein, T. (2001). Wing disc development in the fly: the early stages. *Curr. Opin. Genet. Dev.* **11**, 470-475.
Lawrence, P. A. and Struhl, G. (1996). Morphogens, compartments, and pattern: lessons from *Drosophila*? *Cell* **85**, 951-961.
Lecourtois, M. and Schweisguth, F. (1995). The neurogenic Suppressor of Hairless DNA-binding protein mediates the transcriptional activation of the *Enhancer of Split* complex genes triggered by Notch signaling. *Genes Dev.* **9**, 2598-2608.
Lecuit, T., Brook, W. J., Ng, M., Calleja, M., Sun, H. and Cohen, S. M. (1996). Two distinct mechanisms for long-range patterning by Decapentaplegic in the *Drosophila* wing. *Nature* **381**, 387-393.
Lunde, K., Biehs, B., Nauber, U. and Bier, E. (1998). The *knirps* and *knirps-related* genes organize development of the second wing vein in *Drosophila*. *Development* **125**, 4145-4154.
Lunde, K., Trimble, J. L., Guichard, A., Guss, K. A., Nauber, U. and Bier, E. (2003). Activation of the *knirps* locus links patterning to morphogenesis of the second wing vein in *Drosophila*. *Development* **130**, 235-248.
Marty, T., Muller, B., Basler, K. and Affolter, M. (2000). Schnurri mediates Dpp-dependent repression of *brinker* transcription. *Nat. Cell Biol.* **2**, 745-749.
Minami, M., Kinoshita, N., Kamoshida, Y., Tanimoto, H. and Tabata, T. (1999). *brinker* is a target of Dpp in *Drosophila* that negatively regulates Dpp-dependent genes. *Nature* **398**, 242-246.
Mohler, J., Seecoomar, M., Agarwal, S., Bier, E. and Hsai, J. (2000). Activation of *knot* (*kn*) specifies the 3-4 intervein region in the *Drosophila* wing. *Development* **127**, 55-63.
Morel, V. and Schweisguth, F. (2000). Repression by Suppressor of Hairless and activation by Notch are required to define a single row of *single-minded* expressing cells in the *Drosophila* embryo. *Genes Dev.* **14**, 377-388.
Morel, V., Le Borgne, R. and Schweisguth, F. (2003). Snail is required for Delta endocytosis and Notch-dependent activation of *single-minded* expression. *Dev. Genes Evol.* **213**, 65-72.
Muller, B., Hartmann, B., Pyrowolakis, G., Affolter, M. and Basler, K. (2003). Conversion of an extracellular Dpp/BMP morphogen gradient into an inverse transcriptional gradient. *Cell* **113**, 221-233.
Nellen, D., Burke, R., Struhl, G. and Basler, K. (1996). Direct and long-range action of a DPP morphogen gradient. *Cell* **85**, 357-368.
O'Neill, J. W. and Bier, E. (1994). Double-label in situ hybridization using biotin and digoxigenin-tagged RNA probes. *Biotechniques* **17**, 870-875.
Rusch, J. and Levine, M. (1996). Threshold responses to the dorsal regulatory gradient and the subdivision of primary tissue territories in the *Drosophila* embryo. *Curr. Opin. Genet. Dev.* **6**, 416-423.
Sanson, B. (2001). Generating patterns from fields of cells. Examples from *Drosophila* segmentation. *EMBO Rep.* **2**, 1083-1088.
Shivdasani, R. A. (2002). Molecular regulation of vertebrate early endoderm development. *Dev. Biol.* **249**, 191-203.
Sivasankaran, R., Vigano, M. A., Muller, B., Affolter, M. and Basler, K. (2000). Direct transcriptional control of the Dpp target *omb* by the DNA binding protein Brinker. *EMBO J.* **19**, 6162-6172.
Strigini, M. and Cohen, S. M. (1997). A Hedgehog activity gradient contributes to AP axial patterning of the *Drosophila* wing. *Development* **124**, 4697-4705.
Strigini, M. and Cohen, S. M. (1999). Formation of morphogen gradients in the *Drosophila* wing. *Semin. Cell Dev. Biol.* **10**, 335-344.
Struhl, G. and Basler, K. (1993). Organizing activity of wingless protein in *Drosophila*. *Cell* **72**, 527-540.
Sturtevant, M. A. and Bier, E. (1995). Analysis of the genetic hierarchy guiding wing vein development in *Drosophila*. *Development* **121**, 785-801.
Sturtevant, M. A., Roark, M. and Bier, E. (1993). The *Drosophila rhomboid* gene mediates the localized formation of wing veins and interacts genetically with components of the EGF-R signaling pathway. *Genes Dev.* **7**, 961-973.
Sturtevant, M. A., Biehs, B., Marin, E. and Bier, E. (1997). The *spalt* gene links the A/P compartment boundary to a linear adult structure in the *Drosophila* wing. *Development* **124**, 21-32.
Sun, Y. H., Tsai, C. J., Green, M. M., Chao, J. L., Yu, C. T., Jaw, T. J., Yeh, J. Y. and Bolshakov, V. N. (1995). *white* as a reporter gene to detect transcriptional silencers specifying position-specific gene expression during *Drosophila melanogaster* eye development. *Genetics* **141**, 1075-1086.
Teleman, A. A. and Cohen, S. M. (2000). Dpp gradient formation in the *Drosophila* wing imaginal disc. *Cell* **103**, 971-980.
Torres-Vazquez, J., Warrior, R. and Arora, K. (2000). *schnurri* is required for Dpp-dependent patterning of the *Drosophila* wing. *Dev. Biol.* **227**, 388-402.
Vervoort, M. (2000). Hedgehog and wing development in *Drosophila*: a morphogen at work? *BioEssays* **22**, 460-468.
Vervoort, M., Crozatier, M., Valle, D. and Vincent, A. (1999). The COE transcription factor Collier is a mediator of short-range Hedgehog-induced patterning of the *Drosophila* wing. *Curr. Biol.* **9**, 632-639.
Waites, R., Selvadurai, H. R., Oliver, I. R. and Hudson, A. (1998). The PHANTASTICA gene encodes a MYB transcription factor involved in growth and dorsoventrality of lateral organs in *Antirrhinum*. *Cell* **93**, 779-789.
Wharton, K. A., Cook, J. M., Torres-Schumann, S., de Castro, K., Borod, E. and Phillips, D. A. (1999). Genetic analysis of the bone morphogenetic protein-related gene, *gbb*, identifies multiple requirements during *Drosophila* development. *Genetics* **152**, 629-640.
Williams, J. A., Paddock, S. W., Vorwerk, K. and Carroll, S. B. (1994). Organization of wing formation and induction of a wing-patterning gene at the dorsal/ventral compartment boundary. *Nature* **368**, 299-305.
Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-1237.