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

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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 brklomb 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; Klei, 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 (sarl) (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 (knl; 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 interven 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 interven 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} - FlyBase) genes in a narrow stripe of neighboring cells expressing high levels of ab induce expression of knirps (kni) and knirps related (knl; 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 interven 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 interven 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.

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.

Clonal analysis

Homozygous loss-of-function clones were generated by hsFLP-FRT recombination (Xu and Rubin, 1993). y w brk_{P^{f6a}}^{FRT18a/FM7a} and omb^{P}\wedge w^{FRT18a/FM6} stocks were recombined to generate the w omb^{f6a}\wedge FRT18a/FM0 and w omb^{f6a}\wedge FRT18a/FM0 stocks. Each of these stocks was crossed with w^{1118} P[Ubi-GFP(S65T)nls]X P[neoFRT18A; MKRS, P[hsFLP]/86E/TM6B, Tb[11]] flies. These 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 the omb expression.

Flip-out clones ectopically expressing ab were generated in larvae of the genotype y w hsFLP^{f6a}\wedge ab^{P}\wedge GAL4-lacZ/UASt(>)-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 interven 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 interven 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.
**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 ab1, 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 ab1 allele is likely to be a regulatory mutation, as ab expression is greatly reduced in ab1 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 (Biels 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 (Biels 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. 1H,I), 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. 1LM) and net (data not shown), is lost in cells corresponding to the L5 primordium in ab1 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 MS109G-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, 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*/*ab* 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*/*ab* mutant wing disc. (E) Double labeling of *ab* RNA (blue) and DI 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*/*ab* 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) DI is expressed in the L1 and L3-L5 wing vein primordia. (J) DI expression is lost in the L5 primordium of *ab*/*ab* mutant wing discs. (K) DI expression in all vein primordia is greatly reduced in discs ubiquitously expressing *ab* in *MS1096-GAL4; UAS-ab* wing discs. Weak DI 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*/*ab* 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 DI (blue). DI 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) DI 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*<sup>%36a</sup> (outlined).
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<sup>m68</sup> allele or a deficiency of brk, and trans-heterozygous brk<sup>+;</sup> ab<sup>+;</sup> 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 brk<sup>+/+</sup>; ab<sup>1</sup> ab<sup>1</sup> 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<sup>1</sup> ab<sup>1</sup> ab<sup>1</sup> flies exhibit truncations in the distal portion of L5 (with 3% penetrance, Fig. 3F), whereas neither ab<sup>1</sup> nor omb<sup>1</sup> heterozygotes ever show any L5 phenotype (Fig. 3B,C). Moreover, the omb<sup>1</sup> allele, which causes notching of the wing margin when homozygous but has no associated L5 phenotype (Fig. 3D), strongly enhances the ab<sup>1</sup> ab<sup>1</sup> L5 truncation phenotype. This interaction is evident in omb<sup>1</sup> ab<sup>1</sup> females (Fig. 3G), and is very pronounced in omb<sup>1</sup> omb<sup>1</sup> ab<sup>1</sup> ab<sup>1</sup> double homozygous females (data not shown) or hemizygous omb<sup>1</sup> Y; ab<sup>1</sup> ab<sup>1</sup> 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...
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*<sup>B</sup>-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*<sup>B</sup>-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*<sup>B</sup>-GAL4 driver is the creation of a new *brk*/*omb* border posterior to L5. This border forms between the narrow strip of *Vg*<sup>B</sup>-GAL4 expressing cells and the posterior edge of the endogenous *brk* expression domain, as can be observed in *brk*-lacZ; *Vg*<sup>B</sup>-GAL4; UAS-GFP wing discs (Fig. 4C, arrow). In a fraction of *Vg*<sup>B</sup>-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*<sup>B</sup>-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

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Fig. 3. *omb* interacts genetically with *ab*. (A) A homozygous *ab<sup>1</sup>/*ab<sup>1</sup>* mutant wing lacks the distal section of L5. (B) Heterozygous *ab<sup>1</sup>/* flies have a fully penetrant wild-type wing phenotype. (C) Heterozygous *omb<sup>1</sup>/∗ flies also have a fully penetrant wild-type wing phenotype. (D) An *omb<sup>1</sup>/∗ mutant female wing has a notched wing margin, but no L5 truncation. (E) A *brk*<sup>ab<sup>1</sup>/<sup>ab<sup>1</sup></sup>*; *ab<sup>1</sup>/* wing has a phenotype similar to that of *ab<sup>1</sup>/ab<sup>1</sup>* homozygotes. (F) A trans-heterozygous *omb<sup>1</sup>/∗; *ab<sup>1</sup>/ab<sup>1</sup>* female wing with a distal L5 truncation. This is a reproducible phenotype that occurs with low penetrance (3%). (G) An *omb<sup>1</sup>/∗; *ab<sup>1</sup>/ab<sup>1</sup>* female wing has an enhanced L5 vein truncation relative to that observed in *ab<sup>1</sup>/ab<sup>1</sup>* controls (compare with A). (H) An *omb<sup>1</sup>/Vg<sup>B</sup>*; *ab<sup>1</sup>/ab<sup>1</sup>* double mutant male wing has a greatly enhanced L5 truncation phenotype relative to that observed in *ab<sup>1</sup>/ab<sup>1</sup>* flies (compare with A).
development, we generated somatic \textit{brk} or \textit{omb} mutant clones or double-mutant clones lacking both \textit{brk} and \textit{omb} function (see Materials and methods for details). We first examined the requirement for \textit{omb} by generating \textit{omb}^{-} null clones in different regions of the wing. Such \textit{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 \textit{omb} in wing discs. This result indicates that simply having a border between \textit{omb} expressing and non-expressing cells is not sufficient to induce vein formation. Moreover, although \textit{omb} expression also extends into the L2 primordium (Fig. 2D-F), \textit{omb}^{-} clones located in this region of the wing did not disrupt formation of the L2 vein or expression of the L2 organizer gene \textit{kni} in wing discs (Fig. 5A,B). By contrast, \textit{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 \textit{omb}^{-} clones crossing the L5 primordium in third instar wing discs failed to express the vein marker \textit{Dl} (Fig. 5D), whereas \textit{omb}^{-} clones located in central regions of the wing disc had no effect on \textit{Dl} expression in the L3 or L4 primordia (data not shown). These data indicate that \textit{omb} is required specifically for the formation of the normal L5 primordium, although a border of \textit{omb} expression domain is not sufficient on its own to induce vein formation.

\textbf{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 \textit{brk} in this process by generating \textit{brk}^{-} mutant clones. These \textit{brk}^{-} mutant cells also misexpress \textit{omb} as a result of relieving repression by Brk (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a; Minami et al., 1999). Such \textit{brk}^{-} clones create an ectopic border between \textit{brk} and \textit{omb} if located within \textit{brk}-expressing peripheral regions of the wing disc. When \textit{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 \textit{brk} expression in these cells. By contrast, \textit{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 \textit{brk}^{-} clones, ectopic veins formed strictly within the clone abutting wild-type cells outside the clone (Fig. 5E). Similarly, \textit{brk}^{-} clones examined in third instar wing discs ectopically expressed the vein marker \textit{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 \textit{omb} expression adjacent to \textit{brk}-expressing cells.

Anterior \textit{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 \textit{kni} within the clone (Fig. 5H). However, \textit{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 \textit{brk}^{-} clones as a function of distance from the L2 vein is discussed below.
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 clone 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 ab1/ab1 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 ab1/ab1 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 ab1/ab1 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...
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 \(krnl\), 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 \(kni\)/\(krnl\) 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 Di. 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 \(ab^{+}\)/\(ab^{+}\) 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. Di) 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 \(krnl\) (Bier, 2000; Lunde et al., 1998). In addition, analysis of an L2-specific cis-regulatory element of the \(kni\)/\(krnl\) 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 \(kni\)/\(krnl\) 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 \(sal\) and/or \(sal\) 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 Di, 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 \(kni\)/\(krnl\) 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 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 mesoderm 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 Sip 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 Shviddasani, 2002); and formation of the DV border of leaves in plants controlled by the PHANASTICA 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.

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