The knirps and knirps-related genes organize development of the second wing vein in Drosophila

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SUMMARY

The neighboring homologous knirps (kni) and knirps-related (knrl) genes in Drosophila encode transcription factors in the steroid hormone receptor superfamily. During early embryogenesis, kni functions as a gap gene to control expression of segmentation genes within the abdominal region of the embryo. In this study, we present evidence that kni and knrl link A/P positional information in larval wing imaginal discs to morphogenesis of the second longitudinal wing vein (L2). We show that kni and knrl are expressed in similar narrow stripes corresponding to the position of the L2 primordium. The kni and knrl L2 stripes abut the anterior border of the broad central expression domain of the Dpp target gene spalt major (salm). We provide evidence that radius incompletus (ri), a well-known viable mutant lacking the L2 vein, is a regulatory mutant of the kni/knrl locus. In ri mutant wing discs, kni and knrl fail to be expressed in the L2 primordium. In addition, the positions of molecular breakpoints in the kni/knrl locus indicate that the ri function is provided by cis-acting sequences upstream of the kni transcription unit. Epistasis tests reveal that the kni/knrl locus functions downstream of spalt major (salm) and upstream of genes required to initiate vein-versus-intervein differentiation. Mis-expression experiments suggest that kni and knrl expressing cells inhibit neighboring cells from becoming vein cells. Finally, kni and knrl are likely to refine the L2 position by positively autoregulating their own expression and by providing negative feedback to repress salm expression. We propose a model in which the combined activities of kni and knrl organize development of the L2 vein in the appropriate position.

Key words: Pattern formation, Imaginal disc, Wing vein, Boundary, Positional information, Steroid hormone, knirps, radius incompletus, spalt, rhomboid, Drosophila melanogaster

INTRODUCTION

A major problem in development is how positional information leads to the formation of morphological structures in the organism. The patterning of longitudinal veins along the anterior-posterior (A/P) axis of the Drosophila wing is a particularly well-suited system for forging such a link between primary patterning events and morphogenesis. A variety of evidence suggests that wing veins form at boundaries between discrete sectors, which subdivide the A/P axis of the wing imaginal disc (Sturtevant and Bier, 1995; Sturtevant et al., 1997; Biehs et al., 1998). The clearest example is the second longitudinal wing vein (L2) primordium, which forms just anterior to a domain of cells expressing the transcription factor encoded by the spalt major (salm) gene in wild-type third instar wing discs (Sturtevant et al., 1997). In mutant discs containing clones of cells lacking salm function, ectopic branches of L2 are induced that track along and inside the salm- clone borders (Sturtevant et al., 1997). These observations indicate that salm expressing cells induce their salm non-expressing neighbors to become the L2 primordium. In addition to the L2 vein forming along the salm boundary, it is likely that the L3 and L4 veins form, respectively, along the anterior and posterior borders of a narrow central domain of anterior compartment cells engaged in Hedgehog signaling (Phillips et al., 1990; Johnson et al., 1995; Sturtevant et al., 1997; Mullor et al., 1997; Biehs et al., 1998).

The position of the L2 vein is determined by a chain of known developmental events, beginning with the primary subdivision of the wing imaginal disc into anterior versus posterior lineage compartments (see below and Lawrence and Struhl, 1996, for review). The subdivision of body segments such as the wing primordium into anterior and posterior compartments, in turn, can be traced back to early A/P patterning of the blastoderm stage embryo (Lawrence and Struhl, 1996; Sturtevant et al., 1997). To summarize these events briefly, the posterior compartment fate is defined by...
expression of *engrailed* (*en*), which activates expression of the short-range Hedgehog (*Hh*) signal in posterior compartment cells (Tabata et al., 1992, 1995; Lee et al., 1992; Mohler and Vani, 1992; Zecca et al., 1995) and prevents posterior compartment cells from responding to *Hh* (Sanciola et al., 1995; Zecca et al., 1995; Tabata et al., 1995). Secreted *Hh* travels a short distance (6-8 cells) into the anterior compartment where it initiates a sequence of signaling events, culminating in the activation of several *Hh* target genes including *decapentaplegic* (*dpp*) (Tabata and Kornberg, 1994; Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Capdevila et al., 1994; Zecca et al., 1995; Ingham and Fietz, 1995; Tabata et al., 1995), which encodes a secreted protein (*Dpp*) in the TGF-β superfamily (Padgett et al., 1987). *Dpp* synthesized in this narrow strip of cells travels significant distances in both the anterior and posterior directions to activate expression of *Dpp* target genes such as the neighboring *salm* and *spalt-related* (*salm*) genes (Reuter et al., 1996) in a threshold-dependent fashion (Nellen et al., 1996; Lecuit et al., 1996; de Celis et al., 1996; Singer et al., 1997). Juxtaposition of *salm* expressing and *salm* non-expressing cells induces expression of the *rhomboid* (*rho*) gene in a stripe 1-2 cells wide, corresponding to the L2 vein primordium (Sturtevant et al., 1997). *rho* then promotes differentiation of all longitudinal veins during late larval and early pupal development by potentiating signaling through the EGF-R/RAS pathway (Sturtevant et al., 1993; Noll et al., 1994; Sturtevant and Bier, 1995).

An important unanswered question is whether the signal(s) passing between *salm* expressing and *salm* non-expressing cells directly induces formation of the L2 primordium, or functions indirectly through an intermediary gene(s). If the *salm* border functioned directly to induce the L2 fate, the anterior *salm* border would be expected both to activate expression of vein-promoting genes such as *rho*, and to repress expression of intervein genes. Alternatively, the *salm* border might activate an intermediate tier of genetic control, which would then organize expression of vein and intervein gene expression in the vicinity of a narrow L2 stripe. In this study, we provide evidence for the latter alternative. We show that the neighboring *knirps* (*kni*) and *knirps-related* (*knrl*) genes, which encode related transcription factors in the hormone receptor superfamily, are expressed in narrow stripes at the position of the L2 primordium, and are required for formation of the L2 vein. We provide evidence that *radius incompletus* (*ri*), a well-known wing vein mutant lacking most of the L2 vein, is a regulatory allele of the *kni/knrl* locus, which specifically eliminates expression of *kni* and *knrl* in the L2 primordium. Epistasis experiments reveal that the *kni/knrl* locus functions upstream of *rho* and downstream of *salm*. *kni* and *knrl* are likely to function by organizing gene activity in the position of the L2 primordium rather than by promoting vein fates over intervein fates per se. We discuss several models by which *kni/knrl* locus genes may link the anterior *salm* border to the L2 vein fate.

**MATERIALS AND METHODS**

**Fly stocks**

All genetic markers and chromosome balancers used are described in Lindsley and Grell (1968) and Lindsley and Zimm (1992). We thank Joan Hooper (University of Colorado Health Science Center, Denver) for the hh*fl* stock, Walter Gehring (Biozentrum, University of Basel, Basel, Switzerland) for the A405.1M2 *sal-lacZ* enhancer trap stock, Doug Ruden (University of Kansas, Lawrence) for providing the hs-*kni* stock (=*kni*[hs.PR]; Oro et al., 1988) and several *kni* alleles, Ruth Lehmann (Skirball Institute, New York) for the Df(3L)ri*NT2* allele (Lehmann, 1985), and Fotis Kafatos (Harvard University, Cambridge) for providing the *UAS-salm* and *UAS-salr* lines. Other balancers and chromosomal markers (Lindsley and Zimm, 1992) were obtained from either the Bloomington Indiana Stock Center or the Bowling Green Stock Center.

**Mosaic analysis**

Clones were generated using the FLP-FRT recombinase system (Golic, 1991). Larvae of the genotypes HS-*Flp*; ck *salm*[*HA* FRT40A/FRT40A] (Sturtevant et al., 1997), HS-*Flp*; ck *salm*[*HA* FRT40A/FRT40A]; *ri*, or HS-*Flp*; *mwh* *kni*[*I* FRT80E/M FRT80E were heat-shocked during the first and second instar stages to generate *salm* or *kni* mosaic clones. Clone boundaries were scored by the recessive *ck* or *mwh* trichome markers under a compound microscope.

**UAS transformation constructs**

The full coding region of a *kni* cDNA (Nauber et al., 1988; kindly provided by Steve Small), which is carried on a *KpnI*-XbaI fragment, was subcloned into the corresponding sites of the pUAST vector (Brand and Perrimon, 1993). The full coding region of the *knrl* cDNA carried on an *EcoRI* fragment (Oro et al., 1988; kindly provided by Ron Evans) was cut out of a pBluescript vector with *NolI* and *XhoI* and subcloned into the corresponding sites of pUAST. These constructs were transformed into flies by P element-mediated germline transformation according to standard procedures.

**Mapping of *kni* and *ri* breakpoints**

Restriction fragments isolated from a lambda phage walk covering over 70 kb of the *kni* upstream region were used as probes to determine the locations of various chromosomal breakpoints on Southern blots.

**Mounting fly wings**

Wings from adult flies were dissected in isopropanol and mounted in 100% Balsam Canada mounting medium (Aldrich #28,292-8).

**In situ hybridization to whole-mount embryos or discs**

In situ hybridization using digoxigenin-labeled antisense RNA probes (O’Neill and Bier, 1994) was performed alone or in combination with antibody labeling, as described in Sturtevant et al. (1993). The anti-DI antibody (Kooh et al., 1993) was kindly provided by Marcus Affolter and the anti-Bs antibody (Montagne et al., 1996) was kindly provided by Marcus Affolter.

**RESULTS**

*radius incompletus* is a likely regulatory allele of the *knirps/knirps-related* locus

*radius incompletus* (*ri*) is a well-known mutant that has a severely truncated L2 vein (Fig. 1, compare B with A). *ri* maps (Aräjärvi and Hannah-Alava, 1969) very close to the neighboring and functionally equivalent *kni* and *knrl* genes (Oro et al., 1988; Nauber et al., 1988; Rothe et al., 1992; González-Gaitán et al., 1994). We observed that four different embryonic lethal *kni* alleles fail to complement *ri* when the *ri* mutation is carried on a chromosome (e.g. *TM3 ri*) that is rearranged with respect to the *kni* mutant chromosome (Fig. 1C). The failure of
multiple kni alleles to complement ri indicates that ri is likely to be an allele of the kni/knrl locus. These same kni alleles fully complement ri, however, when the ri and kni alleles are carried on non-rearranged chromosomes (data not shown). In Drosophila, regulatory and coding region mutations in the same gene frequently complement, a phenomenon referred to as transvection (Lewis, 1954; Geyer et al., 1990; Wu, 1993; Goldsborough and Kornberg, 1996). Unlike other forms of inter-allelic complementation, transvection requires that the two mutant chromosomes be co-linear and can be blocked by inverting one chromosome with respect to the other. The failure of ri and kni point mutations to complement when transvection is blocked by chromosomal rearrangement suggests that ri is a cis-acting regulatory mutation in the kni/knrl locus. As the L2 vein-loss phenotype is more variable and typically less complete in kni/TM3 ri trans-heterozygous flies than in ri/ri homozygotes, it is likely that both kni and knrl contribute to ri function. Consistent with kni and knrl providing overlapping functions in promoting L2 development, the L2 vein forms normally in wings containing knir single mutant clones, which cover the L2 vein on both the dorsal and ventral wing surfaces (Fig. 1D). Allelism between ri and the kni/knrl locus is further supported by the observation that low level ubiquitous expression of a kni cDNA transgene in UAS-kniEP flies can rescue the ri L2 truncation phenotype (Fig. 1, compare E with B), although the position of the ‘rescued’ L2 vein is displaced anteriorly relative to the wild-type L2 vein (Fig. 1F).

Consistent with kni and knrl playing a role in L2 vein formation, kni (Fig. 1G) and knrl (Fig. 1H) are expressed in similar narrow stripes corresponding to the position of the L2 primordium. kni-expressing cells abut the anterior border of strong sal-lacZ expression and express little or no detectable lacZ (see also below, Fig. 4A). For convenience, we hereafter refer to these kni expressing cells as salm non-expressing cells. Consistent with the genetic evidence that ri is a regulatory mutant of the kni/knrl locus, the L2 stripes of kni and knrl expression are absent in ri mutant discs (Fig. 1, compare I with G; knrl data identical, not shown). Outside the wing pouch of ri discs, however, kni and knrl are expressed normally (arrow in Fig. 1I).

In support of the genetic evidence suggesting that ri is a cis-acting regulatory allele of the kni/knrl locus, we have mapped ri function to a region lying immediately upstream of the kni transcription unit (Fig. 2). The viable deletion Df(3L)riXrT2, which exhibits a strong ri phenotype when homozygous or in trans to ri (Lehmann, 1985), lacks approximately 50 kb of DNA upstream of the kni transcription unit and defines the limits of ri function. The 3′ breakpoint of Df(3L)riXrT2 maps to a 1.7 kb
The kni/knrl locus acts upstream of rho in initiating L2 vein development

ri function is required to initiate expression of the vein-promoting gene rho in the L2 primordium, but is not essential for rho expression in other vein stripes (Sturtevant et al., 1995) (Fig. 3, compare B with A). As would be expected if the kni/knrl locus acted upstream of rho, initiation of kni expression in the L2 primordium precedes that of rho (data not shown). Another early marker for the L2 vein primordium is down-regulation of the key intervein gene blistered (bs) (Montagne et al., 1996). In ri mutants, down-regulation of Bs in L2 is not observed (data not shown). Consistent with the kni/knrl locus functioning upstream of rho and EGF-R signaling, kni and knrl are expressed normally in rho\(^{OE}\) kni\(^{1}\) double mutant wing discs (data not shown). rho\(^{OE}\) vn\(^{1}\) mutants, which lack rho expression in vein primordia (Sturtevant et al., 1993) and have reduced levels of the EGF-R ligand encoded by the vn gene (Schnepp et al., 1996), are devoid of veins.

Rescue of ri mutants by a ubiquitously expressed kni transgene (Fig. 1E) also suggests that kni controls rho expression, as rho expression in the L2 primordium is restored, albeit at reduced levels, in UAS-kni\(^{OP}\) ri wing discs (Fig. 3C). In addition, low-level ubiquitous kni expression preferentially induces vein formation in the vicinity of L2 in a wild-type background. Thus, heat induction of hs-kni flies during the third larval instar broadens and intensifies rho expression in the L2 primordium (Fig. 3D, bracket), while heat induction during early pupal stages generates an ectopic vein running parallel and just anterior to L2 (Fig. 3E, arrow). Stronger mis-expression of kni or knrl during early pupal stages, however, overrides factors constraining the response to kni to cells in the L2 region. For example, mis-expression of kni using the GAL4/UAS system (Brand and Perrimon, 1993) on the dorsal surface of GAL4-MS1096; UAS-kni pupal wings results in widespread ectopic expression of the vein marker rho (Fig. 3F) on the dorsal wing surface, but not on the control ventral surface (Fig. 3F, inset). Similarly, the vein marker Delta is broadly mis-expressed on the dorsal but not the ventral surface of GAL4-MS1096; UAS-kni pupal wings, and expression of the intervein marker Bs is eliminated from corresponding regions of the pupal wing (data not shown). This altered pattern of gene expression in GAL4-MS1096; UAS-kni pupal wings leads to the production of solid vein material on the dorsal surface of adult wings (Fig. 3G).

kni and knrl function downstream of salm in defining the position of the L2 primordium

We have shown previously that the salm transcription factor functions upstream of rho in the L2 primordium and that rho expression in L2 is induced at the boundary between salm expressing cells and salm non-expressing cells (Sturtevant et al., 1997). The L2 vein primordium abuts salm-expressing cells but is comprised largely of salm non-expressing cells (Sturtevant et al., 1997). Like rho, expression of kni in the L2 primordium abuts the anterior edge of the broad salm
expression domain in wild-type third instar wing discs (Fig. 4A,B, top panel), and is displaced along with the anterior border of *salm* expression in * hedgehog Moonrat (hh\textsuperscript{Mt})* wing discs (Fig. 4B, bottom panel). In *hh\textsuperscript{Mt}* wing discs, the anterior limit of the *salm* expression domain on the ventral surface is frequently shifted forward relative to the border on the dorsal surface (Sturtevant et al., 1997). Associated with the asymmetry in *sal-lacZ* expression, the dorsal and ventral components of the *kni* L2 stripe are driven out of register (Fig. 4B, bottom panel). The coordinate shift of *salm* and *kni* expression is consistent with *salm* functioning upstream of *kni*. In addition, strong ectopic expression of *salm* or *sarl* using the GAL4/UAS system (Brand and Perrimon, 1993) eliminates *kni* and *knrl* (Fig. 4C) expression, and leads to the production of small wings lacking the L2 and L5 veins (Fig. 4D; see also de Celis et al., 1996). The loss of *kni* and *knrl* expression in discs mis-expressing *salm* or *sarl* and the subsequent elimination of L2 presumably result from obscuring the sharp boundary of endogenous *salm* and *sarl* expression. Clonal analysis also indicates that *salm* acts upstream of *kni/*knrl, *salm*\textsuperscript{-} clones generated in the anterior compartment between L2 and L3 induce ectopic forks of the L2 vein, which lie along the inside edge of the *salm*\textsuperscript{-} clones (Sturtevant et al., 1997) (Fig. 4E). In contrast, *salm*\textsuperscript{-} clones produced in corresponding positions of *ri* mutant wings never induce L2 forks (Fig. 4F). Other phenotypes associated with *salm*\textsuperscript{-} clones, however, such as ectopic islands of triple row bristles at the margin (Fig. 4G), are observed with regularity in an *ri* background (Fig. 4H).

**Strong ubiquitous expression of *kni* or *knrl* eliminates distinctions between vein and intervein primordia**

The genetic evidence and expression data described above suggest that localized expression of *kni* and *knrl* is required to define the position of the L2 primordium. To determine the importance of restricting *kni* expression to the L2 primordium, we used the GAL4/UAS system to mis-express *kni* or *knrl* at high levels in various patterns. The GAL4-MS1096 line drives expression of UAS-target genes ubiquitously throughout the dorsal surface of third instar wing discs (Fig. 5A), and weakly on the ventral surface in the anterior region of the disc (Fig. 5A, arrow). GAL4-MS1096-driven expression of either the UAS-*kni* or UAS-*knrl* transgenes eliminates expression of vein markers such as *rho* (Fig. 5E, compare with Fig. 3A), the provein/proneural gene *caupolican* (*caup*) (Fig. 5F,B), the lateral inhibitory gene *Delta* (*Di*) (Fig. 5, compare G with C), and the proneural gene *achaete* (data not shown) on the dorsal surface of the wing disc. In contrast, these vein markers are expressed in normal patterns on the ventral surface, albeit at reduced levels, presumably reflecting the weak expression of GAL4 in ventral cells of GAL4-MS1096 discs. In addition, modulated expression of *blistered* (*bs*), which is lower in vein than intervein cells of wild-type discs (Montagne et al., 1996), also disappears on the dorsal surface of GAL4-MS1096 wing discs (Fig. 5, compare H with D). Thus, strong expression of *kni* or *knrl* on the dorsal surface of wing discs eliminates expression of both vein and intervein markers. Similarly, when GAL4-71B is used to drive UAS-*kni* or UAS-*knrl* expression in a central domain slightly broader than that of *salm*, distinctions between vein and intervein cells are eliminated within the region of GAL4 expression. In these discs, vein and intervein markers are expressed normally in the L5 primordium, which lies outside of the GAL4-71B expression domain (data not shown). These data reveal that ectopic *kni* or *knrl* expression does not simply favor vein over intervein cell fates. As strong uniform *kni* or *knrl* mis-expression is required

**Fig. 3. *kni/knrl* function upstream of *rho* in establishing the L2 primordium.** (A) *rho* expression in a wild-type mid-third instar wing disc. The L1-L5 vein primordia are labeled 1-5 and the future wing margin is denoted by M. (B) *rho* expression in an *ri*\textsuperscript{1}/*ri*\textsuperscript{1} mid-third instar disc is never initiated in the L2 primordium (arrow). (C) *rho* expression in a UAS-*kni*EP* ri*\textsuperscript{1}/*ri*\textsuperscript{1} third instar disc is partially restored in the L2 primordium (arrow). (D) *rho* expression in a hs-*kni* third instar disc, which was heat-shocked 3 times at 37°C for 1 hour with intervening periods of 45 minutes rest at room temperature between each heat shock treatment. *rho* expression in the L2 stripe (bracket) is broader and stronger than in wild-type discs. (E) A hs-*kni* wing heat shocked as in Fig. 1D during early pupal stages. An ectopic vein runs parallel and anterior to L2 (arrow). (F) *rho* is expressed in large wedges occupying most of the dorsal surface of an early GAL4-MS1096/+; UAS-*kni*/+ pupal wing. The GAL4-MS1096 line expresses GAL4 only in the dorsal compartment during early pupal stages (data not shown). Inset: *rho* is expressed in a normal pattern of vein stripes on the ventral surface of a GAL4-MS1096/+; UAS-*kni*/+ early pupal wing. (G) A GAL4-MS1096/+; UAS-*kni*/+ wing. The dorsal surface appears to be one large amorphous expanse of vein tissue with densely packed trichomes and darkly pigmented cuticle, while the control ventral surface has veins of normal thickness in approximately the correct locations. Because vein cells are more densely packed than intervein cells, the wing assumes an upward curving cup shape. GAL4-MS1096; UAS-*kni* and GAL4-MS1096; UAS-*knrl* flies also lack macrochaete on the thorax with high penetrance and frequently have twisted femurs in the T3 segment. v, ventral surface of wing; d, dorsal surface of wing; M, the wing margin.
to eliminate veins, higher levels of kni/krnl activity are necessary to inhibit vein formation than are required to induce expression of rho in or near the L2 primordium.

In contrast to the dramatic effects of ectopic kni expression on vein and intervein markers, expression of genes such as ptc (Fig. 5I), dpp (data not shown) and hh (Fig. 5J) along the previously formed A/P compartment boundary is unperturbed by strong uniform kni mis-expression. These data indicate that kni and krnl do not function as global repressors of gene expression in the wing primordium. Consistent with this view, when mis-expressing kni using the GAL4-71B driver, in addition to eliminating strong rho expression in the L2, L3 and L4 primordia, a very low but reproducible level of rho expression is induced within the domain of GAL4-71B expression (data not shown). The low generalized expression of rho in the absence of strong vein stripes in GAL4-71B; UAS-kni discs suggests that kni has an intrinsic tendency to activate rho expression, which is largely overridden by the potent lateral inhibitory mechanism induced by strong kni expression. We speculate that the reason kni mis-expression induces strong expression of rho in pupal wings (Fig. 3F), but eliminates rho expression in veins in third larval instar wing discs (Fig. 5E), is that the lateral inhibitory mechanism

Fig. 4. kni and krnl function downstream of salm and upstream of rho. (A) kni mRNA expression (blue) abuts the anterior edge of high-level sal-lacZ expression (brown β-galactosidase) in a wild-type third larval instar wing disc. During the early stages of kni expression, low levels of sal-lacZ are observed in kni expressing cells. However, at later stages, there is little detectable overlap between kni and sal-lacZ expression patterns, consistent with the observation that kni can suppress salm expression (see Fig. 6B). As rho expression in the L2 primordium similarly abuts the L2 boundary (Sturtevant et al., 1997), and because double labeling with kni and rho digoxigenin-labeled probes reveals only a single stripe (data not shown), we infer that the kni stripe corresponds to the L2 primordium. (B) Upper panel: high magnification view of the L2 region of the wild-type sal-lacZ disc shown in A. Lower panel: high magnification view of staggered kni expression at the edge of the distorted sal expression domain in a sal-lacZ; hh[mt] third instar wing disc. Asterisks denote the intersection of the dorsal and ventral components of the kni L2 stripes with the margin. (C) kni expression in a GAL4-MS1096; UAS-salr wing disc. kni expression in this disc is lost in L2 within the wing pouch, but is normal outside of the wing pouch (arrow). In other discs, expression is severely reduced or restricted to small spots (in some such discs, the dorsal component of kni or krnl expression is more severely affected than the ventral component, consistent with there being higher levels of GAL4 expression on the dorsal surface of GAL4-MS1096 discs than on the ventral surface), and in a minority of discs kni or krnl expression appears nearly normal. Similar, but more penetrant, elimination of kni and krnl expression was obtained using the GAL4-71B line, which drives gene expression in a broad central domain slightly wider than that of spalt. (D) An adult GAL4-MS1096; UAS-salr female wing. Note the loss of the L2 and L5 veins. In the great majority of GAL4-MS1096; UAS-salr wings, the L2 vein is either entirely missing or only small islands of residual L2 vein material are observed. In a few percent of the cases, longer segments of L2 are present, but a complete L2 vein never forms. Males of the same genotype have more severely affected smaller wings than females, presumably due to dosage compensation of the X-chromosome carrying the GAL4-MS1096 element. GAL4-MS1096; UAS-salr and GAL4-MS1096; UAS-salm flies also have missing macrochaete on the thorax with high penetrance, and twisted femurs in the T3 segment are frequently observed in GAL4-MS1096; UAS-salm flies. Interestingly, these same phenotypes are also observed in GAL4-MS1096; UAS-kni and GAL4-MS1096; UAS-knrl flies. (E) A wing containing a homozygous ck salm[IIA] clone (outlined in red and marked –) between L2 and L3 has an ectopic L2 fork running within and along the clone boundary (Sturtevant et al., 1997). salm+/+ or +/+ cells are indicated by +. (F) A wing containing a comparable ck salm[IIA] clone in an rt1/rt1 background between L2 and L3 is not bounded by an ectopic vein. 20 similar ck salm[IIA] marked clones were examined in detail and none were bordered by ectopic veins. It is likely that all such ck salm[IIA] clones would induce L2 forks in a wild-type background (Sturtevant et al., 1997). In addition, we estimated the total number of ck salm[IIA] clones generated in our collection of scored wings that would have contained L2 forks had they been produced in a wild-type background, by counting the number of wings having ck marked clones associated with L5 forks (L5 forks are often induced at a distance by salm clones in the posterior compartment; Sturtevant et al., 1997). ck salm[IIA] marked clones, generated in parallel in a wild-type background, generated L2 and L5 forks in a ratio of approximately 5:1 (i.e. 47 L2 forks; 10 L5 forks). We observed 20 L5 forks associated with ck salm[IIA] clones in our collection of ck salm[IIA]; rt1 mosaic wings. If these phenotypes are generated at approximately equal frequencies in wild-type versus rt1/rt1 backgrounds, then we are likely to have generated >90 ck salm[IIA] clones, which would have induced L2 branches had they been produced in a wild-type rather than in an rt1/rt1 background. (G) A wing containing a homozygous ck salm[IIA] clone (outlined in red) which intersects the wing margin. Note the island of ectopic triple row bristles (lower overline, asterisk), which typically form at the junction of L2 with the margin (upper overline, asterisk). (H) A wing containing a comparable ck salm[IIA] clone reaching the wing margin in an rt1/rt1 background. Again, note the island of ectopic triple row bristles (lower overline, asterisk).
operating during larval stages to define sharp boundaries is inactive later during pupal development when boundaries have been firmly resolved. The ability of uniform kni or knrI expression to erase distinctions between vein and intervein cells during larval stages suggests that these genes must be expressed in a narrow linear array of cells in order to perform their normal function in organizing gene expression along the L2 primordium.

**kni and knrI refine the position of L2 via positive and negative feedback loops**

In addition to activating rho expression, kni and knrI also are likely to positively autoregulate. Patterned mis-expression of kni using the GAL4/UAS system (Brand and Perrimon, 1993) induces corresponding expression of the knrI gene (Fig. 5K) and vice versa (data not shown). As kni and knrI appear to share cis-regulatory elements in third instar larval wing disc (this study) and during other stages of development (Oro et al., 1988; Nauber et al., 1988; Rothe et al., 1992; González-Gaitán et al., 1994), the reciprocal cross-regulation observed between kni and knrI is likely to reflect an autoregulatory function of these genes. kni function does not appear to be necessary for activating knrI expression in the L2 primordium, however, since elimination of kni function in large kni- clones covering both the dorsal and ventral components of L2 does not lead to any loss of the L2 vein (Fig. 1D).

Another consequence of high level ectopic kni expression is strong down-regulation of salm expression (Fig. 5L). Since kni and knrI are normally expressed immediately adjacent to the anterior salm border (Fig. 4A), suppression of salm expression by kni may sharpen the anterior salm border and refine the position of the L2 primordium. In support of this possibility, we observed a consistent anterior displacement of rescued L2 veins in UAS-kniEP ri wings relative to wild type (Fig. 1E,F). Similarly, rho expression in the L2 primordium is shifted anteriorly in UAS-kniEP ri wing discs (Fig. 3, compare C with A). This anterior displacement of the L2 primordium may reflect a failure to down-regulate salm expression at its anterior border in late third instar ri wing discs.

**DISCUSSION**

**kni/knrl define the position of the L2 primordium rather than promote a vein fate per se**

Data presented in this study suggest that the kni and knrI genes define a linear position at the anterior edge of the salm expression domain. We propose that juxtaposition of salm expressing and salm non-expressing cells induces expression of kni and knrI in a narrow stripe of cells within the domain of salm non-expressing cells. kni and knrI then organize L2 vein development in a precise linear position. Our analysis suggests that the kni locus acts at the last stage of defining positional information rather than at the first stage of directing vein tissue differentiation. This conclusion derives in part from analysis of discs ubiquitously mis-expressing kni or knrI at high levels. The key difference between the kni and knrI genes and other previously identified vein-promoting genes such as rho or genes of the caup/araucan (ara) locus is that both loss of function and ubiquitous expression of kni/knrl lead to elimination of veins. In contrast, ubiquitous expression of vein-promoting genes such as rho or ara induces the formation of

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**Fig. 5.** kni/knrl organize gene expression in the vicinity of the L2 primordium. All panels show gene expression in mid-third instar wing imaginal discs. (A) A GAL4-MS1096; UAS-lacZ disc double-stained for rho RNA expression (blue) and anti-β-gal protein (brown). Strong β-gal staining is restricted to the dorsal surface and weak expression is observed on the ventral surface (arrow). (B) Wild-type expression of caup mRNA in broad provein stripes corresponding to the odd-numbered veins (labeled 1, 3, 5) (Gomez-Skarneta et al., 1996). (C) Wild-type expression of DI protein, detected with an anti-DI antibody, in the L1, L3, L4 and L5 vein primordia (Kooh et al., 1993). (D) Wild-type expression of Bs protein, detected with an anti-Bs antibody (Montagne et al., 1996), is strong in intervein cells and weak in vein primordia. (E) rho mRNA expression in a GAL4-MS1096; UAS-kni wing disc. (F) caup mRNA expression in a GAL4-MS1096; UAS-kni wing disc. (G) DI protein expression in a GAL4-MS1096; UAS-kni wing disc. (H) Bs protein expression in a GAL4-MS1096; UAS-kni wing disc. (I) ptc mRNA expression in a GAL4-MS1096; UAS-kni wing disc. (J) hh mRNA expression in a GAL4-MS1096; UAS-kni wing disc. (K) knri mRNA expression in a GAL4-MS1096; UAS-kni wing disc. (L) salm mRNA expression in a GAL4-MS1096; UAS-kni wing disc.
Fig. 6. Model for how kni/knrl organizes formation of the L2 primordium and similarities with other mechanisms for generating linear patterns of gene expression. (A) Left: diagram to illustrate how the juxtaposition of anterior and posterior compartment cells leads to the production of the long-range Dpp signal in a narrow strip of anterior compartment cells running along the A/P border in the middle of the wing primordium. Dpp diffuses and functions as a morphogen to induce expression of salm (Sal) in a broad central domain (Nellen et al., 1996; Lecuit et al., 1996; Lawrence and Struhl, 1996; Singer et al., 1997). We propose that a short-range signal X induces expression of kni/knrl along the anterior border of the salm expression domain. No vein is induced along the posterior limit of the salm expression domain, which falls between L4 and L5 in Drosophila (Sturtevant et al., 1997), although a vein does form in this position in primitive insects and in Drosophila mutants which have ectopic veins (Biehs et al., 1998). Right: the four functions that kni and knrl provide in the L2 primordium: (1) to promote expression of genes required for vein development (e.g. rho) in collaboration with another activity (dotted arrow), which is restricted to the vicinity of the anterior salm (Sal) boundary, (2) to suppress vein development in neighboring cells, (3) to promote their own and each other’s expression via a positive auto-regulatory loop, and (4) to sharpen the anterior salm boundary through a negative feedback mechanism. Since we propose that kni and knrl function at the last stage of defining positional information rather than acting as ‘master’ vein promoting genes, we speculate that there might be an unknown vein ‘master’ gene promoting the vein fates in the L2 position. Such an L2 ‘master’ gene would presumably activate vein effector genes such as rho, by analogy to the action of caup and ara in promoting formation of the odd number veins. Alternatively, kni and knrl may function directly to activate expression of rho. (B) Models for the genetic control of gene expression in linear patterns. Left: to induce dpp expression in a central stripe 6-8 cells wide abutting the A/P compartment boundary, En activates expression of the short-range signal Hh, while suppressing the response to Hh by suppressing dpp expression. Middle: to induce kni and knrl expression in the 2- to 3-cell wide L2 primordium abutting the anterior border of salm expression, Salm (Sal) activates expression of a hypothetical very short-range signal X, while suppressing the response to X by suppressing kni and knrl expression. Right: to induce sim expression in a single row of presumptive mesectodermal cells abutting the snail expressing mesoderm, we propose that Snail activates the membrane-bound signal DI (DI*), while suppressing the response to DI/Notch signaling by directly repressing sim expression.

ectopic veins (Sturtevant et al., 1993; Noll et al., 1994; Gomez-Skarmeta et al., 1996). In addition, kni and knrl appear to feedback on the patterning process itself by maintaining their own expression and by suppressing salm expression in the L2 primordium. These data suggest that kni/knrl orchestrate gene expression in a precise linear position by promoting vein development in cells where they are expressed and by suppressing vein development in adjacent intervein cells.

A/P patterning culminates in expression of kni and knrl in the L2 primordium

As summarized previously, it is possible to trace formation of the L2 vein back to early A/P patterning in the embryo (Sturtevant et al., 1997). This chain of events leads to activation of the kni and knrl genes in narrow stripes at the anterior edge of the salm expression domain (Fig. 6A, right), thus linking positional information to morphogenesis. We propose that salm activates expression of a short-range signal X, which induces expression of kni and knrl in adjacent salm non-expressing cells. Since Kni and Knrl are members of the steroid hormone receptor superfamily, it is possible that the signal X could be a lipid-soluble factor, which binds and activates Kni and Knrl. Given the minimal sequence conservation between Kni and Knrl in the putative ligand binding regions of these proteins (Rothe et al., 1989), however, this direct form of signaling seems unlikely. Once activated, kni and knrl organize formation of the L2 primordium.

kni and knrl link A/P patterning to vein development in the L2 primordium

We propose that kni and knrl organize development of the L2 vein primordium through a variety of concerted actions (Fig 6A, left). A key target gene activated by kni and knrl in the L2 primordium is the vein-promoting gene rho, which potentiates signaling through the EGF-R/RAS pathway (Sturtevant et al., 1993; Noll et al., 1994; Sturtevant and Bier, 1995). Because low
levels of ubiquitous kni and knrl expression preferentially promote vein development near the location of L2, another activity provided at the anterior boundary of the salm expression domain is likely to act in parallel with the kni and knrl genes to define the position of the L2 primordium. This parallel genetic function may be supplied by the signal X, hypothesized to induce kni and knrl expression in salm non-expressing cells.

kni and knrl are also likely to suppress vein development in neighboring intervein cells since strong uniform mis-expression of kni or knrl eliminates veins. This result could be explained if kni and knrl normally activate expression of a signal that suppresses vein development in neighboring intervein cells. Such a lateral inhibitory function presumably restricts formation of the L2 primordium to a narrow linear array of cells. To account for the fact that kni and knrl do not turn themselves off in L2 as a consequence of the proposed lateral inhibitory signaling, we imagine that these cells are refractory to the lateral inhibitory mechanism. Alternatively, the hypothetical signal X, which promotes kni and knrl expression in cells adjacent to the salm expression domain (Fig. 6A), might continue to exert an inductive influence that overrides lateral inhibitory signaling in the L2 primordium. This possibility is consistent with low levels of ubiquitous kni expression rescuing rho expression in the vicinity of the normal L2 primordium in ri mutants. Although the nature of the proposed lateral inhibitory mechanism is unknown, the Notch signaling pathway is an obvious candidate, since loss of Notch function during late larval stages results in the formation of much broadened rho expressing stripes (Sturtevant and Bier, 1995). Since Delta is unlikely to be the ligand mediating lateral inhibition, due to its absence in the L2 primordium (Sturtevant and Bier, 1995), which is typical of ligands involved in active signaling. Thus, Snail may regulate expression of some co-factor required for membrane bound Delta to productively activate the Notch signaling pathway in adjacent cells, which are free to respond by activating sim expression.

It is noteworthy that in each of three cases considered above, products of entirely distinct domain-defining genes (e.g. En, Salm and Sna) induce the linear expression of genes in adjacent cells by activating production of short-range signals (e.g. Hh, X, DI) while suppressing response to those signals (Fig. 6B). The width of the target gene stripes presumably depends on the range of the signal and on the level of signal required to activate expression of specific genes. Thus, Hh activates expression of the targets gene dpp in a domain 6-8 cells wide, the hypothetical factor X acts more locally to induce expression of kni and knrl in a stripe 2-3 cells wide, and the putative ‘activated’ form of membrane tethered Delta induces sim expression in a single row of abutting mesectodermal cells. Perhaps this ‘for export only’ signaling mechanism is a general scheme for drawing lines in developing fields of cells.

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REFERENCES


A common strategy for drawing lines in developing fields of cells

As discussed above, the model proposed in Fig. 6A for activating expression of kni and knrl in a narrow stripe of cells is analogous to the earlier induction of dpp in a narrow stripe of anterior compartment cells by the short-range Hh signal emanating from the posterior compartment (Fig. 6B, left). In both cases a domain-defining gene (i.e. en or salm) activates expression of a short-range signal (i.e. Hh or X), while preventing these same cells from responding to the signal. According to such a genetic wiring diagram, only cells that are immediately adjacent to cells producing the short-range signal are competent to respond to it. This set of constraints restricts the expression of target genes to narrow stripes or sharp lines.

An exquisite example of linear gene activation is the initiation of sim expression in a single row of mesectodermal cells abutting the snail expression domain in the mesoderm of blastoderm embryos (Fig. 6B, right; Thomas et al., 1988; Crews et al., 1988). Direct mechanisms contribute to activating sim in this precise pattern as snail represses sim expression in venral cells (Nambu et al., 1990; Kosman et al., 1991; Leptin, 1991; Rao et al., 1990) and Dorsal and Twist collaborate to define a relatively sharply defined threshold for activating sim, which extends a short distance beyond the snail border (Kasai et al., 1992; Kasai et al., 1998). However, these direct transcriptional mechanisms alone do not seem sufficient to explain the absolutely faithful linear path of sim expression in a single row of cells along the irregular contour of snail expressing mesodermal cells. Perhaps communication between snail expressing cells and their immediate dorsal neighbors plays a role in achieving the invariant registration of the sim and snail expression patterns. In support of a role for cell-cell communication in this process, initiation of sim expression in the blastoderm embryo requires signaling through the Notch/Delta/E(spl) pathway (Menne et al., 1994; S. Crews, personal communication). Furthermore, in the mesoderm, ubiquitously supplied maternal Delta protein is rapidly retrieved from the surface in the form of multi-vesicular bodies (Kooh et al., 1993), which is typical of ligands involved in active signaling. Thus, Snail may regulate expression of some co-factor required for membrane bound Delta to productively activate the Notch signaling pathway in adjacent cells, which are free to respond by activating sim expression.

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