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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 auto-regulating 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 (Sanicola 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* (*salr*) 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^{Mrt} 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 $Df(3L)ri^{XT2}$ 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* $salm^{IIA}$ FRT^{40A}/FRT^{40A} (Sturtevant et al., 1997), HS-*Flp*; *ck* $salm^{IIA}$ FRT^{40A}/FRT^{40A}; *ri*, or HS-*Flp*; *mwh* kni^{9} FRT^{80E}/M FRT^{80E} 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 *Kpn*I-*Xba*I 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 *Eco*RI fragment (Oro et al., 1988; kindly provided by Ron Evans) was cut out of a pBluescript vector with *Not*I and *Xho*I 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-Dl antibody (Kooh et al., 1993) was kindly provided Marc Muskavitch 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 (Arajärvi 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 kni^{-} 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-kni^{EP} 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 $Df(3L)ri^{XT2}$, 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 $Df(3L)ri^{XT2}$ maps to a 1.7 kb

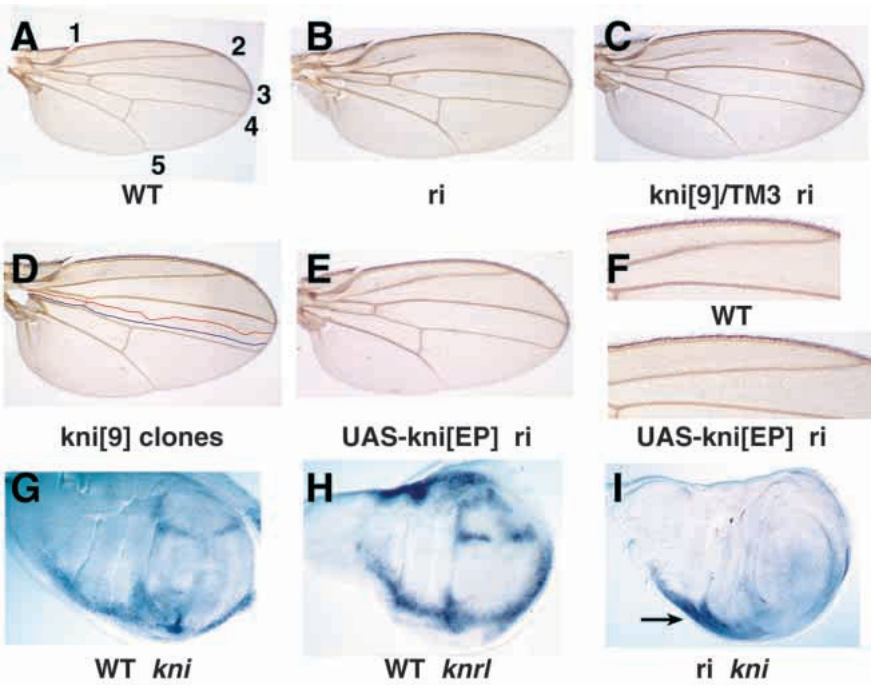

Fig. 1. *ri* is a regulatory allele of the *kni/knrl* locus. (A) A wild-type adult wing. Longitudinal veins L1-L5 are labeled 1-5. L1 is continuous with the wing margin vein. (B) A ri^1/ri^1 adult wing. (C) A $kni^9/TM3\ ri^1$ wing. The L2-loss phenotype in these trans-heterozygotes is fully penetrant, although weaker and more variable than that observed for ri^1/ri^1 wings. We also observed partial failure to complement the $TM3\ ri^1$ L2 vein loss phenotype by kni^1 (= kni^{5F}), kni^3 (= kni^{14F}) and kni^8 (= kni^{FC13}), which were less penetrant (kni^1, kni^8) or less extreme (kni^1, kni^3 and kni^8) than observed for kni^9 (= kni^{IL}) (see Lindsley and Zimm, 1992 for origins of *kni* alleles). The same *kni* alleles that failed to fully complement $TM3\ ri^1$ also failed to complement another rearranged chromosome carrying ri^1 (*In(3L) LD6, fz st cp in* ri^1), although the penetrance and expressivity of the vein-loss phenotype were less than observed with $TM3\ ri^1$. To our knowledge, mutant alleles of *knrl* have not yet been recovered. (D) An adult wing with normal venation containing two large anterior compartment kni^{-} clones, which cover the L2 vein on both the dorsal (red line) and ventral (blue line) surfaces of the wing. (E) The *ri* phenotype is rescued by a single copy of a *UAS-kni* cDNA transgene in the UAS-kni^{EP} *P* element insertion line, which is expressed ubiquitously throughout the wing pouch with elevated levels observed in future proximal regions of the wing blade and in a broad longitudinal strip in the vicinity of L3 (data not shown), presumably as a consequence of chromosomal position effects or 'enhancer piracy' (Noll et al., 1994). The L2 vein truncation phenotype is rescued with high penetrance in UAS-kni^{EP} ri^1/ri^1 flies, but the rescued L2 vein is consistently displaced anteriorly relative to the normal position of the L2 vein. Anterior displacement of L2 is even more pronounced in *GAL4-MS1096*/+; *UAS-kni*/+; ri^1/ri^1 wings, which express higher levels of *kni* than those produced in the UAS-kni^{EP} line (data not shown). (F) High magnification views of the relative position of the L2 vein in the wild-type wing shown in A (top) relative to the anteriorly displaced L2 vein in the UAS-kni^{EP} ri^1/ri^1 wing shown in E (bottom). (G) *kni* expression in a wild-type mid-third instar larval disc. The stripe of *kni* expression slightly precedes and then coincides with L2 *rho* expression (see legend to Fig. 4A). Prolonged staining reveals, in addition, weaker stripes of *kni* and *knrl* expression in the approximate position of the L5 primordium and low levels of ubiquitous expression throughout the wing pouch (data not shown). This low level staining is unlikely to be background as it is largely confined to the wing pouch, is observed reproducibly, and is not observed with various other probes made and used in parallel. (H) *knrl* expression in a wild-type mid-third instar larval disc. (I) *kni* expression in an ri^1/ri^1 mid-third instar larval disc. Although L2 expression is completely absent, *kni* expression outside of the wing pouch is normal (arrow).

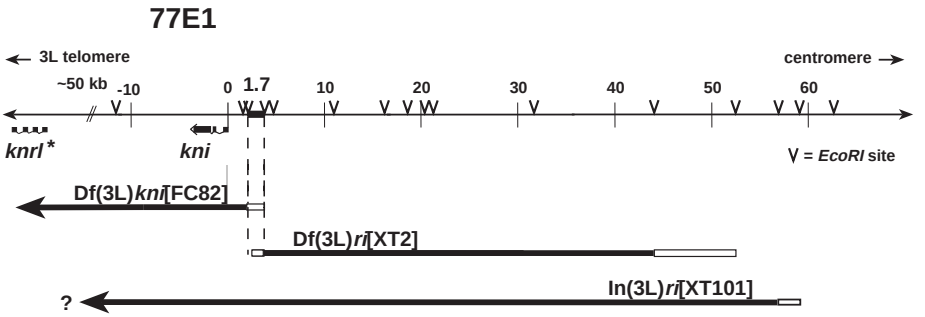

Fig. 2. *ri* maps upstream of the *kni* and *knrl* transcription units. The upper line in the diagram indicates the positions of key deletion breakpoints eliminating *ri* function relative to the *kni* and *knrl* transcription units. The positions of relevant breakpoints were determined by Southern blot analysis using genomic fragments from the *kni/knrl* locus upstream region as probes. The 3′ and 5′ limits of the *ri* function lie between the corresponding breakpoints of *Df(3L)ri*^{XT2}. The 5′ breakpoint of *Df(3L)ri*^{XT2} lies just downstream of the 5′ breakpoint of the deletion associated with the *In(3L)ri*^{XT101}. Since *ri*^{1}/*Df(3L)ri*^{XT2} and *ri*^{1}/*In(3L)ri*^{XT101} have a strong *ri* phenotype it is likely that the *ri* phenotype of *Df(3L)ri*^{XT2} is caused by the deletion mapped here and not by some second site molecular lesion. There may be an element required for *ri* function in the small region of overlap between *Df(3L)kni*^{FC82} and *Df(3L)ri*^{XT2}, since trans-heterozygotes have a strong *ri* phenotype. In addition, putative regulatory DNA, including the 5′ end of the *kni* transcription unit and extending over 5 kb beyond it, which includes the region of potential overlap between *Df(3L)kni*^{FC82} and *Df(3L)ri*^{XT2}, is not sufficient to drive expression of a *lacZ* reporter gene in the L2 primordium or to rescue the *ri* phenotype when driving expression of a *kni* transgene (data not shown). The exact distance between the *kni* and *knrl* genes and the relative orientations of these two genes is not known. Also, the *knrl* transcript, which comprises 23 kb of genomic DNA (Rothe et al., 1992), is not drawn to scale with respect to the right portion of the figure.

*Eco*R1 fragment, which lies only 2.5 kb upstream of the *kni* transcription unit, and the 5′ breakpoint lies 45-50 kb further upstream. Another deletion, *Df(3L)kni*^{FC82} (Nauber et al., 1988), which removes both the *kni* and *knrl* transcription units, has its 5′ breakpoint within the same 1.7 kb *Eco*RI fragment as *Df(3L)ri*^{XT2} and overlaps *Df(3L)ri*^{XT2} by less than 1.0 kb. Since flies trans-heterozygous for the *Df(3L)ri*^{XT2} and *Df(3L)kni*^{FC82} deletions have a strong *ri* phenotype, and because *Df(3L)ri*^{XT2}/*Df(3L)kni*^{FC82} trans-heterozygous larval wing discs lack expression of the *kni* and *knrl* genes in the L2 stripe (B. Biehs, unpublished observations), the 1.7 kb *Eco*RI fragment may contain sequences necessary for *ri* function. The 1.7 kb *Eco*RI fragment does not contain any transcription unit active in wing imaginal discs (B. Biehs, unpublished observations), suggesting that any *ri* function provided by this fragment must be regulatory in nature. It also is possible that the extensive deletions and relatively small overlap between the *Df(3L)ri*^{XT2} and *Df(3L)kni*^{FC82} disrupt transvection between these two chromosomes in trans-heterozygotes, thus preventing wild-type *ri* regulatory sequences present on the *Df(3L)kni*^{FC82} chromosome from activating expression of the intact *kni* and *knrl* genes present on the *Df(3L)ri*^{XT2} chromosome. Both scenarios, however, support the conclusion that *ri* is a cis-acting regulatory mutation of the *kni/knrl* locus.

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*^{ve} *vn*^{1} double mutant wing discs (data not shown). *rho*^{ve} *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*^{EP} *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*^{Mrt}) wing discs (Fig. 4B, bottom panel). In *hh*^{Mrt} 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 *salr* 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 *salr* and the subsequent elimination of L2 presumably result from obscuring the sharp boundary of endogenous *salm* and *salr* expression. Clonal analysis also indicates that *salm* acts upstream of *kni*/*knrl*. *salm*$^{-}$ 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*$^{-}$ clones (Sturtevant et al., 1997) (Fig. 4E). In contrast, *salm*$^{-}$ clones produced in corresponding positions of *ri* mutant wings never induce L2 forks (Fig. 4F). Other phenotypes associated with *salm*$^{-}$ 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* (*Dl*) (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

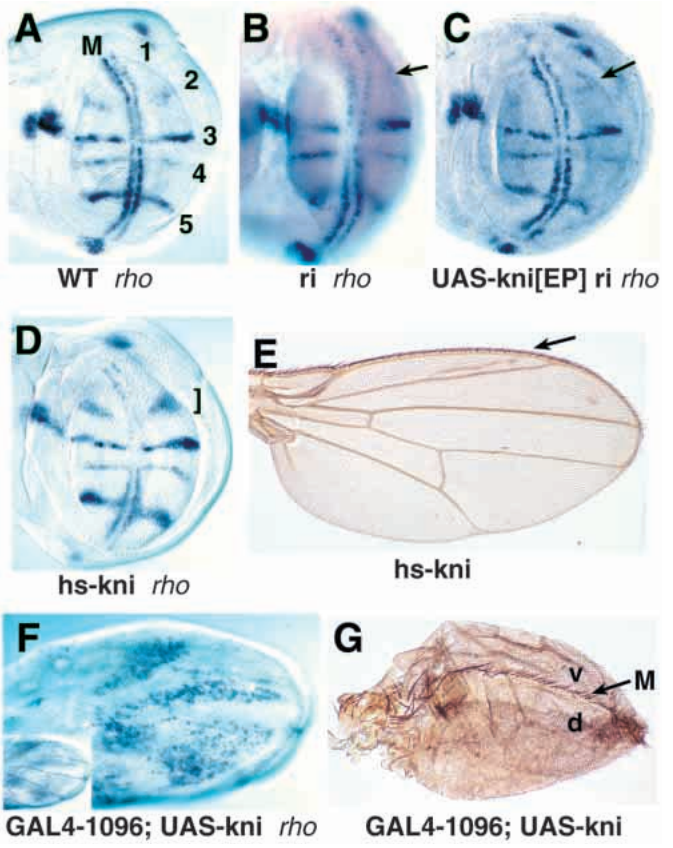

Fig. 3. *kni/knrl* function upstream of *rho* in establishing the L2 primordium. (A) *rho* expression in a wild-type mid-third instar wing disc. The L1-L5 vein primordia are labeled 1-5 and the future wing margin is denoted by M. (B) *rho* expression in an *ri*^{1}/*ri*^{1} mid-third instar disc is never initiated in the L2 primordium (arrow). (C) *rho* expression in a UAS-*kni*^{EP} *ri*^{1}/*ri*^{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.

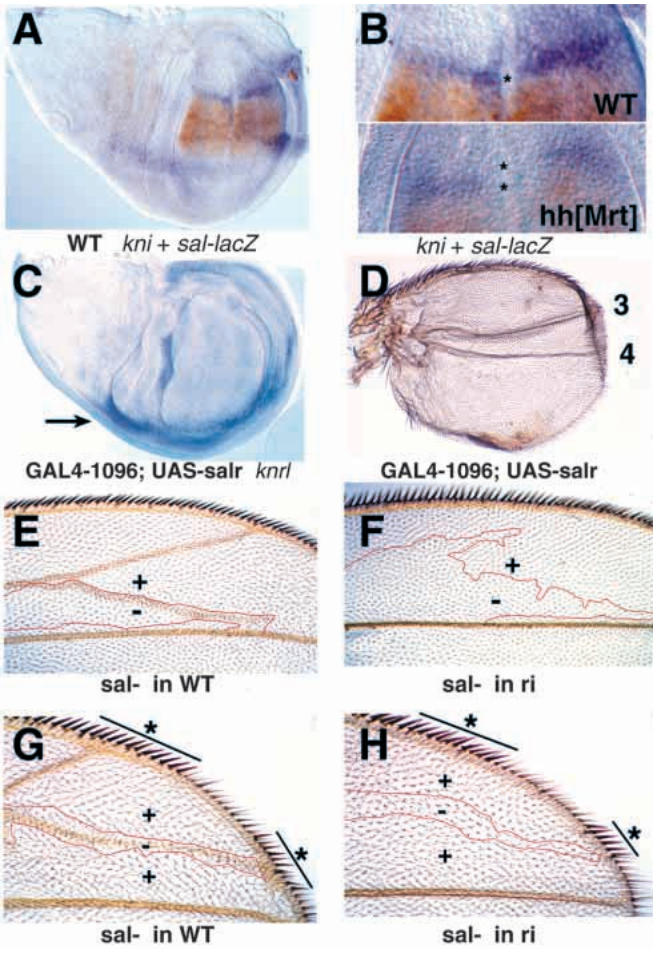

Fig. 4. *kni* and *knrl* function downstream of *salm* and upstream of *rho*. (A) *kni* mRNA expression (blue) abuts the anterior edge of high-level *sal-lacZ* expression (brown β-galactosidase) in a wild-type third larval instar wing disc. During the early stages of *kni* expression, low levels of *sal-lacZ* 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^{Mrt} third instar wing disc. Asterisks denote the intersection of the dorsal and ventral components of the *kni* L2 stripes with the margin. (C) *knrl* expression in a *GAL4-MS1096*; *UAS-salr* wing disc. *knrl* 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 *knrl* 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 *knrl* expression appears nearly normal. Similar, but more penetrant, elimination of *kni* and *knrl* 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 ri^1/ri^1 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* $salm^{IIA}$; ri^1 mosaic wings. If these phenotypes are generated at approximately equal frequencies in wild-type versus ri^1/ri^1 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 ri^1/ri^1 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 ri^1/ri^1 background. Again, note the island of ectopic triple row bristles (lower overline, asterisk).

to eliminate veins, higher levels of *kni*/*knrl* 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 *knrl* 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

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

In addition to activating *rho* expression, *kni* and *knrl* 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 *knrl* gene (Fig. 5K) and vice versa (data not shown). As *kni* and *knrl* appear to share cis-regulatory elements in third instar larval wing discs (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 *knrl* is likely to reflect an autoregulatory function of these genes. *kni* function does not appear to be necessary for activating *knrl* 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 *knrl* 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-kni^{EP} *ri* wings relative to wild type (Fig. 1E,F). Similarly, *rho* expression in the L2 primordium is shifted anteriorly in UAS-kni^{EP} *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 *knrl* 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 *knrl* in a narrow stripe of cells within the domain of *salm* non-expressing cells. *kni* and *knrl* 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 *knrl* at high levels. The key difference between the *kni* and *knrl* 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

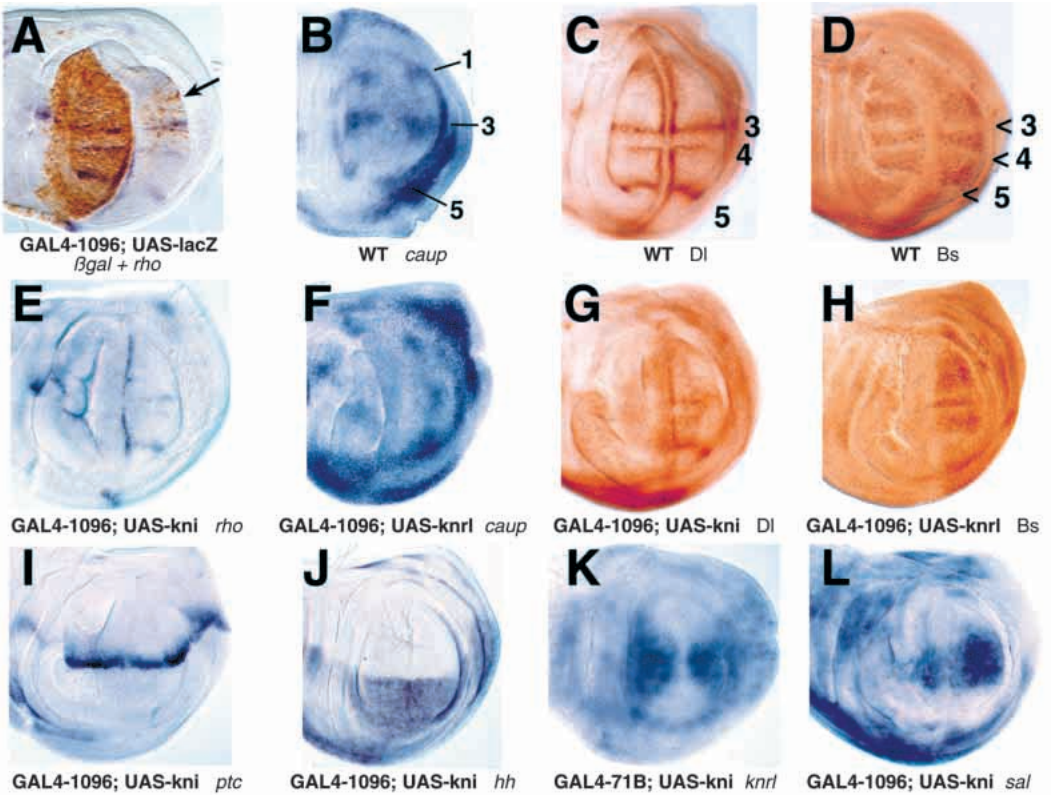

Fig. 5. *kni/knrl* organize gene expression in the vicinity of the L2 primordium. All panels show gene expression in mid-third instar wing imaginal discs. (A) A *GAL4-MS1096; UAS-lacZ* disc double-stained for *rho* RNA expression (blue) and anti-β-gal protein (brown). Strong β-gal staining is restricted 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-Skarmeta et al., 1996). C) Wild-type expression of Dl protein, detected with an anti-Dl 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) Dl 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) *knrl* mRNA expression in a *GAL4-71B*; *UAS-kni* wing disc. (L) *salm* mRNA expression in a *GAL4-MS1096; UAS-kni* wing disc.

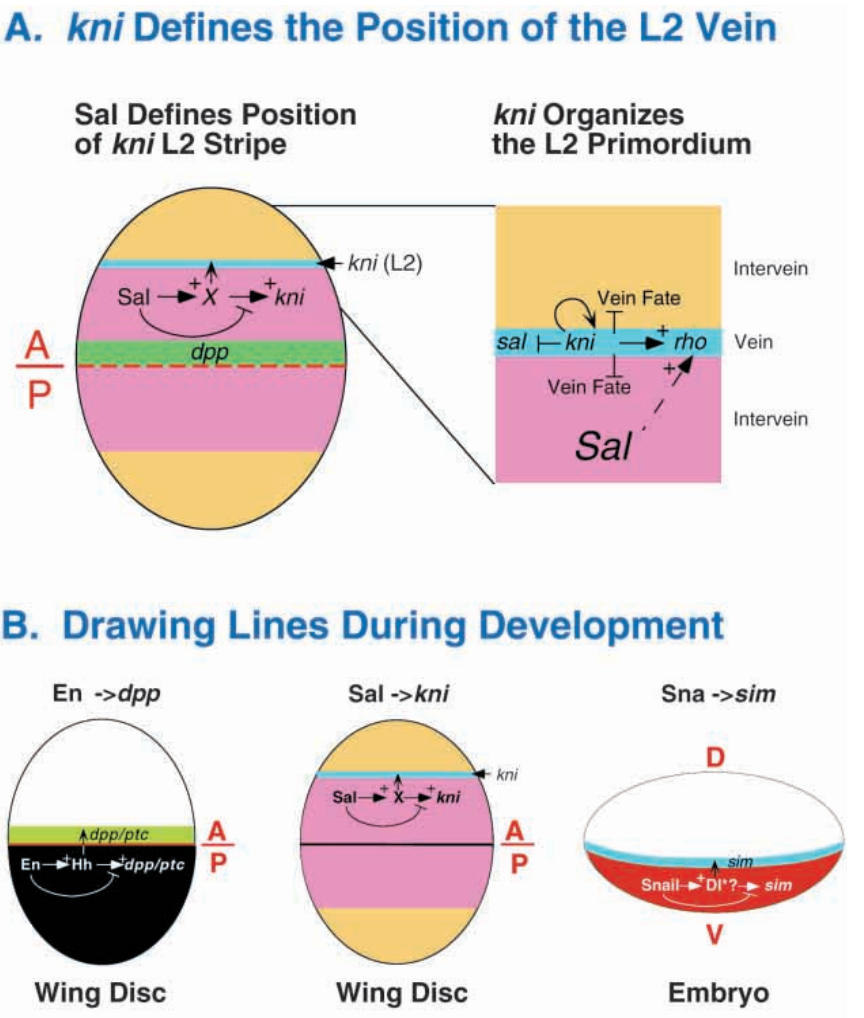

Fig. 6. Model for how *kni/knrl* organizes formation of the L2 primordium and similarities with other mechanisms for generating linear patterns of gene expression. (A) Left: diagram to illustrate how the juxtaposition of anterior and posterior compartment cells leads to the production of the long-range 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 Dl (Dl*), while suppressing the response to Dl/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, another Notch ligand might be activated in response to *kni* and *knrl* to suppress the vein fate in neighboring cells. It is also possible that a different type of signaling pathway is involved in this process.

Finally, *kni* and *knrl* are likely to maintain and sharpen the anterior *salm* border through a combination of autoactivation and negative feedback on *salm* expression. Kni and Knrl may repress *salm* expression directly or could function indirectly through an intermediate tier of regulation. The ability of ectopic *kni* or *knrl* expression to suppress expression of *salm* as well as vein markers, but not to suppress expression of genes involved in defining the A/P organizing center (i.e. *hh*, *dpp* and *ptc*), is consistent with *kni* and *knrl* functioning at the last step in defining positional information required for placement of the L2 primordium. It will be interesting to determine whether there are genes functioning analogously to *kni* and *knrl*, that specify the positions of other longitudinal veins along the A/P axis of wing imaginal discs.

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 ventral cells (Nambu et al., 1990; Kosman et al., 1991; Leptin, 1991; Rao et al., 1990) and Dorsal and Twist collaborate to define a relatively sharp 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.

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, Dl) 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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