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The role of *dAP-2* and *dve* in leg segmentation *in Drosophila melanogaster*

by

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Abstract

The formation of joints between segments in the developing leg of *Drosophila melanogaster* is mediated by the localized activation of the Notch pathway. Rings of cells in each segment expressing the ligands Delta and Serrate activate Notch signalling in the adjacent distal cells. The activation of the Notch pathway is sufficient to induce joint formation and is associated with the expression of several target genes. It has recently been shown that the dAP-2 transcription factor acts downstream of Notch activation and is required for joint formation (Kerber et al., 2001, Monge et al., 2001). Flies homozygous for this mutation survive until the pharate adult stage and the legs lack joints and are much shorter than wild type. In this study, I have identified several new EMS induced *dAP-2* mutations and shown that the mutation *l(3)1215* is an allele of *dAP-2*. By examining the expression patterns of Notch pathway components and other genes expressed in the leg, I have identified targets of dAP-2. I have shown that the expression of the Notch ligand Serrate and the homeobox gene *defective proventriculus (dve)* require dAP-2 function. *dAP-2* and *dve* are expressed in a similar pattern in the leg and are required to repress the expression of *Ser*. Although both transcription factors affect *Ser* expression similarly, they have opposite effects on adult leg phenotype, as dAP-2 promotes joint formation, while DVE inhibits it. I have proposed models incorporating these data which provide an explanation for the process of leg segmentation.

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List of abbreviations

EMS	Ethyl methane sulfonate
PBS	phosphate buffer saline
DAB	Diaminobenzydene
GFP	Green Fluorescent Protein
GMM	Gary's Magic Mount (mounting solution)
ta	tarsal segment
α	anti

Chapter1: Introduction

Cell interactions play an important role in patterning of a developing organism. I am studying the leg of *Drosophila melanogaster* as a model system for cell interactions involved in pattern formation. Specifically, I am interested in the process of leg segmentation and joint formation. The fruit fly leg is segmented along the proximal-distal axis. Each of the segments, coxa, trochanter, femur and tarsae is unique and separated from the other by joints. The process of joint formation is mediated by local cell interactions that require the activity of the Notch signal transduction pathway.

Studies of the process of segmentation in *Drosophila* have focused extensively on segmentation of the embryo (Wolpert et al., 1998). Embryonic segmentation occurs early, in the syncytial blastoderm. Patterning along the anterior-posterior axis is established by the formation of concentration gradients of transcription factors, which are free to diffuse throughout the acellular environment. Leg segmentation, on the other hand, is faced with a different challenge as this process occurs in a cellularized imaginal disc. Patterning of this structure is also coupled with the growth of the disc. This work will examine the roles of two genes, that appear to have opposing effects on leg segmentation and joint formation. Mutations in *dAP-2* result in the loss of all joint structures in homozygous mutant flies. Mutations in *defective proventriculus (dve)* give rise to ectopic joints, associated with clones of mutant tissue.

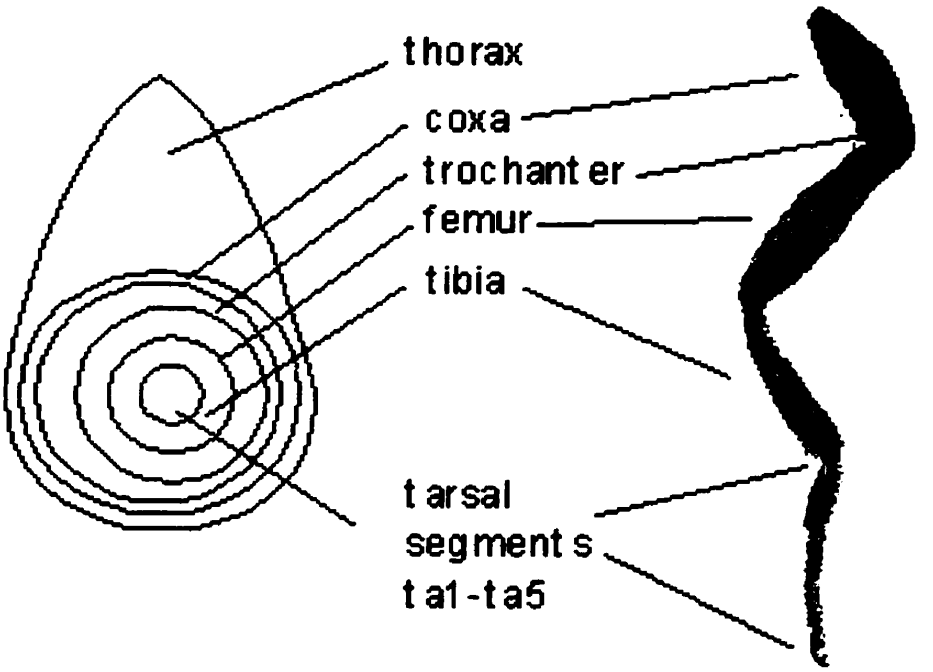
1.1 Imaginal Discs

The head and thoracic cuticle of the adult fly develops from groups of cells called imaginal discs. Imaginal discs are set aside early in development as clusters of epithelial cells that grow and divide throughout development, giving rise to folded epithelial sacs. At pupation, discs evert to give rise to the adult structures. Leg imaginal discs develop in such a way that the center of the disc gives rise to the most distal segments of the leg, while more proximal segments develop from cells closer to the periphery of the disc (Fig 1.), (Cohen, 1993). Patterning of leg discs begins early in development through the action of several genes that are required to specify cell fates along the anterior-posterior, dorsal-ventral and proximal-distal axes.

1.2 Pattern Formation in the Leg: Anterior-Posterior and Dorsal-Ventral Axis Formation

Initially, the leg disc is divided into anterior and posterior compartments, through the action of the homeobox selector genes *engrailed (en)* and *invected (inv)*. The posterior-specific expression of *en* is inherited from its earlier segmental ectoderm expression established during embryogenesis. (Cohen, 1993). EN expression then allows the expression of the signalling molecule Hedgehog. This occurs by repressing the transcription factor Ci, thus de-repressing *hh* (Brook et al., 1996). HH in turn functions to set up the expression of *wg* and *dpp*, which are required for patterning along the dorsal-

Figure 1. Leg imaginal disc and the corresponding adult leg segments.


thorax
coxa
trochanter
femur
tibia
tarsal
segments
ta1-ta5

ventral as well as the proximal-distal axis (Fig 2.) (Lecuit and Cohen, 1997; Basler and Struhl, 1994).*wg* and *dpp* are expressed in two wedge-shaped domains in the leg discs. *wg* is expressed in a ventral anterior wedge and is necessary and sufficient to specify ventral cell fates, while *dpp* is expressed in a dorsal anterior wedge and is necessary and sufficient for development of dorsal leg structures. These two domains of expression, which are kept separate by mutual repression result in concentration gradients, that specify dorsal and ventral fates independently (Jiang and Struhl, 1996; Brook and Cohen, 1996). The expression of *wg* and *dpp* meet at the center of the disc where both are required to direct the expression of transcription factors which are responsible for establishing the proximal-distal axis (Campbell and Tomlinson, 1995; Diaz-Benjumea et al., 1994; Abu-Shaar and Mann, 1998).

1.3 Proximal-Distal Axis Formation

Initially, the action of WG and DPP divides the leg disc into proximal and distal domains by directly specifying the expression of two homeobox transcription factors, Distalless (DLL) and homothorax (HTH). High concentrations of WG and DPP induce the expression of *Dll* in a circular domain at the center of the leg disc (Lecuit and Cohen, 1997). The *Dll* expression domain corresponds to the region of the imaginal disc that give rise to the most distal leg segments and *Dll* expression is required for normal

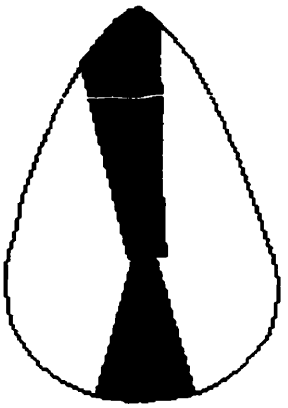
Figure 2. Proximal-distal patterning of the leg.

a. *dpp* and *wg* are expressed in two wedge shaped concentration gradients in the dorsal-anterior and ventral anterior regions of the leg disc respectively.

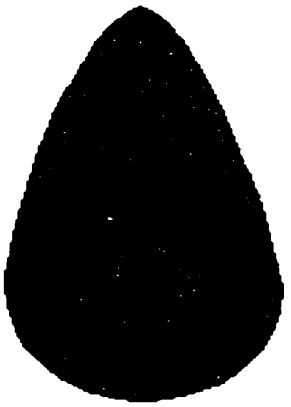
b. High DPP and WG concentrations induce expression of *Dll* and inhibit *hth* expression. The expression of HTH refines the expression of *dac*. Together these genes divide the leg into broad domains along the P-D axis.

c. DAC expression then helps to set up the proper expression of *Bar*, whose function is required for development of the tarsal segments. BAR also patterns the proper expression of *bab*, *ap*, *al* and *Lim1*, which regulate each other and specify the unique identities of each tarsal segment.

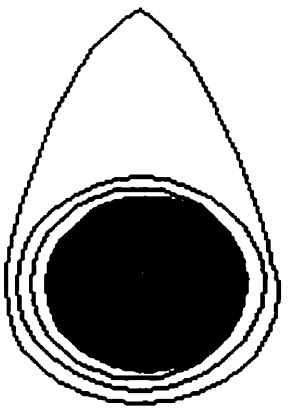
a. D-V Patterning



b. P-D Patterning



c. Tarsal Patterning



development of these structures. Strong hypomorphic alleles of *Dll* lead to the deletion of all tarsal segments as well as shortening of the tibia (Cohen and Jürgens, 1989). However, analysis of null *Dll* alleles as well as clonal analysis revealed that DLL is required for the development of all segments distal to the coxa (Campbell and Tomlinson, 1998). Indeed, by taking advantage of the relatively high protein perdurance of a *Dll* reporter transgene, it was shown that *Dll* is expressed throughout the leg disc early and is later restricted to the most central regions as the disc matures (Campbell and Tomlinson, 1998). This approach was used to determine whether any evidence of earlier *Dll* transcription could be detected at stages when the DLL protein is no longer present. A *Dll-GAL4* construct was used to drive the expression of *UAS-GFP* or *UAS-lacZ* with the possibility that either the GFP or β-galactosidase protein will remain and be detectable. This result was also confirmed by generating mitotic clones under the control of *Dll-GAL4* and *UAS-Flp*. Using this approach, clones will only be induced in *Dll* expressing cells. Clones could be found throughout the leg disc, indicating that at some stage *Dll* is expressed throughout the leg disc (Campbell and Tomlinson, 1998; Weigmann and et al., 1999).

homothorax is also a homeobox gene that functions to promote the nuclear localization of its cofactor Extradenticle (EXD), a PBX-like homeobox protein (Rieckhof et al., 1997). Together HTH and EXD are required for normal development of the coxa and trochanter. High concentrations of WG and DPP act to prevent the expression of homothorax (Abu-Shaar and Mann, 1998; Wu and Cohen, 1999). Since the initial expression of *Dll* and *hth* are exactly complementary, the possibility that WG and DPP

act through DLL to repress *hth* expression was examined. However, clones of cells, located in the distal domain of leg imaginal discs that are mutant for *Dll* function do not ectopically express *hth*, indicating that WG and DPP do not repress *hth* through the activation of *Dll* (Wu and Cohen, 1999). As a result of this, the leg disc is divided into proximal and distal regions, with *hth* expression being restricted to the proximal, outer regions of the disc and *Dll* being expressed in the distal, central regions of the disc (Fig 2.).

Homothorax further plays a role in refining the proximal-distal pattern. It functions to reduce the sensitivity of cells that express *hth* to receiving the WG and DPP signal (Wu and Cohen, 1999). This activity is required for the proper expression of another target gene of WG and DPP signalling, *dachsund (dac)*. Null alleles of *dac* show truncation and fusion of the femur, tibia and proximal three tarsi (Mardon et al., 1994). Accordingly, *dac* is expressed in a ring, which corresponds to the deleted segments (Fig 2.). Its expression begins slightly later in the development of the disc and is induced directly by lower levels of WG and DPP signalling and is repressed in the center of the disc by high levels of WG and DPP signalling (Lecuit and Cohen, 1997). Further refinement of *Dll*, *dac* and *hth* expression occurs through their mutual antagonism. (Dong et al., 2000).

1.4 Tarsal Segmentation

The genes described above initially subdivide the leg into broad domains. However, additional genes are required to further define the segments and their identities, within these broad domains. The distal region of the leg, the tarsus, is composed of five distinct segments. Initially, the tarsus is divided into the proximal and distal tarsus by the expression of *dac*, in tarsal segments ta1-ta2 and the homeobox transcription factor BAR in the distal most segments, ta3-ta5 (Mardon et al., 1994; Kojima and Saigo, 2000). Consequently, *dac* mutants exhibit fusion of the three proximal tarsal segments, while *Bar* mutant clones give rise to fusions of the three distal tarsal segments. Shortly after, this pattern is further refined. The distal end of *dac* expression moves proximally and the proximal end of *Bar* expression moves distally. *dac* expression now ends in tarsal segment ta1 and *Bar* expression begins in tarsal segment ta4 and is at its highest levels in segment ta5 (Kojima and Saigo, 2000). This generates a region where neither *dac* nor *Bar* is expressed, corresponding to tarsal segments ta2-ta3.

bric à brac (bab), a gene encoding a BTB domain nuclear factor, is expressed in the region which corresponds to tarsal segments ta1-ta4 (Godt et al., 1993). Its expression is strongest in ta3 and ta4, slightly weaker in ta2 and weakest in ta1. *bab* expression has been shown to be dependent on the early expression of *spineless*, a bHLH-PAS domain transcription factor (Duncan et al., 1998). The strongest levels of *bab* expression are found in the medial tarsal segments where both BAR and DAC are absent. Here, the activity of bric à brac is responsible for repressing ta1 tarsal fate. Analysis of *bab* mutant

legs showed the presence of transformations of tarsal segments ta2-ta4 to a ta1 fate, based on bristle pattern including the formation of an ectopic sex comb (Godt et al., 1993). This observation suggests the involvement of *bab* in specifying tarsal segment identity.

The Lim homeodomain transcription factor, Apterous (AP) is required for the formation of tarsal segment ta4. *apterous* expression is dependent upon *Bar* expression as shown by genetic mosaic and misexpression analyses. Clones of cells mutant for BAR function present in ta4 do not express *ap*. Conversely, misexpression of *Bar* results in the ectopic expression of *ap* (Kojima and Saigo, 2000). BAR is involved not only in regulating *ap* in ta4 but its later strong expression is required for proper ta5 identity. *Bar* mutant clones in ta4 and ta5 were often associated with sensilae companiformae, sensory structures representative of ta3, suggesting that BAR function serves to repress ta3 fate. Misexpression of *Bar* in the *ap* domain showed transformation of the ta4 segment into a ta5-like fate, indicating that strong *Bar* expression in the ta5 segment is required for its proper identity.

The identity of the distal most leg structure, the pretarsus is also regulated by BAR function. In tarsal segment ta5, BAR represses the activity of *aristaless (al)* and *Lim1* (Kojima and Saigo, 2000; Tsuji et al., 2000). However, in the pretarsus, LIM1 regulates the expression of *al* and together the activity of both LIM1 and AL represses *Bar* (Tsuji et al., 2000).

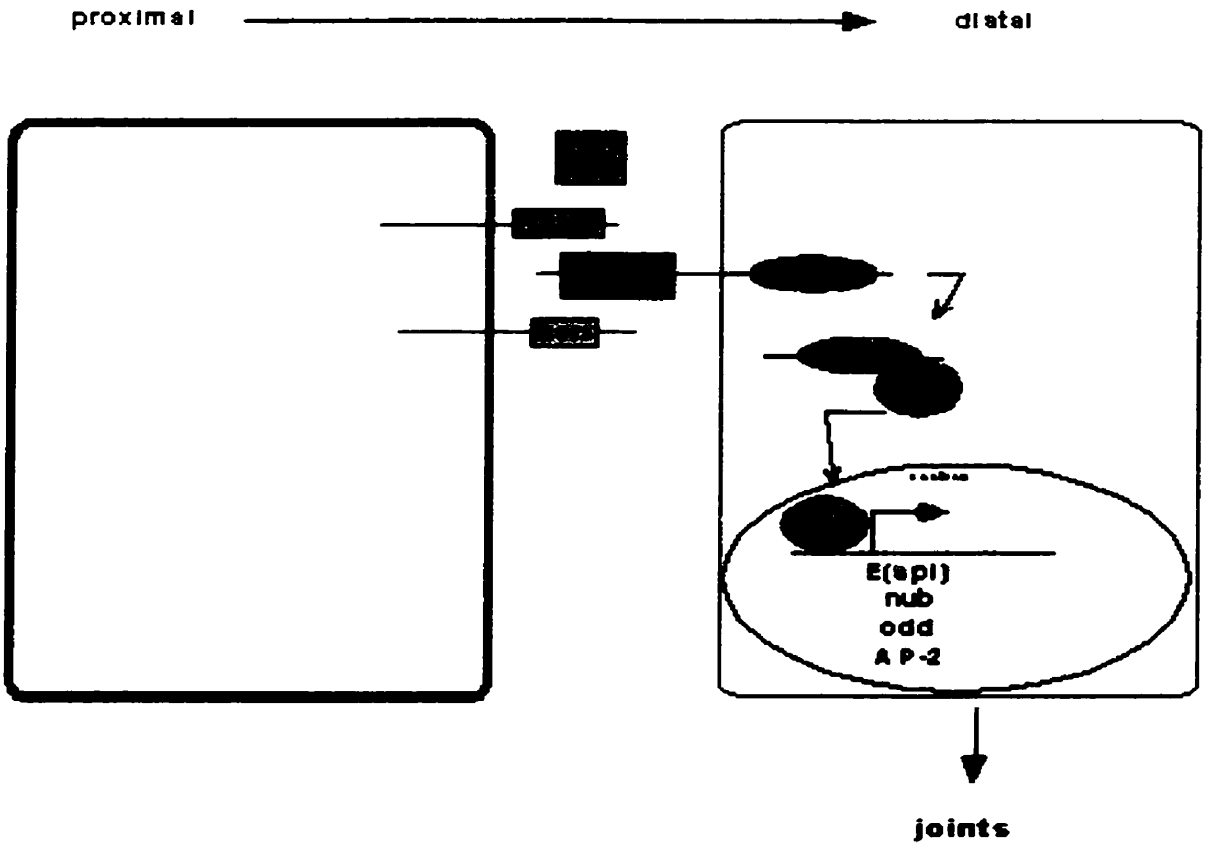
1.5 Segmentation: the Notch signalling pathway

Another group of genes required to subdivide the P/D axis are members of the Notch (N) signalling pathway. Unlike *dac, bab, ap,* and *Bar* the Notch pathway does not contribute to the unique identity or growth of particular leg segments. Rather Notch signalling is required for the proper growth of all segments and the specification of the joints that separate the segments. Hypomorphic mutants or somatic mosaics of null mutants for Notch pathway elements fail to form joints and result in fusions of the leg segments, suggesting an involvement for Notch signaling in leg development (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). *Notch* encodes a transmembrane receptor, that responds to signals from two transmembrane ligands, Delta (DL) and Serrate (SER) (Fig. 3). Another protein, Fringe encodes a glycosylase that modifies the affinity of the Notch receptor for its two ligands, so that in the presence of FNG, Notch can bind to Delta but not Serrate (Bruckner, et al., 2000). Once the DL/SER signal is received by Notch expressing cells, Notch becomes activated. Upon activation, the Notch intracellular domain is cleaved and interacts with the transcription factor Suppressor of Hairless, (Su(H)). The complex of Su(H) and N_{intra} activates downstream target genes of Notch signalling (Reviewed in Artavanis-Tsakonas et al., 1999). The expression of Notch ligands and other Notch pathway elements in ring of cells in each segment suggested that the Notch pathway may be involved in leg. In late third instar leg discs, Delta, Serrate and Fringe are expressed in a series of concentric circles, each circle representing part of one segment of the future leg. Detailed inspection of the staining

pattern in developing pupal legs revealed that *fng, Dl* and *Ser* are expressed in cells proximal to the joint of each segment (Fig 4.), (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). The expression of *fng*, *Ser* and *Dl* were largely overlapping, however sometimes *Dl* expression was observed in more proximal regions where neither SER nor *fng* were present. Indeed, Bishop et al., (1999) report that *Dl*, detected by either antibody or a

Figure 3. The Notch signalling pathway.

DL or SER signal to adjacent cells by binding to the Notch receptor. In the presence of FNG, only DL is able to bind N. Upon ligand binding, the intracellular domain of N is cleaved and binds to Su(H). Su(H) together with N translocate to the nucleus, where Su(H) regulates the expression of downstream target genes such as *E(spl)*, *nub*, *odd* and dAP-2.


proximal
distal
E(spl)
nub
odd
AP-2
joints

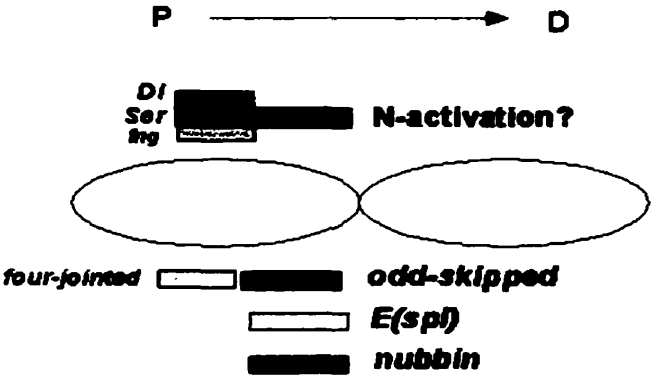
lacZ reporter, was found at low levels throughout the leg discs and upregulated at sites where joints will later form. The *Notch* expression pattern was visualized by in situ hybridization to *N* RNA. *N* RNA was seen uniformly throughout larval leg imaginal discs. However, 24 hours after pupation, the levels of *N* RNA were upregulated at segment boundaries, in places were joints are beginning to form (de Celis et al., 1998). Notch activation was also monitored by the expression of known Notch signalling target genes, such as the Enhancer of split complex. *E(spl)* RNA expression was also seen in a series of concentric circles, representing each segment. In the developing leg *E(spl)* is expressed in distal cells at each segment boundary, corresponding to the cells in which *N*RNA becomes upregulated (de Celis et al., 1998).

These expression patterns are consistent with the data indicating that Notch signalling is required in cells that will form joints of the adult leg. In the leg, Notch signalling appears to be involved in directing the formation of joints. Hypomorphic mutations of *Notch* result in fusions of leg segments and similar fusions were observed in mutations of *Ser* and *Dl* (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). However, closer examination of *Dl*, *Ser* and *Notch* mutants revealed a difference in their phenotypes. Legs of *Ser* null mutant adults lack all joints, but maintain the boundaries between segments, as represented by the presence of apical bristles, which are characteristic of the distal end of each segment. *Dl* hypomorphs, lack all the joints and have apical bristles. However all segments are shortened in length, suggesting a greater requirement for DL function.

Figure 4. Expression patterns of Notch pathway components in the developing tarsal joints.

DL, SER and FNG are expressed in rings of cells in each segment. This leads to N-activation in adjacent cells distal to the DL, SER, FNG expression domain. Expression of Notch target genes such as *odd, E(spl)* and *nub* occurs in the domain of N-activation, while *fj* is expressed in the DL, SER and FNG domain.

Gene expression in developing tarsal joints



Hypomorphic *Notch* mutants, exhibit a combination of both of these phenotypes, where the legs fail to form all joints and are also shortened. *Notch* mutant legs do not form segment boundaries, as they lack apical bristles (Bishop et al., 1999). Furthermore, regulatory alleles that abolish specific segment expression of *Dl* lose those segments completely. This suggests an absolute requirement for Notch signalling for segment specification or survival (Mishra et al., 2001).

Several other lines of evidence show more directly that the activation of the Notch receptor in cells adjacent to the cells expressing the Notch ligands lead to segmentation and joint formation. Somatic clones of cells mutant for *Notch* show that Notch function has a cell autonomous role only at the boundaries between leg segments, as clones located only in cells which normally contribute to the formation of a joint were able to impair this process. Notch function is required for the formation of all joints in the leg. Notch is also required for the proper growth of the leg, as large clones caused the segments in which the clone was located to be shorter (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999).

A similar clonal analysis was performed with mutations of other components of the Notch signaling pathway such as *Serrate*, *Delta* and *fringe*. As with *Notch* mutant clones, it was determined that *Ser, Dl* and *fng* clones only disrupt joint formation when the clone spans the area where a joint would normally form (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). SER and DL are required in all the joints, however the participation of FNG is necessary in all joints except for the tibia-tal joint

(Rauskolb and Irvine, 1999). Clones of cells mutant for both *Dl* and *Ser*, or *fng* that span more than one segment cause defects in joint formation, but also affect the length of the segment. Smaller clones, on the other hand, which are located in one segment only have no effect on either the joint structure or the size of the segment (de Celis et al., 1998; Rauskolb and Irvine, 1999). Some non-autonomous effects are also observed in *fng* mutant and *Notch* mutant clones, where the loss of joints affects the wild-type tissue surrounding the clone (Rauskolb et al., 1999).

Additional information of how Notch signalling functions to specify the position of joints was obtained from studies of the expression of joint markers (Table 1). Consistent with Notch being required for proper segmentation of the leg, the expression of the segment boundary marker *disconnected (disco)*, did not form properly in *Delta*, *Serrate* mutant as well as in temperature-sensitive mutants of *Notch*. In *Delta* mutant leg discs, the normal concentric rings of *disco-lacZ* expression fail to resolve properly and fuse. In *Serrate* mutants, the rings of *disco-lacZ* expression form, but are later lost (Bishop et al., 1999). In N_{ts} discs, *disco-lacZ* expression is both not resolved properly and later lost. These results suggest that signaling from both ligands is required for segmentation and that this process may occur in two separate steps. The first step, signalling through DL, may be required for the establishment expression of joint markers. A subsequent step, perhaps signalling though SER may be necessary to refine and maintain the correct expression pattern.

Notch activation is sufficient to induce joint formation. A constitutively active form of Notch, the intracellular domain, expressed in the leg using the *GAL-4/UAS* system in the *ptc* domain gave rise to continuous fold of naked cuticle along the proximal-distal axis (de Celis et al., 1998). This structure, formed in the region where N_{intra} was expressed, was

Table 1: Summary of the effects of mutations of the Notch pathway on joint formation and gene expression.

	Gene expression				**Joint formation**
	nubbin	*fj*	*odd*	*disco*	
Dl/Ser (ectopic)	Induced in cells bordering the clone, inhibited within the clone	Inhibited in cells bordering the clone, induced within the clone		Induced, where a joint has been induced	Inhibited if clone includes normal joint, induced at the border of clone
fng (ectopic)	Induced along the inside of the clone	Inhibited along the inside of the clone			Induced at clone borders, inhibited within clone
N activation (ectopic)	Induced within the clone	Inhibited within clone	Induced within the clone	Induced, where ectopic joint forms.	Induced within the clone
Dl/Ser (loss of function)				Rings of expression fail to resolve, *(Dl-)* and are not maintained *(Ser-)*	Inhibit joint formation and reduce growth.
fng (loss					Inhibit joint

	Gene expression				Joint formation
	nubbin	*fj*	*odd*	*disco*	
of function)					formation and reduce growth.
N- (loss of function)	Loss of nub expression			Rings of expression do not resolve and are not maintained.	Inhibit joint formation and reduce growth.

(Bishop and et al., 1999; Rauskolb and Irvine, 1999)

concluded to be an ectopic joint. The effects of ectopic expression of *Dl*, *Ser* and *fng* have also been examined. Ectopic expression of *Dl*, using the *GAL-4/UAS* system revealed that DL could have a similar effect to a loss of Notch function and cause a fusion of tarsal segments, suggesting that DL serves to limit the effect of Notch activation. This role is consistent with what has been observed in the wing, where high levels of DL and SER function to autonomously inhibit Notch activation (Micchelli et al., 1997). However, ectopic expression of *Dl* was able to induce the formation of an ectopic joint at the boundary of its expression (Bishop et al., 1999; Rauskolb and Irvine, 1999). Experiments examining ectopic *Dl* expression using Flip-out clones further support this. DL was able to inhibit joint formation, when the clone spanned the normal joint, but could also induce joint structures at the borders of the clone (Rauskolb and Irvine, 1999). Mis-expression of *Ser* or *fng*, using either the *GAL-4/ UAS* system, or Flip-out clones, yielded similar results. Ectopic expression could inhibit joint formation and could induce ectopic joints at

the borders of its expression (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999).

The expression of *nubbin* and *four-jointed*, two genes expressed during leg development are particularly informative to the role of N-signalling in leg development. One of these genes *nub*, encodes a POU-domain protein that is normally expressed in a series of concentric rings (Ng et al., 1995). *nub* mutations result in shortened legs (Cifuentes and Garcia-Bellido, 1997). Its relation to the Notch pathway was examined by generating clones of cells that are mutant for components of the Notch pathway or ectopically express them (Table 1). Clones of *Notch*$^{-}$ cells resulted in a loss of *nub* expression within the clone. Similarly, clones of ectopic Notch activation ectopically expressed *nub*, indicating that *nubbin* is positively regulated downstream of Notch. In clones that ectopically express *Delta* or *Serrate*, *nub* expression is observed in cells bordering the clone, but not in the clone itself, suggesting that ectopic expression of ligands generates a novel border where Notch is activated. Additionally, when a clone of ectopic *Ser* or *Dl* is induced in a location where a joint would normally form, its formation is inhibited. Ectopic expression of *fng*, on the other hand, is able to induce *nubbin* just inside the border of the clone. Clones of ectopic *fng* can induce joint structures along clone borders and can inhibit joint formation within it (Rauskolb and Irvine, 1999).

Another gene involved in proper leg development whose relation to the Notch pathway was examined was *four-jointed (fj)*. *fj-lacZ* is also expressed in a series of

concentric rings and mutations of this gene result in the fusion of the t2 and t3 tarsal segments, as well as a reduction in size of the femur tibia and three tarsal segments. *fj-lacZ* expression was shown to be negatively regulated by Notch activation. Clones of ectopic Notch activation were able to inhibit *fj-lacZ* expression. Its expression was also inhibited in cells bordering clones of ectopic *Ser* and *Dl* as well as in cells within ectopic *fng* clones. *fj-lacZ* expression was seen in clones of ectopic *Ser* and *Dl*. Immunohistochemical staining for both *fj-lacZ* and NUB showed that these proteins are seen in a series of exactly complementary rings in leg imaginal discs (Rauskolb and Irvine, 1999). The expression of *odd-skipped (odd)* was also examined and was shown to be positively regulated by Notch-activation.

The Notch signalling pathway is required to regulate the growth of all segments and to specify and induce proper joint formation. However, it is unclear how the correct spatial expression of Notch pathway components is established. Genes patterning the tarsal segments may be required to organize the pattern of the ligands that initiate Notch signalling. The intercalation of specific tarsal fates is required prior to the initiation of joint formation (Galindo and Couso, 2000). An additional gene that this work will show is required to refine the expression pattern of a Notch ligand is *dAP-2*.

1.6 Joint formation: the role of dAP-2

dAP-2 plays a major role in the process of Notch mediated joint formation. This gene was originally cloned as homologue of the vertebrate transcription factor AP-2 (activator protein 2) (Bauer et al., 1998; Monge et al., 1998). This transcription factor is composed of a transactivation domain and a basic region followed by a helix-span-helix domain, which is involved in DNA binding and dimerization (Hilger-Eversheim et al., 2000) The mammalian AP-2 transcription factor family is composed of three members, *AP-2α*, *AP-2β* and *AP-2γ*. Studies in mice have revealed that these genes are expressed in numerous tissues of the developing embryo such as the neural crest and its derivatives, mesynchymal cells of limbs as well as the developing central and peripheral nervous system (reviewed in Hilger-Eversheim et al., 2000). Mice mutant for the *AP-2α* gene show severe craniofacial defects as well as nervous system and limb defects. These embryos also show an increased amount of programmed cell death in the hindbrain, mid brain and in cells derived from the neural crest.

The *Drosophila* homologue shows 42-45% amino acid similarity with the mammalian proteins (Bauer et al., 1998; Monge et al., 1998). Like its mammalian homologues, *dAP-2* is also expressed in various components of the nervous system such as the embryonic brain, ventral nerve cord and maxillary segments. It is also expressed in the imaginal discs, in a series of concentric circles in third instar leg and antenna imaginal

discs (Monge et al., 1998). Mutants of *dAP-2* were isolated in an EMS mutagenesis screen and their phenotype was examined (Monge et al., 2001). Flies homozygous for null alleles of *dAP-2* survive until adulthood, but are unable to eclose from their pupal cases. The most striking phenotype of these flies is that their legs are significantly shorter than their wild-type counterparts. Upon close examination, it is evident that all segments are present in the correct order along the proximal-distal axis. However all joints of the tarsus are missing. In the most severe mutants only the tibia-basitarsus joint forms. Mutant flies also exhibit a shortening of the proboscis, consistent with *dAP-2* expression in the labial imaginal disc (Monge et al., 2001).

The expression pattern of *dAP-2* was also examined. *dAP-2* is expressed in concentric circles in third instar larval imaginal discs. Double staining of everting pupal legs showed that *dAP-2* is expressed at each joint, in cells that also express downstream target genes of Notch signalling such as *E(spl)mβ* and *big-brain (bib)* (Kerber et al., 2001). However, the result of this study will show that dAP-2 does not correspond exactly to cells expressing *E(spl)mβ–CD2* . The observation that dAP-2 is found in the same domain as genes activated by Notch led to the investigation of whether *dAP-2* is also regulated by the Notch pathway (Kerber et al., 2001). Clones of cells in which a *Suppressor of Hairless(Su(H))* mutation has inactivated the Notch signalling pathway, do not express *dAP-2*. Conversely, clones of cells expressing a consituitively-activated form of Notch, N_{intra}, induce *dAP-2*. Therefore, dAP-2 acts downstream of Notch to regulate

joint formation. However, dAP-2 does not regulate the expression of the joint markers *E(spl)mβCD2* and *bib* since their expression remains unaltered in *dAP-2* mutant leg discs.

The function of dAP-2 was examined further using clonal and misexpression analysis. dAP-2 was found to have an autonomous function regulating joint formation and a non-autonomous function regulating growth (Kerber et al., 2001). Small *dAP-2*$^{-}$ clones located in the inter-joint region have no effect. However, if the clone spans more than one segment, it autonomously inhibits the formation of the joint, indicating that dAP-2 is required for joint formation. Legs containing large clones that span more than one segment also show a reduction in the overall length of the leg, suggesting that dAP-2 has a role in regulating growth of the segment. Misexpression of dAP-2 revealed that its activity is sufficient to induce joint formation. The *ptc-GAL4* driver was used to direct ectopic expression of *UAS-dAP-2*. Ectopic joints were observed only in close proximity to the endogenous joint. These results suggest that although dAP-2 is capable of inducing joints, this is only possible in a certain "competent' region, indicating a requirement for genes other than *dAP-2* to direct joint formation. Indeed, additional genes downstream of Notch activation are necessary, as joint formation due to ectopic *dAP-2* expression is inhibited in a *Notch* mutant background.

The effect of dAP-2 on growth was also examined (Kerber et al., 2001). The shortening of leg segments could be due to either a lack of cell proliferation, or cell death. No difference in the number of cell divisions in *dAP-2* mutant leg discs was observed. However, an increased amount of cell death was seen in mutant leg discs, when examined

using TUNEL staining (Kerber et al., 2001). The majority of cell death was occurring in the interjoint region of the leg, where *dAP-2* is not expressed. They interpreted these results as indicating that dAP-2 has an indirect effect on the growth of the leg, inhibiting cell death of the interjoint region. However, this may not be the case as a broader expression domain of dAP-2 will be shown in this study.

The dAP-2 transcription factor plays an important role in leg development. It functions downstream of Notch activation to autonomously induce joint formation. It also has a non-autonomous effect on leg growth by suppressing cell death in the interjoint region. However, much is yet unknown about its function in joint development. Through this work I will show that one function of *dAP-2* in joint development is to block the activation of the Notch ligand SER. I will also demonstrate that dAP-2 acts together with another transcription factor, defective proventriculus (DVE), to regulate joint formation.

1.7 Summary

The process of leg segmentation requires the participation of numerous genes and the communication between differing cell populations. This process begins with the expression of two signaling molecules, WG and DPP, in two concentration gradients. The overlapping expression of *wg* and *dpp* then sets up the expression of various transcription factors, which pattern the leg along the proximal-distal axis. The expression of genes such as *Dll, dac* and *homothorax* divides the leg into proximal and distal domains. *Dll*

and *dac* expression then allows for the expression of such genes as *Bar* and *bab*, that allow for the intercalation of tarsal segments into these broad domains. Later in leg development BAR and BAB in addition to AP, AL and Lim1, function to specify the unique identity of each tarsal segment. Although these genes specify distinct domains along the P-D axis, they do not direct the formation of individual segments. The Notch signalling pathway, including *Delta*, *Serrate* and *fringe*, is required for specifying the position of boundaries between each of the segments. Notch activation then directs the expression of downstream target genes such as *nubbin, four-jointed* and *dAP-2*, which in turn participate in the process of joint formation and leg growth. Notch signalling creates a boundary that causes a local differentiation of a structure and has long range effects on growth.

1.8 Objectives of this study

The initial goal of this study was to characterize the *dAP-2* mutation. This is reflected in the first part of the study, consisting of the mapping of the original *l(3)1215* mutation and mutagenesis to isolate new alleles. However, during the course of this study, two other groups characterized *dAP-2*. Thus my attention shifted to identifying downstream targets of dAP-2. In this process I was also able to examine the role of *dve* in leg segmentation.

Chapter 2: Materials and Methods

2.1 Fly rearing conditions

Flies were grown on Caltech medium, containing agar, dextrose, sucrose, corn meal, yeast and propionic acid/phosohoric acid (Ashburner, 1989). All crosses and stocks were grown at 25°C, unless otherwise specified.

2.2 EMS mutagenesis to isolate new dAP-2 alleles

An isogenic *red e* stock was created by crossing *red e* females to *Oregon-R* males. Single *red e/+* males were then mated to *w; TM3 /TM6B* females (Bloomington Stock Center, balancer described in (Lindsley and Zimm, 1992). Several stocks were established by mating *red e/ TM6B* progeny from the previous cross to generate isogenic *red e/ red e* progeny. The healthiest stock was picked and used for mutagenesis. EMS mutagenesis was performed as described in Grigliatti, (1998). Adult *red e* males were starved 5-8h then allowed to feed overnight (16h) on Kim-wipes soaked in 1% sucrose + 20mM ethanemethylsulfonate (EMS) (Sigma). Optimal EMS concentration was determined by crossing EMS-treated males to *c(1)RM, yw/Y* (attached-X, *X^X/Y*) females and calculating the percentage of lethality induced by different concentrations of EMS. From this cross, there are only two possible viable phenotypes, females that inherit the attached-X chromosome from the female parent and a *Y* chromosome (*X^X/Y*) from the male parent and males that inherit an X chromosome from the male parent and the Y

from the female parent (*XY*). Males that inherit an X chromosome from the male parent that carries a recessive lethal mutation will not survive. Lethality can then be calculated by comparing the proportion of male progeny from an EMS-treated cross and an untreated cross (untreated male progeny/total untreated progeny)-(treated male progeny/total treated progeny). 20mM EMS was found to cause ~20% lethality on a single X chromosome, or approximately 40% of each autosome will carry a lethal mutation (Grigliatti, 1998). EMS treated *red e* males were then mated to third chromosome balancer females (*w; TM3/TM6B*). Approximately 50 females and 25 males were mated in each bottle. Individual *red e/ TM6B* males were collected from this cross, each representing a single mutagenized chromosome and mated with *h th st dAP-2* $^{l(3)1215}$ *red e/ Tm3* females. *dAP-2* $^{l(3)1215}$ is the original allele of dAP-2 (Held et al., 1986). *red e/h th st dAP-2* $^{l(3)1215}$ *red e* progeny from this cross were scored for lethality and dAP-2 phenotype.

2.3 Male recombination

Females of the genotype *ru h p Ki cu sr e ca/Tm3* were crossed to males which carry a source of transposase (Δ2-3), a P-element and *dAP-2*: *CyO Δ2-3/+; P-element/h th st dAP-2* $^{l(3)1215}$ *red e*. The P-elements used to do this are listed in Table 2. All P element stocks were obtained from the Bloomington Drosophila Stock Center. *h* and *e+* or *h+* and *e* recombinants were collected and crossed back to *dAP-2*$^{-}$ flies. The progeny of recombinants were then scored for the dAP-2 phenotype.

2.4 Molecular Marker Analysis - Antibody staining of imaginal discs

Wild type and *dAP-2* mutant pupae were collected ~4-6h after puparium formation. They were dissected in 1x PBS (Phosphate Buffer Saline), and fixed for 15 min in 4% formaldehyde in 1x PBS (Ashburner, 1989). The fix was removed and heads were washed 2x 10 min in BBX-250 (PBS + 0.2% (v/v) Triton X 100 + 1% (w/v) BSA + 250mM NaCl) and blocked 1x 10 min in 5% NGS (Normal Goat Serum) in BBX-250. Primary antibody was then added into a final volume of 100 ul of BBX-250 and allowed to incubate overnight. Primary antibodies and the concentration used are listed in Table 3.

Primary antibody was washed off 5x 20 min in BBX-250 and 1x 30 min in 5% (v/v) NGS in BBX-250. Secondary antibody was then added into a final volume of 1000 µl of 5% (v/v) NGS in BBX-250.The secondary antibodies used and their conjugates are listed in Table 3.

Secondary antibody was washed off 4x 15 min in PBX2 (PBS + 0.2% Triton X 100). Discs were dissected and mounted in SlowFade Anti-Fade Kit (Molecular Probes). Discs were examined and photographed using the Zeiss Axiovert 100M confocal microscope with LSM 510 software. The thickness of optical slices taken was approximately 1.0-1.2 µm. All photographs represent individual slices, rather than projected images using all slices. All figures were assembled using Adobe Photoshop.

DL antibody staining was detected using DAB (Diaminobenzydene). The secondary antibody used for this was goat anti-mouse biotin conjugate at a concentration of 1: 1000 (Vector). Secondary antibody was washed off 4 x 15 min in PBX2. The

detection was done using the Vectastain kit (Vector). Heads were incubated in ABC reagents from the Vectastain kit, for 45 min and washed 4 x 15 min in PBX2. They were then preincubated in 0.5mg/ml DAB in PBX2 for 5 min, after which 5 ml of 0.3% of H_2O_2 (v/v) was added. Colour development was visible within 5 min and the reaction was stopped by washing 5 x 5 min in PBX2. Discs were mounted in 70% glycerol in PBS (v/v) and photographed using brightfield opticts on a Leica DMRE photomicroscope.

2.5 Crossing schemes for generating dAP-2- larvae for molecular marker staining

To generate *dAP-2* mutant larvae for SER, NUB and DL staining *h th st dAP-2* $^{l(3)1215}$ *red e/TM3* were crossed with *mwh dAP-2* $^{l(3)1215}$ *red e/TM3* flies. *h th st dAP-2* $^{l(3)1215}$ *red e/mwh dAP-2* $^{l(3)1215}$ *red e* larvae were selected on the presence of the red malpighian tubule phenotype of *red/ red* homozygous larvae.

2.6 Crossing schemes for generating dAP-2- larvae for molecular marker staining: markers located on chromosome II.

To generate dAP-2 mutant larvae for *dve* ^{01738} *-lacZ* staining, *dve* ^{01738} *-lacZ /CyO* flies were crossed to *h th st dAP-2* $^{l(3)1215}$ *red e/TM3* flies (Bloomington Drosophila Stock Center). *dve* ^{01738} *-lacZ* /+; *h th st dAP-2* $^{l(3)1215}$ *red e/+* flies were crossed to *mwh dAP-2* $^{l(3)1215}$ *red e/TM3* flies. Larvae showing the red phenotype (*dve* ^{01738} *-lacZ* /+; *h th st dAP-*

2 $^{l(3)1215}$ red e/ mwh dAP-2 $^{l(3)1215}$ red e) were picked and stained for βGal. These larvae were also stained for *dve ^{01738}-lacZ* and SER double staining. *red+* larvae were stained as wild-type controls. Alternatively, *dve ^{01738}-lacZ /+; h th st dAP-2 $^{l(3)1215}$ red e/+* flies were crossed to *dAP-2^{15} FRT 80 E(spl)mβ–CD2 /TM6B* flies (Kerber et al., 2001) and *Tb^{+}* larvae were selected and stained for α βGal or α βGal and α CD2. *Tb^{-}* larvae were stained as wild-type controls. The same crossing schemes were used to generate *fj-lacZ /+; h th st dAP-2 $^{l(3)1215}$ red e/ mwh dAP-2 $^{l(3)1215}$ red e* or *fj lacZ /+; h th st dAP-2 $^{l(3)1215}$ red e/ dAP-2^{15} FRT 80 E(spl)mβ–CD2* and *odd-lacZ /+; h th st dAP-2 $^{l(3)1215}$ red e/ mwh dAP-2 $^{l(3)1215}$ red e* or *odd lacZ /+; h th st dAP-2 $^{l(3)1215}$ red e/ dAP-2^{15} FRT 80 E(spl)mβ–CD2* larvae.

2.7 Crossing schemes for generating dAP-2- larvae for molecular marker staining: markers located on chromosome I.

To generate *dAP-2* mutant larvae for *disco-lacZ* staining, *disco-lacZ* females were crossed to *dAP-2^{15} FRT 80 E(spl)mβ–CD2 /TM6B. disco-lacZ; dAP-2^{15} FRT 80 E(spl)mβ–CD2/+* males were then crossed to *h th st dAP-2 $^{l(3)1215}$ red e/TM6B* females. Female, *Tb^{+}* larvae were picked from this cross and stained for βGal and *E(spl)mβ–CD2*. Larvae from the *disco-lacZ* stock were stained as controls.

2.8 Crossing schemes for generating dAP-2$^-$ larvae for molecular marker staining: double staining.

To generate *dAP-2* mutant larvae for staining against SER and *E(spl)mβ–CD2*, *dAP-2^{15} FRT 80 E(spl)mβ–CD2 /TM6B* females were crossed to *h th st dAP-2 $^{l(3)1215}$ red e/TM6B* males. *Tb*$^+$ larvae were selected from this cross and stained for *E(spl)mβ–CD2* and SER. *Tb*$^-$ progeny were stained as wild-type controls.

To generate wild type larvae for staining for *E(spl)mβ–CD2* and dAP-2, *dAP-2^{15} FRT 80 E(spl)mβ–CD2 /TM6B* larvae were picked and stained.

2.9 Mosaic analysis of *dAP-2*

Clones were induced using an *dAP-2^{15} FRT 80 E(spl)mβ–CD2* chromosome (Kerber et al, 2001) and a *hs FLP; FRT 80 GFP* chromosome (Bloomington Drosophila Stock Center). Clones were induced at 36-60 h after egg laying, by shifting larvae to 37°C for 1h. *hs FLP; FRT 80 GFP/ TM6B* females were crossed to *dAP-2^{15} FRT 80 E(spl)mβ–CD2 /TM6B* males, and *Tb*$^+$ larvae were selected and dissected. Discs were stained and analyzed according to the protocol described above. Imaginal disc clones were marked by the loss of GFP.

2.10 Misexpression of *dAP-2*

A *ptc-GAL4* chromosome (Bloomington Drosophila Stock Center) was used to drive *UAS-dAP-2* expression (Kerber et al., 2001). Flies were grown at 18°C for the first 2 days of development and then transferred to 25°C for the remainder of the time. *UAS-GFP(I)* females were crossed with *ptc-GAL4* males. *UAS-GFP (I); ptc-GAL4/+* males were then crossed to *UAS-dAP-2* females. Female larvae from this cross (*UAS GFP (I)/+; ptc-GAL4/+; UAS-dAP-2/+*) were selected. Discs were dissected and analyzed according to the protocol described above.

2.11 Mosaic analysis of dve

All clonal analysis was done using *yw; FRT 42 dve* ^{01738} */SM1* fly strain. This stock was made from *w; neoFRT 42D* w^+ *ubi-GFP/CyO* and *dve* ^{01738}*-lacZ/CyO* parent stocks, by selecting for *w-*, neomycin resistant recombinant progeny. Recombinant progeny were stocked, over the *SM1* balancer and tested for *dve* ^{01738}*- lacZ* staining. *yw hsFLP; FRT 42D* y^+*/CyO* females were mated to *yw; FRT 42 dve* ^{01738} */SM1* , to examine adult cuticle clones. *yw hsFLP; FRT 42GFP+/CyO* females were mated to *yw; FRT 42 dve* ^{01738} */SM1* males to generate imaginal disc clones. Clones were induced at 24h and 36-60h after egg laying, by shifting larvae to 37°C for 1h. Adult legs were dissected and mounted in GMM mounting media. Adult cuticle clones were photographed using bright field and Nomarski

optics on a Leica DMRE photomicroscope. Imaginal disc clones were marked by the loss of GFP. Imaginal discs were dissected and analyzed according to the protocol described above.

2.12 Misexpression of *dve*

An *omb-GAL4* chromosome (Bloomington Drosophila Stock Center) was used to drive the expression of *UAS-dve* (Fuss and Hoch, 1998). *UAS-dve* females were mated to *omb-GAL4* males and adult females were selected to examine adult legs. Female larvae were selected to examine SER expression in imaginal discs. To examine *E(spl)mβ–CD2* expression, *UAS-GFP(II)* females were crossed to *UAS-dve* males, and *omb-GAL4/ FM7* females were crossed to *dAP-2^{15} FRT 80 E(spl)mβ–CD2 /TM6B* males. *UAS-GFP(II)/+;UAS-dve/+* females were then crossed to *omb-GAL4; dAP-2^{15} FRT 80 E(spl)mβ–CD2 /+* males. Female larvae were collected and stained for *E(spl)mβ–CD2* and GFP. Flies were grown at 25°C. Adult legs were dissected and mounted in GMM mounting media. Adult cuticle clones were photographed using bright field and Nomarski optics on a Leica DMRE photomicroscope. Imaginal discs were dissected and analyzed according to the protocol described above.

Table 2: Fly stocks used

STOCK		REFERENCE
EMS mutagenesis		
red e	Mutagenized males	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu
C(1)RM, y w/ Y	Attached X	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu
w; TM3/TM6B	Third chromosome balancer females	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu
mwh (3)1215 red e/ TM3	Original dAP-2 mutant strain	Held et al., 1986
h th st dAP-2 $^{l(3)1215}$ *red e/ TM3*	Original dAP-2 mutant strain	W. Brook
Male Recombination mapping		
P{ry+}CyO Δ2-3/+; P-element/h th st dAP-2 $^{l(3)1215}$ *red e*	Source of transposase, P-element and dAP-2 mutant	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu, this study
y w, P{w^{+}*}l(3) s3246/TM3 (85A01)*	LacW P-element	Bloomington Drosophila Stock Center

		http//:flystocks.bio.indiana.edu
y w, P{w$^+$}l(3) j9A5/TM6B (85E15)	LacW P-element	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu
y w, P{w$^+$}l(3) jB6/ TM3 (86B01)	LacW P-element	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu
y w, P{w$^+$} E6-3-7/TM3 (86E)	LacW P-element	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu
y w, P{w$^+$} Y06, ry (87BC)	LacW P-element	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu
y w, P{w$^+$}l(3) j1E7/TM3 (88A04)	LacW P-element	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu
y w, P{w$^+$}l(3) j14A6/TM6B (88B01)	LacW P-element	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu
y w, P{w$^+$} L1820 (89B11)	LacW P-element	Bloomington Drosophila Stock Center

		http//:flystocks.bio.indiana.edu
y w, P{w$^+$} L4032 (89D01)	LacW P-element	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu
Molecular Marker Analysis		
fj-lacZ [P1]	fj-lacZ	This lab
odd-lacZ	odd-lacZ, PZ enhancer trap	This lab
disco-lacZ	disco-lacZ, PZ enhancer trap	Cohen Lab
dAP-2^{15} FRT80E(spl)mβ–CD2 / TM6B	E(spl)mβ-CD2 enhancer trap, dAP-2 null mutation	deCelis et al., 1998 Kerber et al., 2001
P {ry$^+$} dve^{01738}/CyO;ry	dve-lacZ PZ enhancer trap	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu
dAP-2 mosaic analysis		
dAP-2^{15} FRT80E(spl)mβ–CD2 / TM6B	FRT-dAP-2	deCelis et al., 1998 Kerber et al., 2001
y w P{ry+}hsFLP; P{w+}Ubi-GFP P{ry+}neoFRT80B/TM6B	Source of FLP, GFP	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu

dAP-2misexpression		
ptc-GAL4	GAL4 enhancer trap	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu
UAS-dAP-2	pUAST transgene	Kerber et al., 2001
UAS-GFP(I)	pUAST transgene	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu
dve mosaic analysis		
y w; P{ry+}neoFRT42D P{ry+}dve^{01738}/Sm1		Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu, this study
y w P{ry+}hsFLP; P{ry+}neoFRT42D P{y$^+$ ry$^+$}/ CyO	Source of FLP, y marker	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu
y w P{ry+}hsFLP; P{ry+}neoFRT42D P{w$^+$} Ubi-GFP/CyO	Source of FLP, GFP marker	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu
dve misexpression		
omb-GAL4/Fm7	GAL4 enhancer trap	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.

		edu
UAS-dve	pUAST transgene	Fuss, B. and Hoch, M., 1998
UAS-GFP(II)	pUAST transgene	Bloomington Drosophila Stock Center http//:flystocks.bio.indiana.edu

Table 3: Antibodies used

ANTIBODY USED	CONCENTRATION	REFERENCE
Primary Antibodies		
Mouse anti- βgalactosidase	1:500	Promega
Rabbit anti- βgalactosidase	1:1000	Cappel
Mouse anti- Rat CD2	1:50	Serotec
Mouse anti- DL	1:50	Kooh et al., 1998
Rat anti- SER	1:1000	Papayannopulos et al., 1998
Mouse anti- NUB	1:20	Averof and Cohen, 1997
Rabbit anti- dAP-2	1:200	Monge et al., 2001
Secondary antibodies + conjugates		
Goat anti- mouse Alexa488	1:5000	Molecular Probes
Goat anti- mouse Alexa594	1:5000	Molecular Probes
Goat anti- rabbit Alexa594	1:5000	Molecular Probes
Goat anti- rat Cy3	1:400	Jackson ImmunoResearch
Goat anti- rabbit FITC	1:500	Jackson ImmunoResearch
Goat anti- mouse biotin	1:500	Vector

Chapter 3: Results

3.1 dAP-2 Leg phenotype

The original *dAP-2* mutation was isolated as an EMS generated point mutation and called *l(3)1215*. This mutant has a dramatic effect on the development of the leg, which has been described in great detail in a previous study (Held et al., 1986). The legs of wild-type adult flies are divided along the proximal-distal axis into nine distinct segments. From proximal to distal, the segments are; coxa (co), trochanter (tc), femur (fe), tibia (ti), and tarsal segments ta1-ta5. The distal most structure of the leg is a claw structure. Each of the segments is unique and can be identified by its characteristic bristle pattern. Segments are separated from one another by joints, characterized by constrictions in the cuticle and formation of ball-and-socket structures. Flies homozygous for the *dAP-2* mutation die as pharate adults. That is, adult development is completed, however the flies are unable to eclose from their pupal cases and die as a result of starvation. When pharate adults are examined, it is clear that leg development is grossly affected. Legs of these mutants are significantly shorter than their wild type counterparts (Fig. 5.). It is also evident that all leg joints are missing. The only exception to this is the tibia-ta1 joint, which remains and is also often mirror image duplicated.

Figure 5. *dAP-2* leg phenotype.

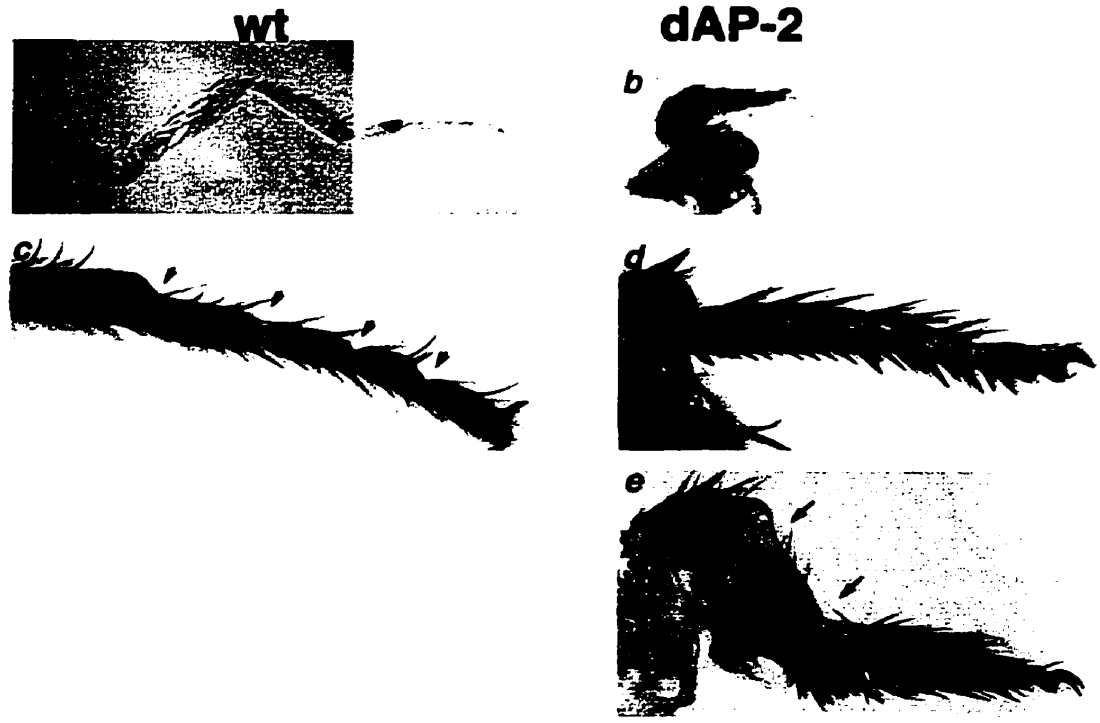
a. Wild type (*Oregon-R*) adult leg.

b. Adult leg from a *dAP-2* mutant (*dAP-2* $^{l(3)1215}$ /*dAP-2* $^{l(3)1215}$), shown at the same magnification as a. to show the difference in size.

c. Higher magnification of the tarsal segments in a wild type adult leg. Arrows point to the joints.

d. Higher magnification of the tarsal segments in a *dAP-2* mutant leg (*dAP-2* $^{l(3)1215}$ /*dAP-2* $^{l(3)1215}$), showing a lack of joints.

e. A leg from a *dAP-2* mutant (*dAP-2* $^{l(3)1215}$ /*dAP-2* ^{joi3}), showing a duplicated tibia-ta1 joint. Arrows point to the duplicated joint.

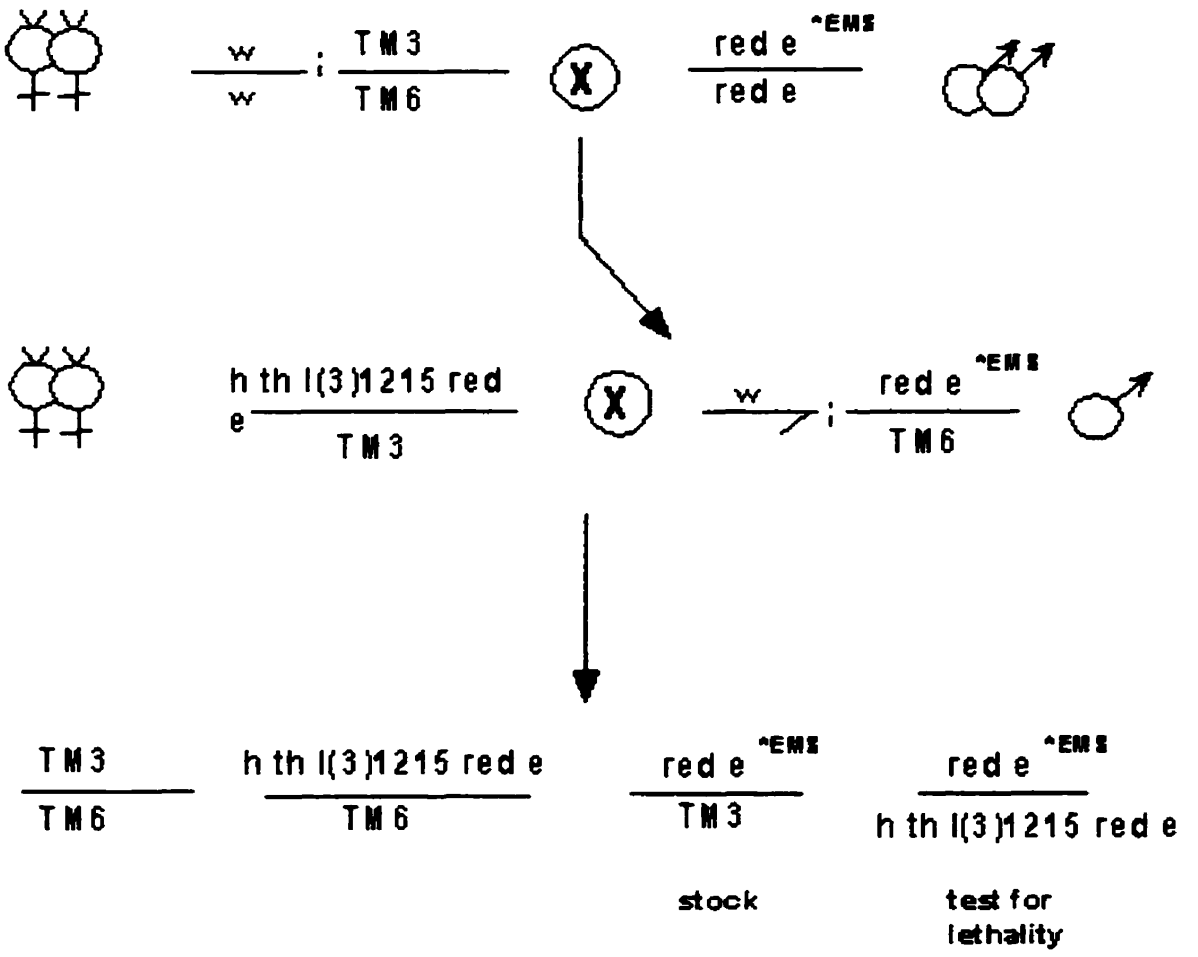

wt
dAP-2
b
c
d
e

3.2 Generating new alleles of *dAP-2*.

At the time that this work was begun, it was not yet known that *l(3)1215* was an allele of *dAP-2*. It also appeared that *l(3)1215*, a gift of Lewis Held, was the only allele available. It was not known whether this allele was a null mutation or a weak hypomorphic mutation, or even if it was definitely a loss-of-function mutation, thus making it unclear if this mutation represented all the possible functions of dAP-2. In order to identify new alleles of *dAP-2*, I performed an F2 EMS (Ethyl Methane Sulfonate) screen. EMS is a mutagen that most frequently causes G:C to A:T transitions and efficiently induces recessive loss-of-function mutations. In this screen, I treated males from an isogenic *red e* stock with EMS and crossed them to females carrying third chromosome balancers, *TM3/TM6* (Fig. 6). Male progeny from this cross of the genotype *red e/TM6*, each representing a single mutagenized chromosome, were crossed to females carrying the original *dAP-2* mutation, *l(3)1215*. I scored the progeny for lethality in flies that carry the mutagenized *red e* chromosome and the original *l(3)1215* mutation, as well as for the presence of joint and leg defects (Fig. 6.). Mutations were then recovered in sibs.

I identified nine new alleles from 5600 mutagenized chromosomes. All nine are lethal and have the *dAP-2* leg phenotype in *trans* to the original *l(3)1215* mutation. Crossing the alleles *inter se* suggests that all are approximately the same strength. All nine new alleles fail to form all the joints of the leg, except for the tibia-tarsus joint, and also show the most severe shortening, as described by Monge et al. (2001). Although,

Figure 6. Crossing scheme for EMS mutagenesis screen


w / w ; TM3 / TM6
X
red e^EMS / red e
h th l(3)1215 red e / TM3
X
w / ; red e^EMS / TM6
TM3 / TM6
h th l(3)1215 red e / TM6
red e^EMS / TM3
stock
red e^EMS / h th l(3)1215 red e
test for lethality

there is little variability of phenotypes between the various alleles, some variability does exist within each allele stock. For example, although each stock results in flies that survive until adulthood, some alleles result in a high number of flies dying as pupae. In addition, none of the novel alleles appear to be temperature-sensitive, as all show identical phenotypes when grown at 25 °C and 18 °C. The frequency with which novel *dAP-2* alleles were isolated is approximately 1 in 622 chromosomes. This is similar to the rate reported by Monge et al., (2001) where 16 *dAP-2* alleles were isolated at a rate of ~ 1 in 800 chromosomes.

The similarity of phenotypes among the various alleles of *dAP-2* generated in this and previous studies suggests that its major role is in the process of leg development. Although some death occurs during pupal development, suggesting an earlier function for dAP-2, the majority of flies, approximately 80%, survive until adulthood with leg defects present, indicating that dAP-2 acts mainly to regulate leg development.

3.3 Genetic and physical mapping of *l(3)1215*

The original *l(3)1215* mutation was mapped to meiotic position 3-50.4, however, there was reason to doubt this location. A series of overlapping deficiencies that uncover the region between polytene map 85D and 87D, uncovering most of the region between 3-50.0 and 3-51.1 were found to complement *l(3)1215*, indicating that this mutation does not fall into a region uncovered by any of the deficiencies. I used P-element mediated male recombination, with precisely mapped P-element inserts, to determine a more

precise location for this gene. Normally meiotic recombination in *Drosophila melanogaster* occurs only in females. However, recent studies have shown that in the presence of a P-element as a well as a source of transposase, recombination will occur in males (Preston et al., 1996). This process results in a crossover specifically at the site of the P-element insertion. This technique allowed me to place *l(3)1215* to either the right or left of a series of P-elements for which the physical map location is known. The P-elements I chose for this mapping were located between 85A05 and 89D0, the physical region where *l(3)1215* was thought to be located, based on the published meiotic map position of 3-50.4. I crossed females carrying the markers *ru h p Ki cu sr e ca/Tm3* to *CyO P[ry$^+$,Δ2-3]/+; P-element/h th st l(3)1215 red e* males (Fig. 7.). These males carry the *Δ2-3* transposase source and one of the P-elements in the interval of interest in trans to a *l(3)1215* chromosome carrying the flanking markers *hairy (h)* and *ebony (e)*. Recombinant offspring were collected and crossed back to *l(3)1215* mutant flies and progeny of recombinants and *l(3)1215* flies was then scored for the presence of the *l(3)1215* phenotype (Fig. 7.). In this scheme, when a particular P-insertion generates h^+ *e* recombinants bearing the *l(3)1215* marker and *h l(3)1215*$^+$ e^+ recombinants, it indicates that the P-element is located to the right of *l(3)1215*. The reciprocal marker combinations, *h l(3)1215* e^+ and h^+ *l(3)1215*$^+$ *e* indicate that the insertion is to the left of *l(3)1215*. All *h* and e^+ recombinants obtained from all of the P-elements carried the *l(3)1215* marker, whereas all h^+ and *e* recombinants did not. This result indicated that *l(3)1215* is located to the left of all P-elements used, or to the left of 85A05 (Fig. 8.).

Figure 7. Crossing scheme for P-element mediated male recombination mapping.

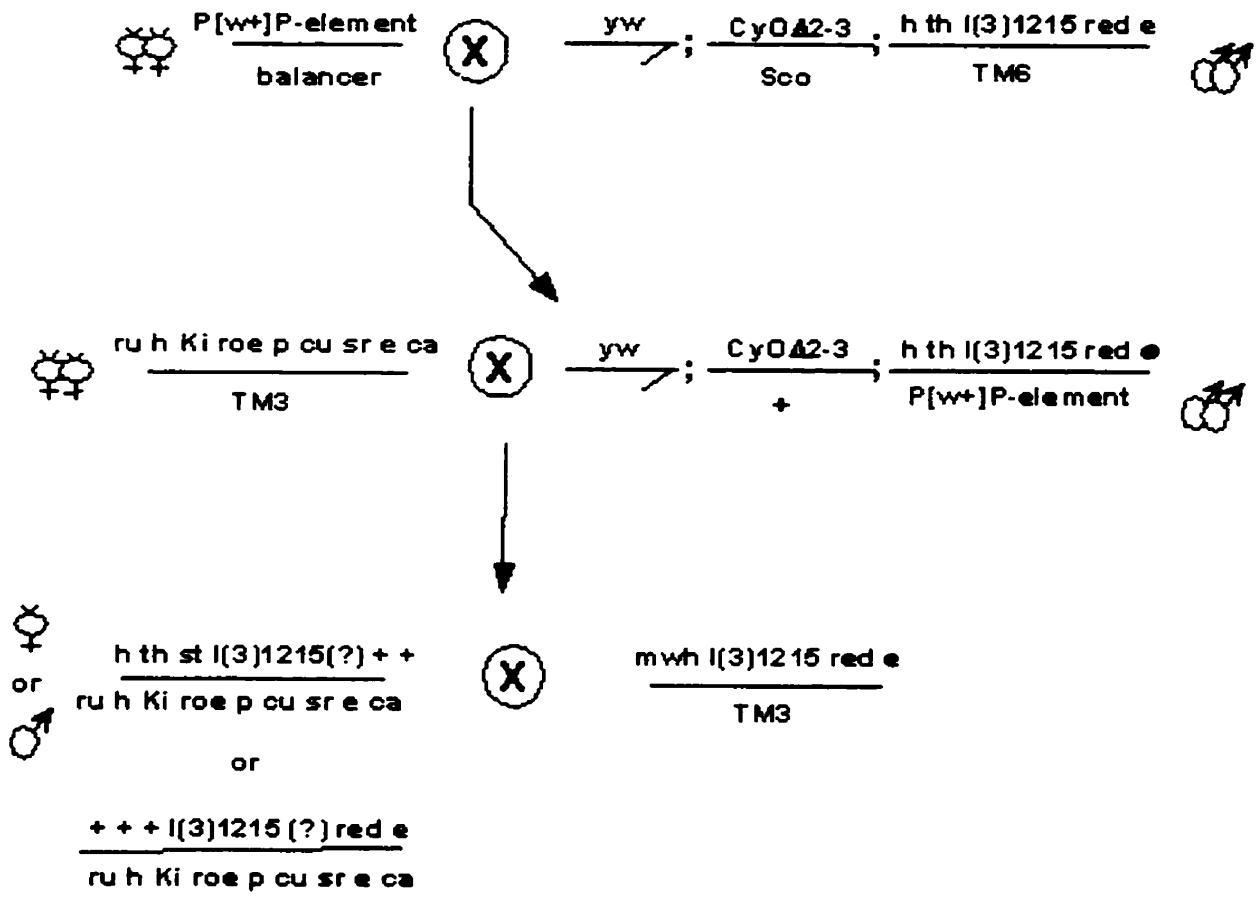

P[w+]P-element
balancer
X
yw
CyOΔ2-3
Sco
h th l(3)1215 red e
TM6
ru h Ki roe p cu sr e ca
TM3
X
yw
CyOΔ2-3
+
h th l(3)1215 red e
P[w+]P-element
or
h th st l(3)1215(?) + +
ru h Ki roe p cu sr e ca
or
+ + + l(3)1215 (?) red e
ru h Ki roe p cu sr e ca
X
mwh l(3)1215 red e
TM3

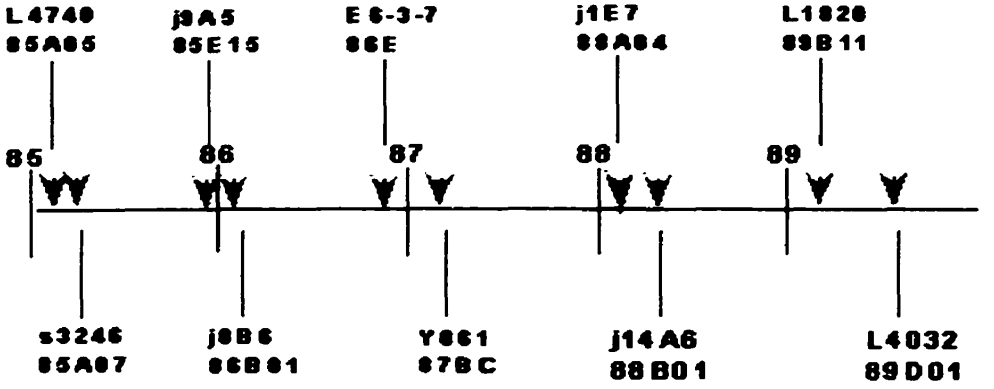
Figure 8. Diagramatic representation of P-element mediated male recombination mapping.

a. Map showing cytological location of P-elements used

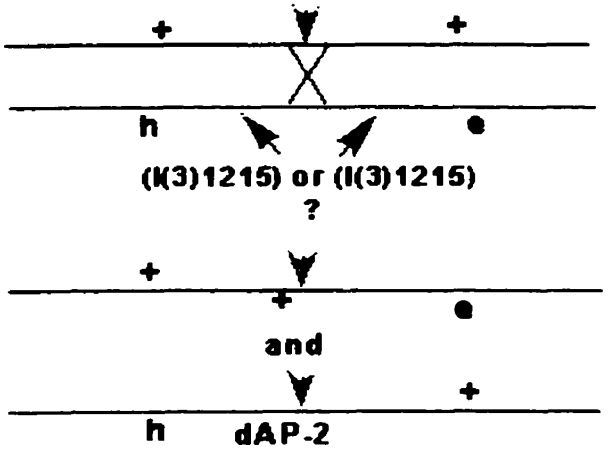
b. Crossover event at a P-element

c. Table showing recombinants and each P-element and whether each complements *l(3)1215*

a



b



+ + e and h l(3)1215 + recombinants indicate that l(3)1215 maps to the left of the P-element

c

P-element	h + recombinants (# recombinants)	+ e recombinants (# recombinants)
L4740	l(3)1215 (1)	none obtained
s3246	none obtained	none obtained
j9A5	none obtained	none obtained
j9B6	none obtained	none obtained
E6-3-7	l(3)1215 (1)	l(3)1215+ (1)
Y061	l(3)1215 (6)	none obtained
j1E7	none obtained	l(3)1215+ (1)
j14A6	l(3)1215 (7)	none obtained
L1820	l(3)1215 (8)	l(3)1215+ (6)
L4032	l(3)1215 (2)	l(3)1215+ (2)

The region to the left of 85A05 on the right arm of the third chromosome extending through the centromere into the proximal region of the left arm of chromosome three has reduced meiotic recombination. Since the published meiotic map position was clearly in error and since the reduction in meiotic crossover in the region can greatly exaggerate small errors, I crossed *l(3)1215* mutant flies to deficiencies that uncover the region between the centromere and 85A05. All deficiencies in this region complemented the *l(3)1215* mutation, indicating that it was not located in this region. In attempting to identify its location, I began to search for this mutation further, on the left arm of the third chromosome. I crossed *l(3)1215* flies to a series of deficiencies uncovering most of the left arm, the 3L deficiency kit (DK3L, Bloomington Drosophila Stock Center). One deficiency, *Df(3L)Pc-2q* failed to complement *l(3)1215*, indicating that this mutation was located in the region uncovered by this deficiency, 78C05-79A01. Of all the genes found in this region of the chromosome, *dAP-2* appeared to be the most likely candidate to be allelic to *l(3)1215* (*dAP-2*$^{l(3)1215}$). *dAP-2* was recently reported to be involved in leg development and show joint defects comparable to *dAP-2*$^{l(3)1215}$ (Monge et al., 2001). Indeed, when I obtained an allele of *dAP-2* and crossed it to flies carrying the *dAP-2*$^{l(3)1215}$ mutation, the two failed to complement each other, confirming that *dAP-2*$^{l(3)1215}$ is an allele of *dAP-2*.

The phenotype of *dAP-2*$^{l(3)1215}$ flies in trans to *Df(3L)Pc-2q* was very similar to what is observed in *dAP-2*$^{l(3)1215}$ homozygous flies, in terms of leg length and the degree to which leg joints are lost. This argues that the original *dAP-2* mutation is either a strong hypomorphic or null mutation. Although not all novel *dAP-2* alleles generated in this

study were tested in *trans* to the deficiency, all alleles were tested *inter se* and the resulting phenotypes were indistinguishable. The legs of flies from various allelic combinations all exhibited similar defects to *dAP-2*$^{l(3)1215}$*/Df(3L)Pc-2q* flies and to flies carrying null *dAP-2* mutations, as described by Monge et al., (2001). These similarities also suggest that the novel *dAP-2* alleles also represent strong hypomorphic or null mutations.

3.4 Expression of Notch target genes in *dAP-2*$^{-}$ legs

Recent studies have begun to address the role of the transcription factor dAP-2 in leg development (Kerber, 2001; Monge, 2001). *dAP-2* is expressed in a ring pattern in all the segments of the leg. Kerber et al.,(2001) report that its expression coincides with the expression domain of *E(spl)mβ-CD2*. The expression of *dAP-2* is dependent on Notch activation and it is required cell autonomously to promote joint formation. However, since no downstream targets of dAP-2 have been identified, I began to examine the expression patterns of various downstream targets of Notch signalling and known joint markers to determine whether any are affected by a *dAP-2* mutation. All expression patterns were examined in imaginal discs taken from pupae at 2-4h after puparium formation. At this stage of development, leg discs have stopped cell division and are beginning to evert. As a result the leg is extended and less folded, facilitating the examination of distinct segments.

The genes of the *Enhancer of split complex* (*E(spl)*) are direct downstream targets of Notch activation. The *E(spl)* complex member *E(spl)mβ* is an excellent read-out of Notch activation. *E(spl)mβ* expression is activated by the binding of the Su(H) transcription factor together with the intracellular domain of Notch. It has been shown to be expressed in stripes of cells positioned at the distal end of each leg segment (de Celis et al., 1998; Rauskolb et al., 1999). Although its expression depends on Notch, *E(spl)mβ* mutants do not affect joint formation. Clones of cells mutant for *E(spl)mβ* function have no effect on joint development (de Celis et al., 1998).

I examined *E(spl)mβ* expression in both wild-type and *dAP-2*⁻ pupal legs using an *E(spl)mβ-CD2* enhancer trap (de Celis et al., 1998). Its expression is clearly visible at the distal end of each segment. In *dAP-2* mutant developing legs, stripes of *E(spl)mβ-CD2* expression still remain (Fig. 9.) (Kerber et al., 2001). The stripes are closer together in *dAP-2* mutants than in wild-type legs. This can in part be explained by the large amount of cell death occurring in *dAP-2* mutant legs (Kerber et al., 2001). The fact that *E(spl)mβ-CD2* expression is still expressed in distinct rings in an *dAP-2* mutant indicates that the Notch receptor is still activated, confirming that dAP-2 functions downstream of Notch and either downstream of *E(spl)mβ* or in a parallel pathway

Another joint marker whose expression I examined was *disconnected* (*disco- -lacZ*). This gene encodes a transcription factor required for axonal migration in the visual system (Heilig et al., 1991). However, its expression pattern has been shown to be closely associated with all developing joints and has been shown to be dependent on Notch

Figure 9. Expression patterns of *E(spl)m*β-*CD2*, *disco-lacZ* and NUB in wt and *dAP-2* legs.

a. Discs from *dAP-2*^{15} *FRT 80 E(spl)m*β-*CD2 /+* and *dAP-2*^{15} *FRT 80 E(spl)m*β-*CD2 /dAP-2* $^{l(3)1215}$ pupae (2-4h after puparium formation) stained with a mouse α rat CD2 antibody and goat α mouse AlexaFluor 488 secondary conjugate. *E(spl)m*β-*CD2*expression is seen in rings of cells proximal to the constriction in wt tarsal segments.

b. In *dAP-2*$^{-}$ legs, distinct rings of *E(spl)m*β-*CD2* expression remain visible.

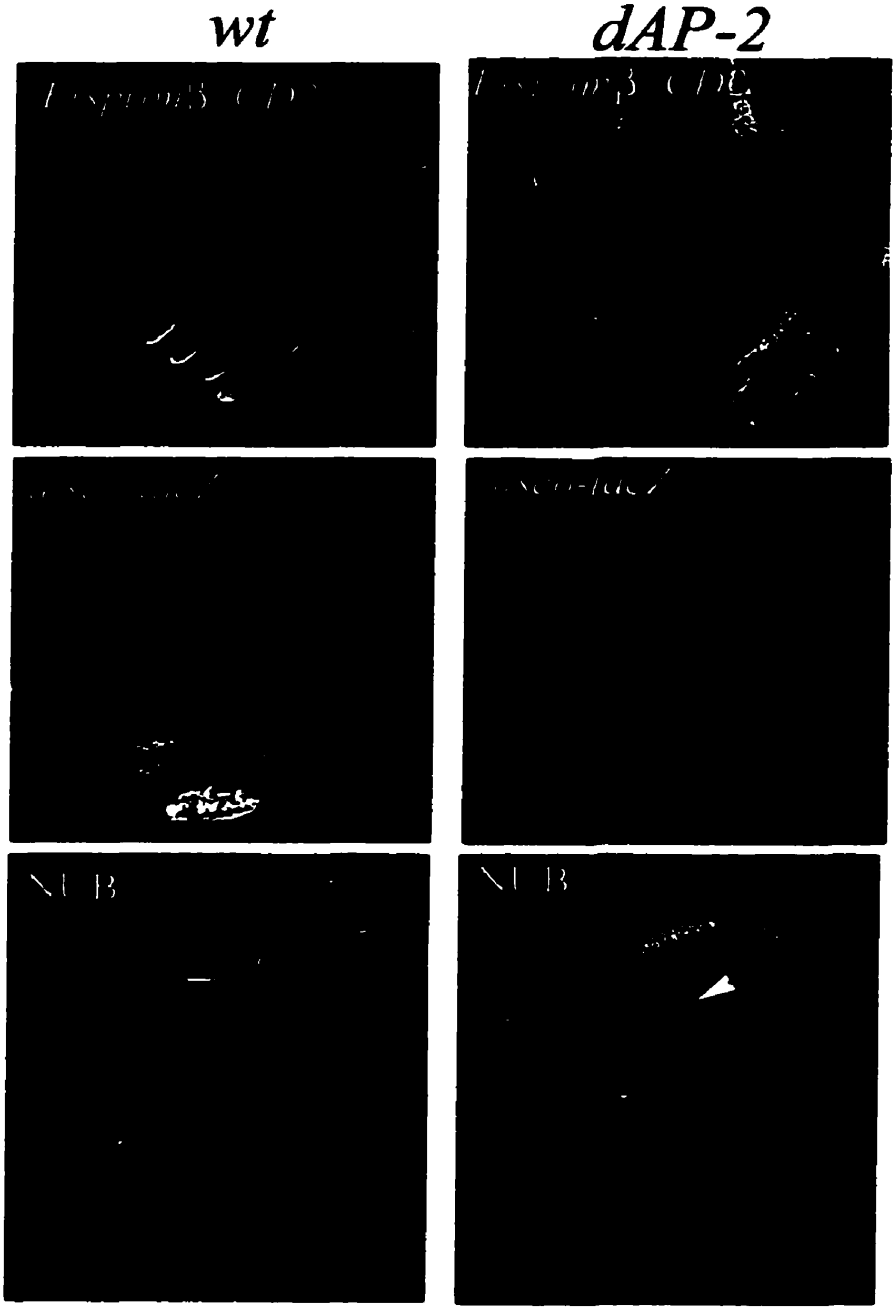
c. Discs from *disco-lacZ* and *disco-lacZ*; *dAP-2*^{15}*FRT 80 E(spl)m*β-*CD2 /dAP-2* $^{l(3)121}$pupae (2-4h after puparium formation) stained with a rabbit α βGal antibody and with a goat α rabbit AlexaFluor 594 secondary conjugate.

d. Wt *disco-lacZ* expression is seen in all tarsal segments. In *dAP-2*$^{-}$ legs, distinct rings of *disco-lacZ* expression are visible.

e. Discs from *Oregon-R* and *dAP-2* $^{l(3)1215}$ */dAP-2* $^{l(3)1215}$ pupae (2-4h after puparium formation) stained with a mouse α NUB antibody and a goat α mouse AlexaFluor

488 secondary conjugate. In wt legs NUB expression is seen in the femur-tibia, tibia-ta1 and ts5-pretarsus joints.

f. In *dAP-2* mutant legs NUB expression remains unchanged. Ectopic NUB is detected in duplicated tibia-ta1 joint. (Arrowhead points to ectopic NUB).


wt
dAP-2

signalling (Bishop et al., 1999). Similarly to *E(spl)mβ-CD2* expression, *disco-lacZ* expression also remains in distinct rings in *dAP-2* mutant legs (Fig. 9).

A second downstream target of Notch signalling is the gene *nubbin*, which encodes a POU-domain protein (Ng et al., 1995). *nub* is expressed at the boundaries of the proximal leg segments as well as at the ta1 and ta5 joints of the tarsus. *nubbin* expression has been shown to correspond to the domain of Notch activation (Rauskolb and Irvine, 1999). It was also shown to be positively regulated by Notch. In *dAP-2* mutant legs, the rings of *nub* expression remain unaltered, indicating that Notch activation is not prevented by the *dAP-2* mutation (Fig. 9). It should be noted however, that in several leg discs examined an ectopic spot of *nub* was found, near the existing tibia-ta1 ring of expression (Fig. 9). This observation is consistent with the phenotype of *dAP-2* mutants. Legs of *dAP-2* mutants often exhibit a duplicated tibia-ta1 joint.

The Notch target gene, *four jointed (fj),* encoding a type 2 transmembrane protein, is also expressed in stripes near segment boundaries (Villano and Katz, 1995). However, unlike *nub*, *four-jointed* expression is negatively regulated by Notch signalling. It is therefore found in a domain of cells proximal to the domain, which expresses *E(spl)mβ-CD2* and *nub* (Rauskolb and Irvine, 1999). In wild type pupal leg disc, I was able to detect two stripes of *fj-lacZ* expression, using a *fj[P1]-lacZ* enhancer trap (Brodsky and Steller, 1996). These two stripes correspond to the tibia-femur and tibia-ta1 joints (Fig.. 10). Previous reports indicate *fj* expression in the tarsal segments, however I was unable to detect this pattern using the *fj[P1]* enhancer trap line. As a result, it was not possible to

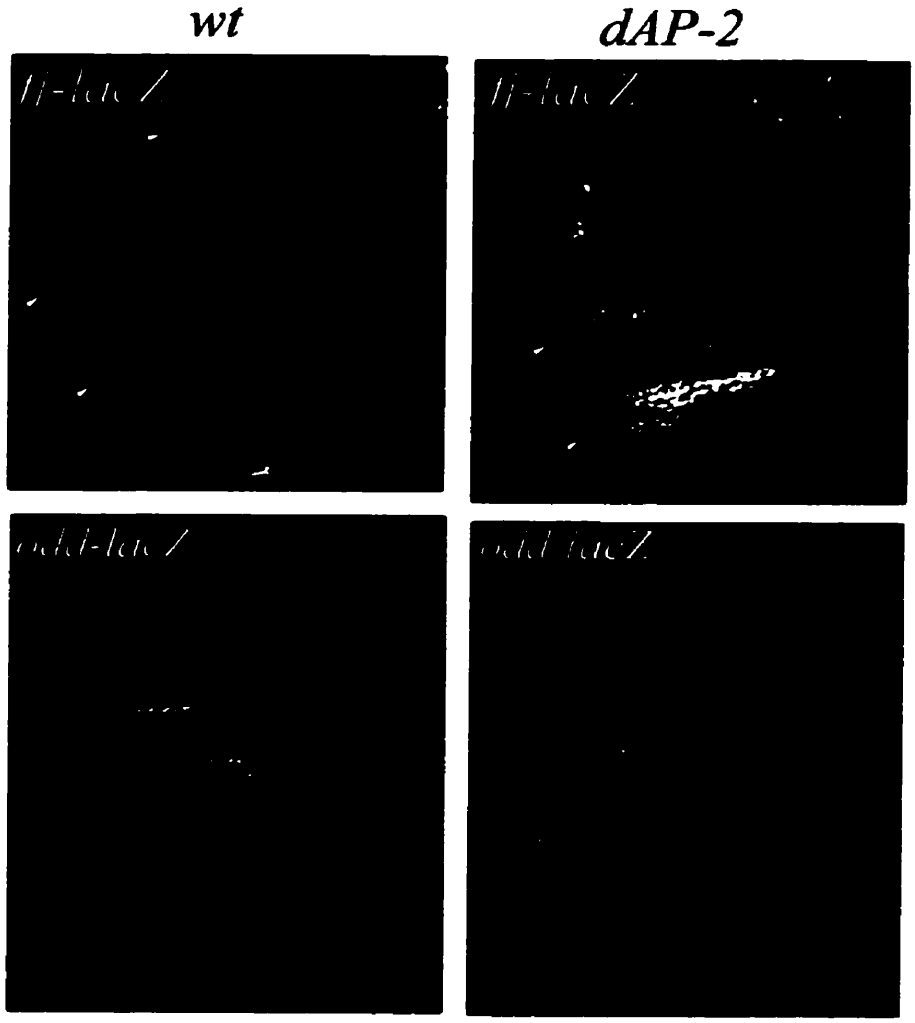
Figure 10. Expression patterns of *fj-lacZ* and *odd-lacZ* in wt and *dAP-2* leg discs.

a. Discs from *fj-lacZ* and *fj-lacZ*/+; *dAP-2*^{15}*FRT 80 E(spl)m*β*-CD2 /dAP-2* $^{l(3)1215}$ pupae (2-4h after puparium formation) stained with a rabbit α βGal antibody and with a goat α rabbit AlexaFluor 594 secondary conjugate. In wt discs, *fj-lacZ* is detected in the trochanter-femur, femur-tibia joint and the tibia-ta1 joints. Arrow points to these rings of expression.

b. In *dAP-2* legs, only the femur-tibia ring of expression remains. Arrow points to the trochanter-femur and tibia-ta2 rings.

c. Discs from *odd-lacZ* and *odd-lacZ*/+; *dAP-2*^{15} *FRT 80 E(spl)m*β*-CD2 /dAP-2* $^{l(3)1215}$ larvae (third instar) stained with a rabbit α βGal antibody and with a goat α rabbit AlexaFluor 594 secondary conjugate. In wt discs, *odd-lacZ* expression is detected in rings.

d. In *dAP-2* discs, the expression pattern remains unaltered, however it is weaker.


wt
dAP-2

determine whether *fj* expression in the tarsal segments of *dAP-2* mutants is altered. However, I was able to observe that in mutant leg discs, the tibia-femur stripe is lost, while the basitarsus joint remains unchanged (Fig. 10). This result corresponds well to the adult phenotype of *dAP-2* mutants. Legs of these adults fail to form the femur joint properly, while the formation of the endogenous tibia joint is unaffected. These results indicate that dAP-2, acting downstream of Notch activation, in part controls the expression of genes known to be expressed during joint formation.

Another target of Notch signalling whose expression is altered in *dAP-2* mutant leg discs, is *odd-skipped* (Rauskolb and Irvine, 1999). This gene is expressed in the proximal segments of the leg, as well as the fifth tarsal segment. However its role in leg development is not understood. The expression of *odd* is also under positive control from Notch activation. In *dAP-2* mutant leg discs the pattern of *odd-lacZ* expression remains unchanged, however its expression is weaker than in wild type legs (Fig. 10).

Loss of dAP-2 activity appears to not have a dramatic effect on genes positively regulated by Notch. The expression of *E(spl)mβ-CD2, disco-lacZ* and *nub* remains unaltered in *dAP-2* mutants, while the expression of *odd-lacZ* is downregulated, but spatially correct. The expression of *fj-lacZ*, a gene negatively regulated by Notch, is lost in the femur-tibia ring, in *dAP-2* mutants. These results are consistent with dAP-2 acting downstream of Notch.

3.5 Expression of Notch ligands in *dAP-2*⁻ legs

It has been clearly demonstrated that dAP-2 acts downstream of Notch activation and regulates the expression of joint markers (Kerber et al.,2001). However, I wanted to confirm that the *dAP-2* mutation has no effect on the expression of genes that function upstream of Notch, namely the ligands DL and SER. *Dl* and *Ser* have previously been shown to be expressed at every joint of the leg, in a domain that lies just proximal to the *E(spl)mβ-CD2* domain of expression (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine 1999). Although *Ser* and *Dl* expression overlap, lower levels of *Dl* expression extend throughout the leg, in regions where SER is not found (Bishop et al., 1999). *Dl* expression is still present in segmental rings in *dAP-2* mutants, consistent with *dAP-2* being a downstream target of Notch signalling (Fig. 11). Surprisingly however, *dAP-2* mutants expressed *Ser* ectopically throughout the medial tarsus (Fig. 11). Individual stripes of *Ser* expression were visible in the proximal leg segments, up to the basitarsus (ta1). In the medial tarsal segments, ta2-ta4, *Ser* expression was present as a continuous block in all *dAP-2*⁻ legs examined. A defined stripe of *Ser* expression was again detected in the distal-most tarsal segment ta5.

The expression of *Ser* in *dAP-2*⁻ legs could be explained as either *Ser* becoming ectopically expressed throughout the medial tarsus, or individual stripes of *Ser* expression becoming fused as a result of cell death occurring in this region. To distinguish between these two possibilities, I examined the SER expression together with *E(spl)mβ-CD2* expression in *dAP-2* mutant legs (Fig. 12). In wild type legs, *Ser* and

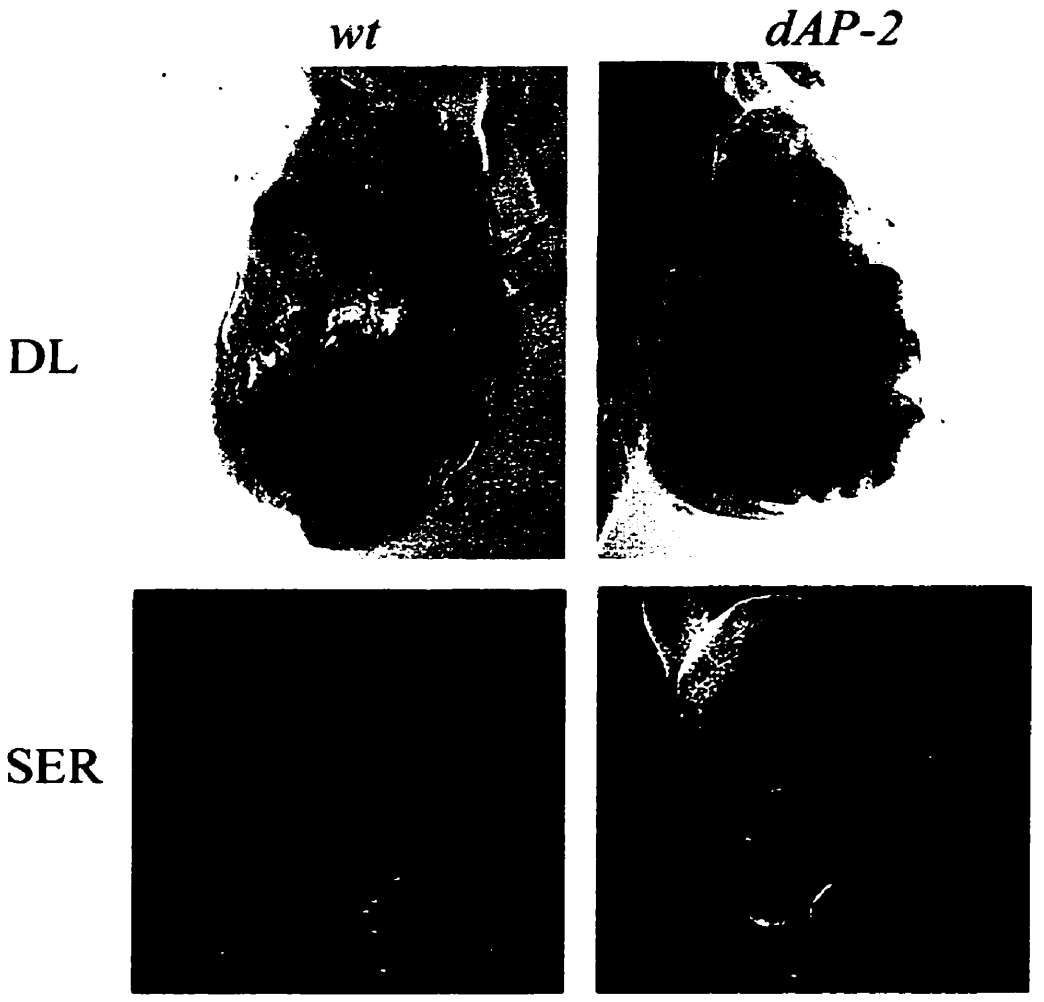
Figure 11. Expression patterns of *Dl* and *Ser* in wt and *dAP-2* leg discs.

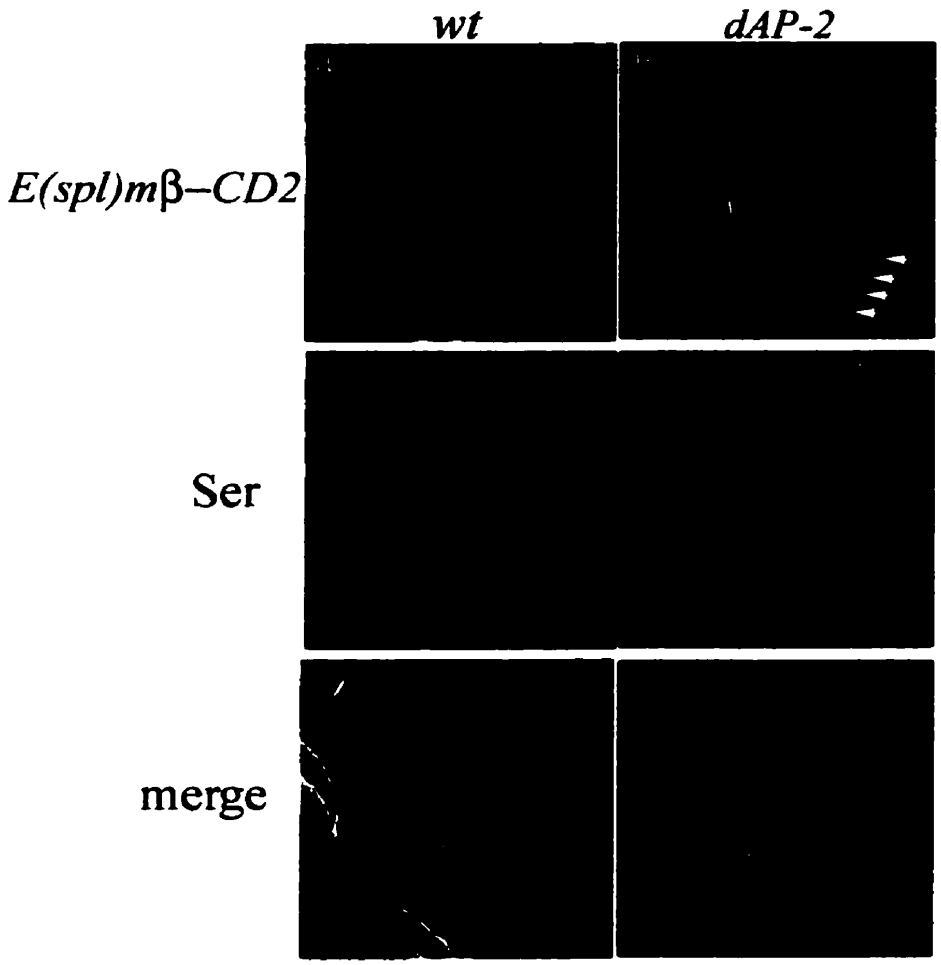
a. Discs from *OR* and *dAP-2* $^{l(3)1215}$ */dAP-2* $^{l(3)1215}$ pupae (2-4h after puparium formation) stained with a mouse α DL antibody and detected histochemically using DAB. In wt legs DL expression is seen in all segments of the leg, in cells distal to the constriction in all tarsal segments.

b. In dAP-2 legs, all rings of DL expression remain visible.

c. Discs from OR and *dAP-2*^{15}*FRT 80 E(spl)m*β*–CD2 /dAP-2* $^{l(3)1215}$ pupae (2-4h after puparium formation) stained with a rat α SER antibody and a goat α rat Cy3 secondary conjugate. In wt legs SER expression is seen in all segments of the leg, in cells distal to the constriction in all tarsal segments. Arrows point to rings of expression.

d. In *dAP-2* legs SER expression remains as in wt legs in proximal leg segments, coxa-ta1. Throughout segments ta2-ta4 SER expression is seen as a uniform region of expression. A distinct ring of SER expression remains in segment ta5. Arrows point to the ta1 and ta5 rings of expression.


wt
dAP-2
DL
SER


wt
dAP-2
E(spl)mβ–CD2
Ser
merge

E(spl)mβ-CD2 are found in adjacent but not overlapping domains. Stripes of *E(spl)mβ-CD2* expression can be detected in the tarsus of *dAP-2* mutant legs, suggesting that domains other than the normal *Ser* domain of expression still remain in *dAP-2* mutants. Indeed, stripes of *E(spl)mβ-CD2* expression are found overlapping with SER indicating that *Ser* becomes ectopically expressed in the *E(spl)mβ-CD2* region. This result suggested that dAP-2 is not only an effector of Notch signalling, but also regulates the expression of one of the Notch ligands.

3.6 Expression of *dve-lacZ* in *dAP-2*⁻ legs

An additional joint marker that was examined is the gene *defective proventriculus* (*dve*), which has not been previously shown to have a role in leg development (Fuss and Hoch, 1998; Nakagoshi et al., 1998). I analyzed the expression of this gene using a series of lacZ enhancer trap lines. This gene is also expressed in a series of rings in segments of the developing leg (Fig. 13). The expression of *dve-lacZ* is partially dependent on dAP-2. In *dAP-2* mutant pupal legs, *dve-lacZ* expression is downregulated throughout the medial tarsal segments. The stripes of *dve-lacZ* located in the first and fifth tarsal segments remain unchanged. However, the stripes located in ta2-ta4 are much weaker or missing (Fig. 13). These results are variable with approximately 75% of the leg discs examined showing a loss of dve and 25% showing weaker expression throughout the tarsal segments ta2-ta4. I also tested whether dAP-2 was sufficient to induce *dve-lacZ* expression. This was performed using the *GAL4/UAS* system. When ectopic *UAS-dAP-2*

Figure 13. Expression pattern of *dve-lacZ* in wt and *dAP-2* legs.

a. Discs from *dve*^{01738} *lac Z* /+ pupae (2-4h after puparium formation) stained with a rabbit α βGal antibody and with a goat α rabbit AlexaFluor 594 secondary conjugate. In the tarsal segments expression is visible just proximal to the constriction and through out the constriction.

b. Discs from *dve*^{01738} *lac Z* /+; *dAP-2* $^{l(3)1215}$ /*dAP-2* $^{l(3)1215}$ pupae (2-4h after puparium formation) stained with a rabbit α βGal antibody and with a goat α rabbit AlexaFluor 594 secondary conjugate. *dve-lacZ* expression remains in segment ta1. Its expression is not detected in tarsal segments ta2-ta4 and a distinct ring of *dve-lacZ* expression is seen in segment ta5.

c. Discs from *dve*^{01738} *lac Z* /+; *dAP-2*^{15} *FRT 80 E(spl)m*β*–CD2* /*dAP-2* $^{l(3)1215}$ pupae (2-4h after puparium formation) stained with a rabbit α βGal antibody and with a goat α rabbit AlexaFluor 594 secondary conjugate. *dve-lacZ* expression is seen in all tarsal segments, however it is weaker.

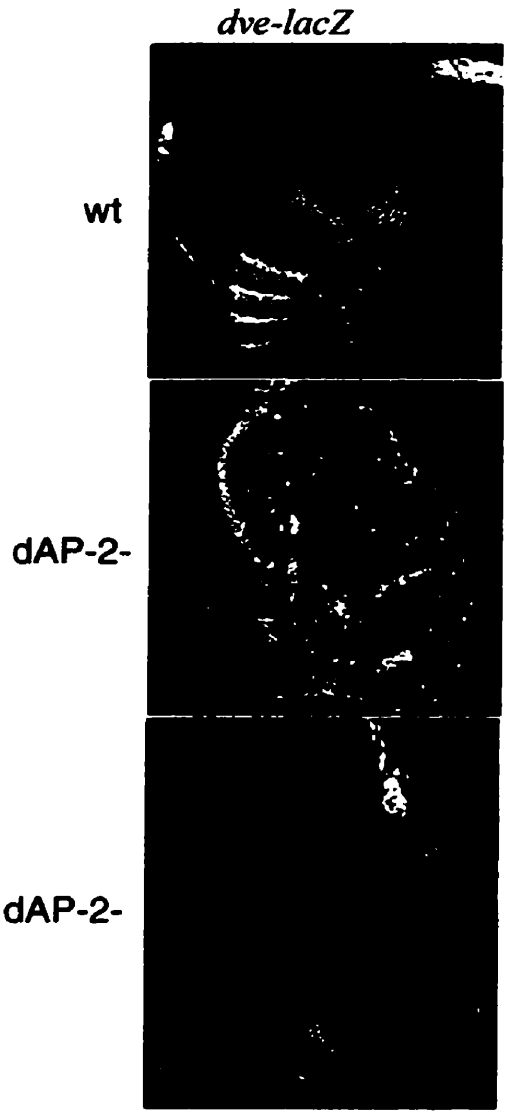
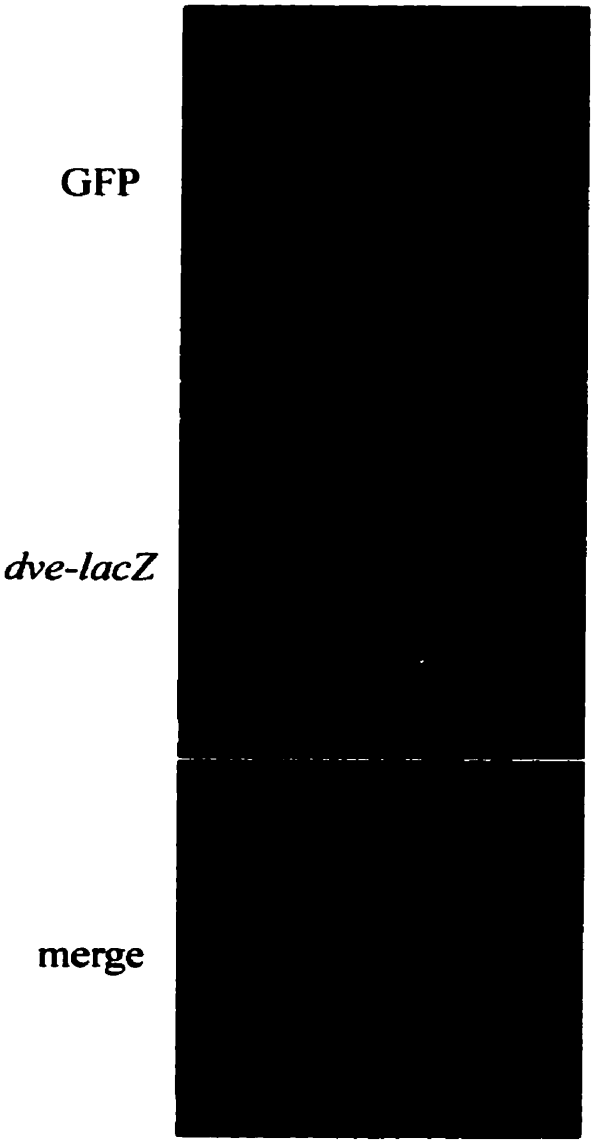

dve-lacZ
wt
dAP-2-
dAP-2-

Figure 14. The expression of *dve-lacZ* in the presence of ectopic dAP-2.

Leg disc from a *UAS-GFP/+; ptc-GAL4/dve-lacZ; UAS- dAP-2/+* pupa (2-4h after puparium formation) stained with rabbit α βGal and goat α rabbit AlexaFluor 594 secondary conjugate. The *ptc* domain, where *dAP-2* is ectopically expressed is visualized with GFP. No change in *dve-lacZ* expression is seen in the presence of ectopic dAP-2.


GFP
dve-lacZ
merge

was expressed perpendicular to its normal domain, using the *ptc-GAL4* driver, no ectopic *dve-lacZ* expression was detected (Fig. 14). Such results suggest that dAP-2 may have some role in regulating *dve-lacZ* expression, however *dve* is not a direct target of dAP-2.

3.7 Gene Expression Domains in Wild-type Legs

Since the expression pattern of *dve* in developing legs has not been analyzed, I examined its expression in comparison to the expression domains of *E(spl)mβ-CD2* , *Ser* as well as dAP-2. In wild-type pupal legs, *dve-lacZ* is expressed at the distal end of each leg segment (Fig. 15). In the tarsus, the proximal edge of the *dve-lacZ* expression domain overlaps slightly, approximately four cell lengths, with the distal edge of *E(spl)mβ-CD2* expression. *dve-lacZ* continues to be weakly expressed further distally in the constriction visible between each segment (Fig. 15). The most distal edge of *dve-lacZ* expression borders on the proximal edge of *Ser* expression, such that the two are found in non-overlapping domains (Fig. 15). The expression of *dAP-2* corresponds to the domain of *dve-lacZ* expression (Fig. 15). This is different than the pattern described by Kerber et al., (2001) who report that *dAP-2* expression overlaps exactly with *E(spl)mβ-CD2* expression. To test this, I examined the expression of *dAP-2* and *E(spl)mβ-CD2* in pupal leg discs (Fig. 16). *dAP-2* expression like *dve-lacZ*, was not found exactly in the

Figure 15. Expression pattern of *dve-lacZ* together with *E(spl)mβ-CD2*, SER and dAP-2 in wt leg discs.

a. Discs from *dve^{01738} -lac Z /+; dAP-2^{15}FRT 80 E(spl)mβ–CD2 /+* pupae (2-4h after puparium formation) stained with a rabbit α βGal antibody and with a goat α rabbit AlexaFluor 594 secondary conjugate and a mouse α rat CD2 antibody and with a goat α mouse AlexaFluor 488 secondary conjugate. *dve-lacZ* expression is seen partially overlapping with *E(spl)mβ-CD2* expression and extending distally into the constriction.

b. Higher magnification of a disc from *dve^{01738} -lac Z /+; dAP-2^{15} FRT 80 E(spl)mβ–CD2 /+* pupae (2-4h after puparium formation) stained with a rabbit α βGal antibody and with a goat α rabbit AlexaFluor 594 secondary conjugate and a mouse α rat CD2 antibody and with a goat α mouse AlexaFluor 488 secondary conjugate. *dve-lacZ* expression overlaps with *E(spl)mβ-CD2* expression over 4 cell diameters. Arrows point to the regions of overlap.

c. Discs from *dve^{01738}-lac Z /+; dAP-2 $^{l(3)1215}$ /+* pupae (2-4h after puparium formation) stained with a rabbit α βGal antibody and with a goat α rabbit FITC secondary conjugate and a rat α SER antibody and a goat α rat Cy3 secondary conjugate. *dve-lacZ* expression is seen in non-overlapping domains with *Ser* expression.

d. Discs from *dve*$^{01738\text{-}}$ *lac Z /+; dAP-2* $^{l(3)1215}$ /+ pupae (2-4h after puparium formation) stained with mouse α βGal antibody and with a goat α mouse AlexaFluor 488 secondary conjugate and a rabbit α dAP-2 antibody and a goat α rabbit AlexaFluor 594 secondary conjugate. *dve-lacZ* expression overlaps with dAP-2 expression throughout the tarsal segments.

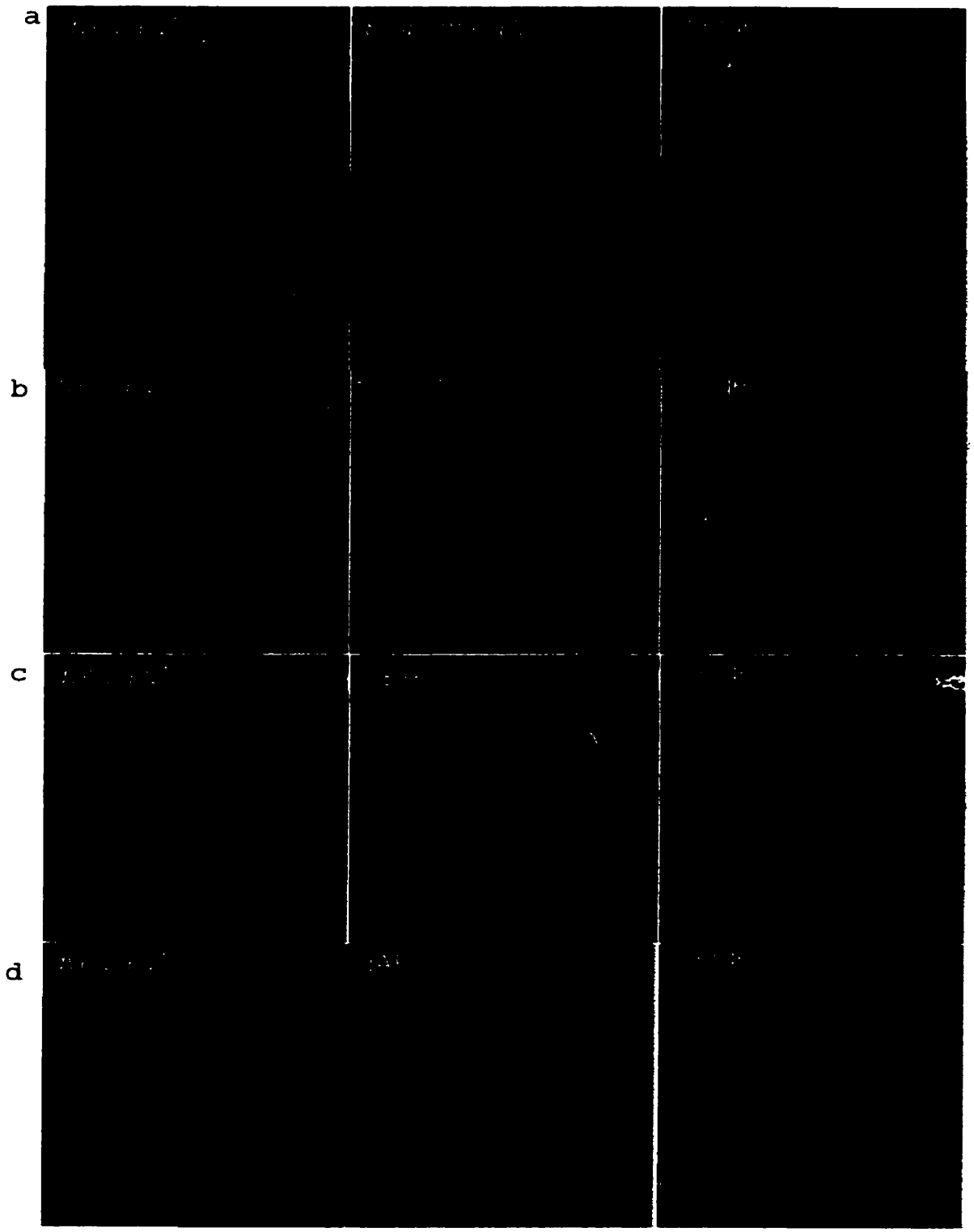
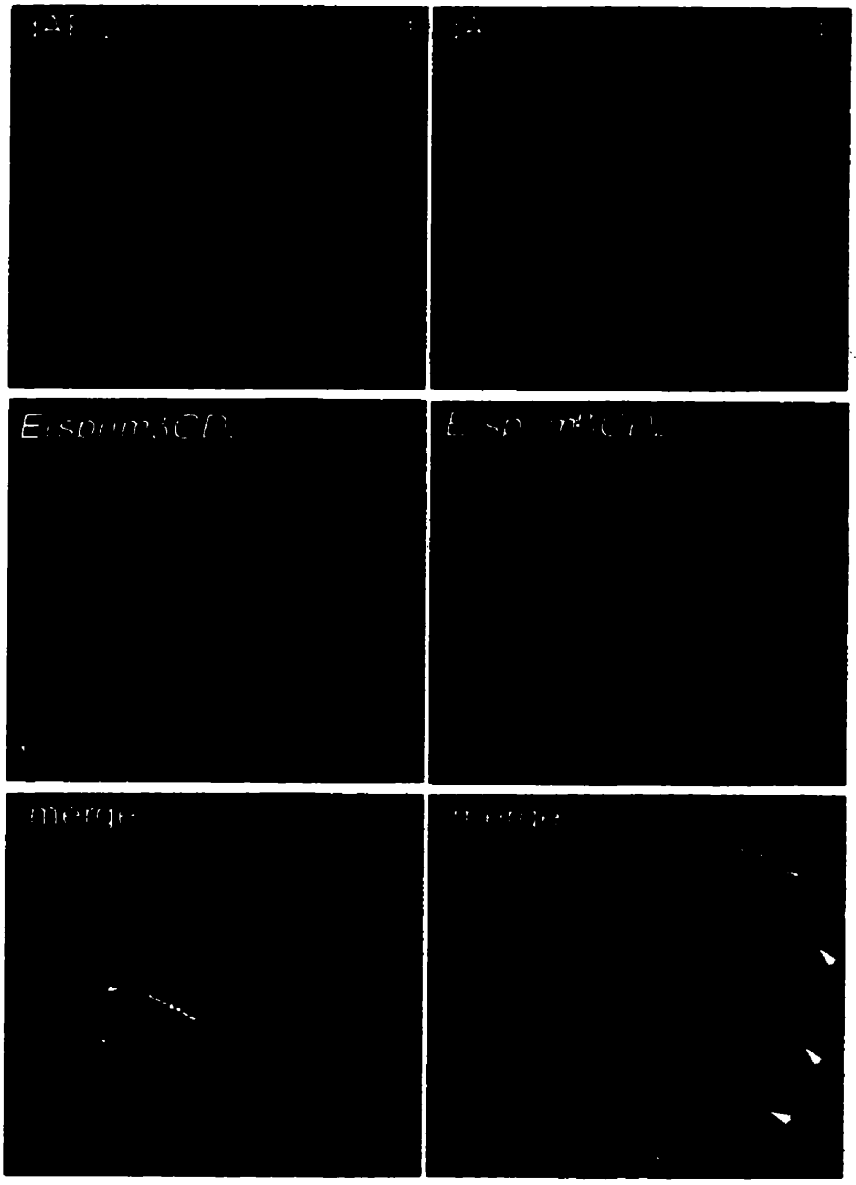

a
b
c
d

Figure 16. Expression pattern of dAP-2 together with *E(spl)mβ–CD2* in wt leg discs.

a. Disc from *dAP-2^{15} FRT 80 E(spl)mβCD2* /+ pupa (2-4h after puparium formation) stained with a mouse α rat CD2 antibody and goat α mouse AlexaFluor 488 secondary conjugate and with a rabbit α dAP-2 antibody and a goat α rabbit AlexaFluor 594 secondary conjugate. dAP-2 expression partially overlaps with *E(spl)mβ–CD2* expression in the tarsal segments.

b. Higher magnification of the disc in a. *E(spl)mβ–CD2* expression alone is seen for approximately 3 cell diameters. *E(spl)mβ–CD2* and dAP-2 expression overlaps for approximately 4-5 cell diameters. Weaker dAP-2 expression continues distally in the constriction for approximately 3 cell diameters. Arrowheads point to regions of overlap.



E(spl)mβ-CD2 domain. Rather, its expression began three cells from the proximal edge of the *E(spl)mβ-CD2* domain. Weaker *dAP-2* expression also continued beyond the distal edge of the *E(spl)mβ-CD2* domain, several cell lengths towards the *Ser* domain (Fig. 16).

From this study, several distinct domains can be identified along the proximal-distal axis of the tarsus. First, starting at the distal edge the constriction of the ta1 segment, there is a domain of *Ser* expression that continues until the middle of the ridge of the ta2 segment. Starting at the distal border of *Ser* expression, *E(spl)mβ-CD2* becomes expressed and continues until the proximal edge of the next constriction. Next, starting approximately three cell lengths from the proximal edge of *E(spl)mβ-CD2* domain, *dAP-2* and *dve-lacZ* expression can be detected. Strong *dAP-2* and strong *dve-lacZ* expression is found overlapping with the domain of *E(spl)mβ-CD2* expression, approximately four cell lengths. The domain that includes *E(spl)mβ-CD2, dAP-2* and *dve-lacZ* expression is followed by a domain in which only *dAP-2* and *dve-lacZ* is found. This domain stretches across the entire length of the constriction and borders on the proximal edge of the next domain of *Ser* expression. Such domains of expression generate a repeating pattern in tarsal segments ta2-ta4. It should be noted that the distal edge of *dAP-2* expression may not correspond exactly to the distal domain of *dve-lacZ* expression. However, this was difficult to determine due to high background from the *dve-lacZ* enhancer trap.

Examination of molecular joint markers in mutant legs yielded several potential targets for regulation by dAP-2, such as *fj, odd, dve* and *Ser*. However, for the remainder of this study I chose to focus on two of the potential targets, *dve* and *Ser*. These two markers present most intriguing results as dAP-2 exerts its effect on both markers in the same region, the medial tarsus ta2-ta4. In addition, *dAP-2* affects the expression of *dve-lacZ* and *Ser* in opposite ways, as *dve-lacZ* expression is downregulated in *dAP-2* mutants and *Ser* is ectopically expressed in *dAP-2* mutants. Since these two genes represent the clearest targets of dAP-2, I wished to further understand the interplay between these genes and their contributions to the process of joint formation.

3.8 Regulation of *Ser* by dAP-2

The ectopic expression pattern of *SER* in *dAP-2* mutant developing legs suggests that dAP-2 represses *Ser*. I used genetic mosaic analysis to further examine this as well as the autonomy with which *dAP-2* affects *Ser* expression. This was performed using the *FLP/FRT* system, which induces mitotic recombination and generates clones of *dAP-2*$^-$ cells. In this experiment Green Fluorescent Protein (GFP) was used as a cell marker. Heterozygous cells expressed one copy of *GFP*, homozygous twin spots expressed two copies of *GFP*, while *dAP-2*$^-$ clones were marked by a loss of *GFP* (Fig. 17.). Clones were induced at 36-60 h of development, during imaginal disc growth and prior to the process of segmentation and joint formation. Clones of cells mutant for *dAP-2* that extend outside the normal *Ser* expression domain, express *Ser* in a cell autonomous

Figure 17. Schematic representation of generating somatic clones using the FLP/FRT system.

Cells heterozygous for the GFP marker and the dAP-2 mutation will undergo mitotic recombination at FRT sites, in the presence of the FLP recombinase. This will result in cells homozygous for dAP-2 (clones) that do not express GFP, and cells lacking dAP-2 but expressing two copies of GFP (twins).

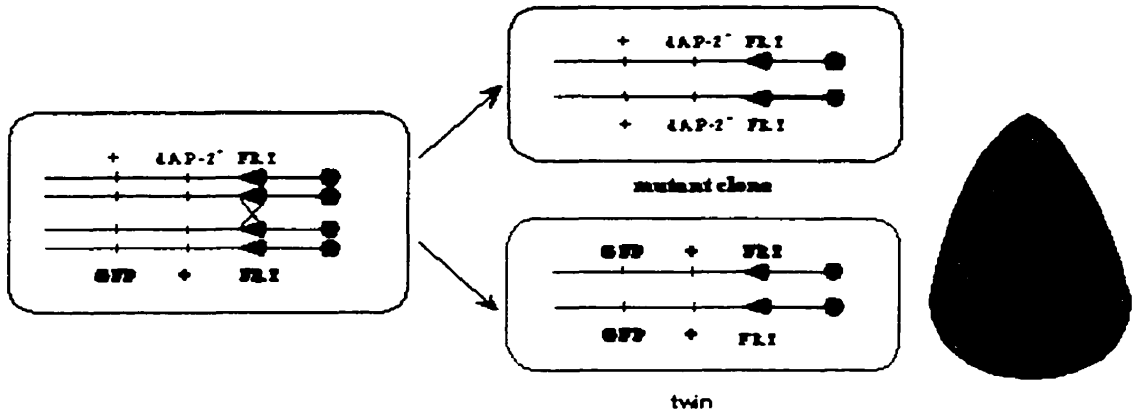
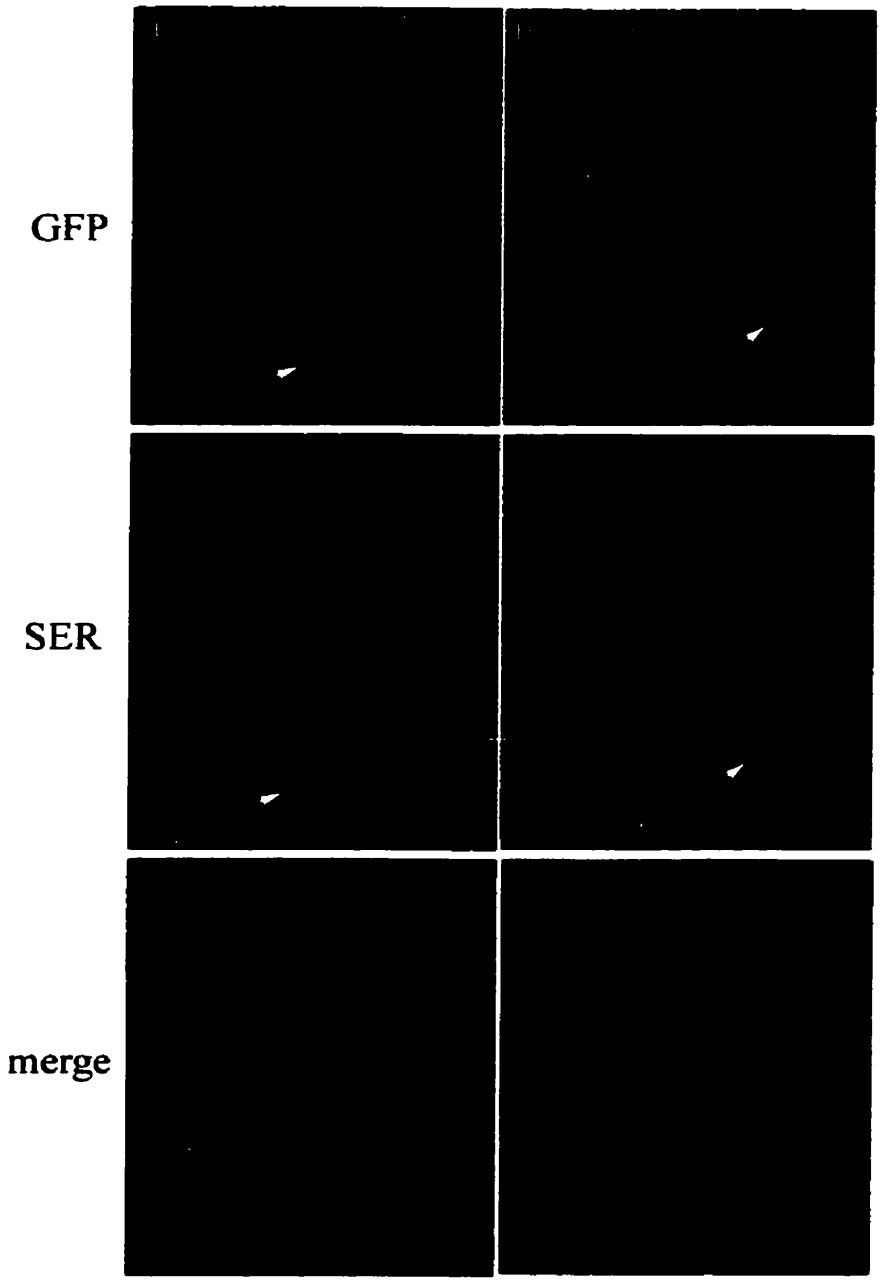

+ dAP-2⁻ FLI
GFP + FLI
+ dAP-2⁻ FLI
+ dAP-2⁻ FLI
mutant clone
GFP + FLI
GFP + FLI
twin

Figure 18. SER expression in *dAP-2*$^{-}$ leg clones

a. Leg disc from a *yw hs FLP; dAP-2*^{15} *FRT 80 E(spl)m*β*–CD2 /FRT 80 GFP* pupa (2-4h after puparium formation) stained with a rat α SER antibody and a goat α rat Cy3 secondary conjugate. Clones in the tarsal segments ta3-ta4 and ta1-ta3 (marked by a loss of GFP expression) show autonomous ectopic SER expression. Arrow points to clones in the tarsal segments. A clone in the femur-trochanter joint also shows ectopic SER expression. Arrowhead points to femur-trochanter joint clone.

b. Leg disc from *a yw hs FLP; dAP-2*^{15}*FRT 80 E(spl)m*β*–CD2 /FRT 80 GFP* pupa (2-4h after puparium formation) stained with a rat α SER antibody and α goat a rat Cy3 secondary conjugate. Clone in the tarsal segments ta2-ta4 (marked by a loss of GFP expression) shows ectopic SER expression. Arrow points to the clone.


GFP
SER
merge

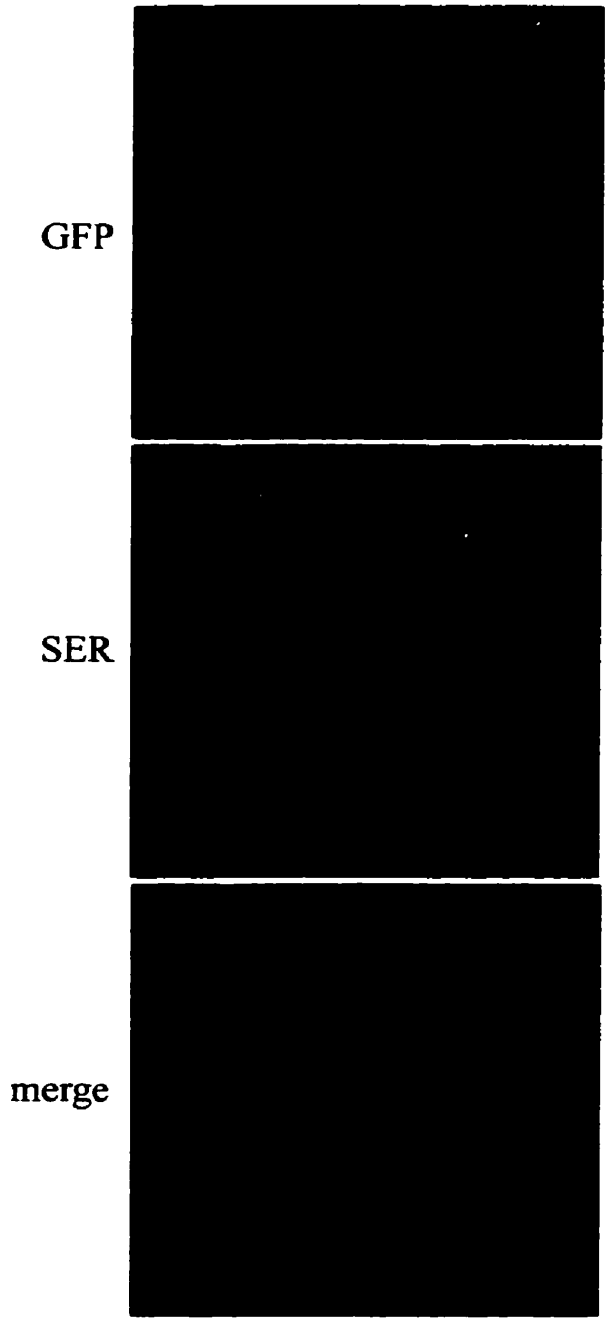
manner (Fig. 18). Large clones that span several segments express *Ser* uniformly throughout the entire segment and throughout the clone. This is true not only for clones located in the medial tarsal segments, but also in the more proximal segments of the leg. This result indicates that dAP-2 functions to prevent *Ser* expression in cells where dAP-2 is found. To test whether dAP-2 is sufficient to block SER expression, I expressed *dAP-2* ectopically in the *Ser* domain. To do this, I used the *GAL4/UAS* system. The *ptc-GAL4* driver was used to direct the expression of *UAS-dAP-2*. The *ptc* expression domain runs along the anterior/ posterior boundary of the leg and therefore perpendicular to the *Ser* expression domain. I used a *UAS-GFP* marker to visualize the *ptc* domain. At each point that *UAS-dAP-2* expression crossed an endogenous stripe of *Ser* expression, *Ser* expression was lost (Fig. 19). Consistent with the clonal analysis, *Ser* expression was turned off cell autonomously. Both, the loss and gain of *dAP-2* function experiments have allowed me to conclude that dAP-2 function is required to cell autonomously negatively regulate *Ser* expression.

3.9 The role of dve in leg segmentation

The role of the homeobox transcription factor DVE in leg development has not been previously characterized. Aside from its expression pattern in leg imaginal discs, it was not known whether DVE performed any significant function in the process of limb patterning. The role of DVE in gut development has been the focus of previous studies.

Figure 19. Expression of SER in leg disc containing ectopic *dAP-2*.

Disc from a *UAS-GFP; ptc Gal4/+; UAS-dAP-2/+* pupa (2-4h after puparium formation) stained with a rat α SER antibody and a goat α rat Cy3 secondary conjugate. The *ptc* expression domain, containing ectopic *dAP-2* is marked by GFP expression. SER expression is repressed cell autonomously in cells expressing *dAP-2*.


GFP
SER
merge

DVE is required for the development of the proventriculus and the middle midgut (Fuss and Hoch, 1998; Nakagoshi et al., 1998). Loss-of-function mutations result in gut constrictions, which lead to lethality at the first instar larval stage, as larvae are unable to feed properly. The *dve* ^{01738}, mutation is a hypomorphic allele, caused by an enhancer trap P-element insertion. Since homozygous *dve* ^{01738} mutants are larval lethal, I performed a genetic mosaic analysis, in order to examine its function in leg development, using the *FLP/FRT* system. An FRT chromosome carrying *dve* ^{01738} was put in *trans* to an FRT chromosome carrying a *yellow*$^{+}$ transgene in a *yellow*$^{-}$ background. As a result, after clone induction heterozygous tissue and wild-type twin spots contained wild-type bristles, whereas homozygous mutant cuticle was marked by the presence of yellow bristles.

The most significant observation was that *dve*$^{-}$ clones were frequently associated with ectopic joints (Fig. 20). Legs were scored for the presence of clonal tissue, the position of the clone and the presence of a defect. Clones were induced at either 24-48h or 36-60h of development. Similar results were obtained for both time points. For clones induced at 36-60h, 137 legs were examined and 31 clones were found (Table 4). Of the 31 clones, 14 were associated with an ectopic joint. The ectopic joints were always found in the tarsal segments. None of the clones found in the tibia and femur (12 clones) were associated with defects. Only 5 out of 19 clones found in the tarsus exhibited no phenotype. It should also be noted that most ectopic joints were found in tarsal segments ta2-ta5. The presence of ectopic joints in the basitarsus (ta1) was less frequent than in more distal segments. Ectopic joints were associated both with large clones that spanned

Figure 20. Adult legs expressing ectopic *dve* or lacking *dve* function.

a. Leg from an *omb-Gal4; UAS-dve/+* adult showing incomplete joints. Joint formation is initiated in the ventral half of the leg, however joint formation is blocked in the dorsal half of the leg. Image taken using Nomarski optics. Arrows point to incomplete joints.

b. Higher magnification image of leg in a. showing incomplete joint formation. Image taken using Nomarski optics.

c. Leg from *yw hsFLP; FRT42D* dve^{01738}*-lac Z / FRT 42D y+* adult showing a large clone spanning segments ta2-ta5. Clone is marked by the presence of yellow bristles. Ectopic joints are seen associated with the clone. Only one ectopic joint is seen per segment and the orientation of the ectopic joint is reversed with respect to the endogenous joint. Arrows point to endogenous joints. Arrowheads point to ectopic joints. Image taken using Nomarski optics.

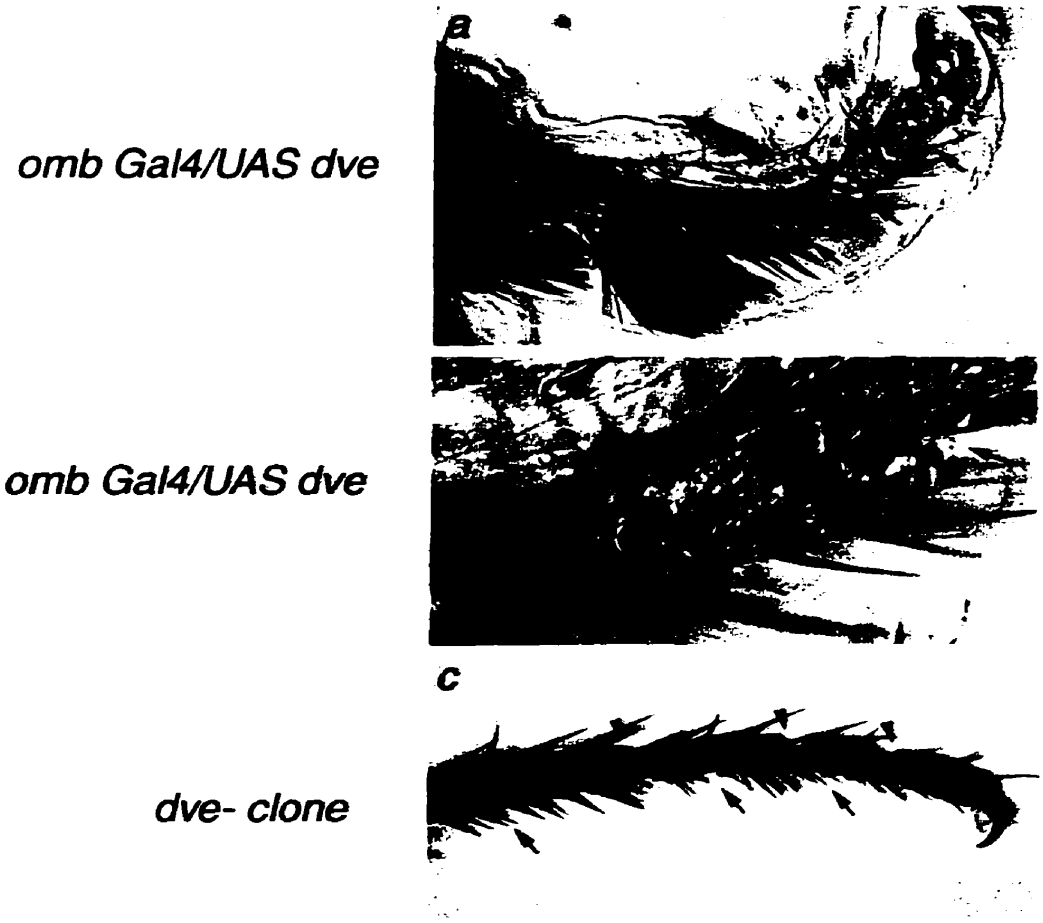

a
omb Gal4/UAS dve
omb Gal4/UAS dve
c
dve- clone

Table 4: Summary of *dve* mosaic analysis

	Tibia and femur clones		Ta1 clones		Ta2-Ta5 clones	
	Ectopic joint present	**No ectopic joint**	**Ectopic joint present**	**No ectopic joint**	**Ectopic joint present**	**No ectopic joint**
Induced at 24-48h n=40	**0**	**14**	**2**	**7**	**14**	**3**
Induced at 36-60h n=31	**0**	**12**	**2**	**2**	**12**	**3**

multiple segments as well as with clones located in only one segment. However, clones located in the endogenous joint had no effect on its morphology. Within a clone, only one ectopic joint was seen per segment. The position of the ectopic joint was also very constant. The joint was always positioned in the middle of the segment rather than close to the endogenous joint. Ectopic joints also exhibited opposite polarity with respect to the endogenous joint. These results indicate that DVE is required during leg patterning to prevent the formation of joints at ectopic locations, but that it is not required for joint formation. To test whether ectopic expression of *dve* was sufficient to block the formation of endogenous joints, I used the *GAL4/UAS* system to drive overexpression of *dve*. I used *omb-GAL4* to drive *UAS-dve* expression in the dorsal half of the leg (Fig. 20). The adults obtained from this cross, showed severe leg defects. The dorsal region of the leg was shortened resulting in curved legs. Closer examination revealed that while joints in the ventral half, outside the *omb-GAL4* domain, formed normally, their formation was blocked in the dorsal compartment, resulting in incomplete joints (Fig. 20). This observation is consistent with DVE function being sufficient to block joint formation.

3.10 Regulation of *Ser* by DVE

Genetic mosaic analysis and overexpression studies just described revealed that *dve* plays an important role in leg patterning, by inhibiting joint formation. To understand how dve modulates N-mediated joint formation, I examined how DVE influences the

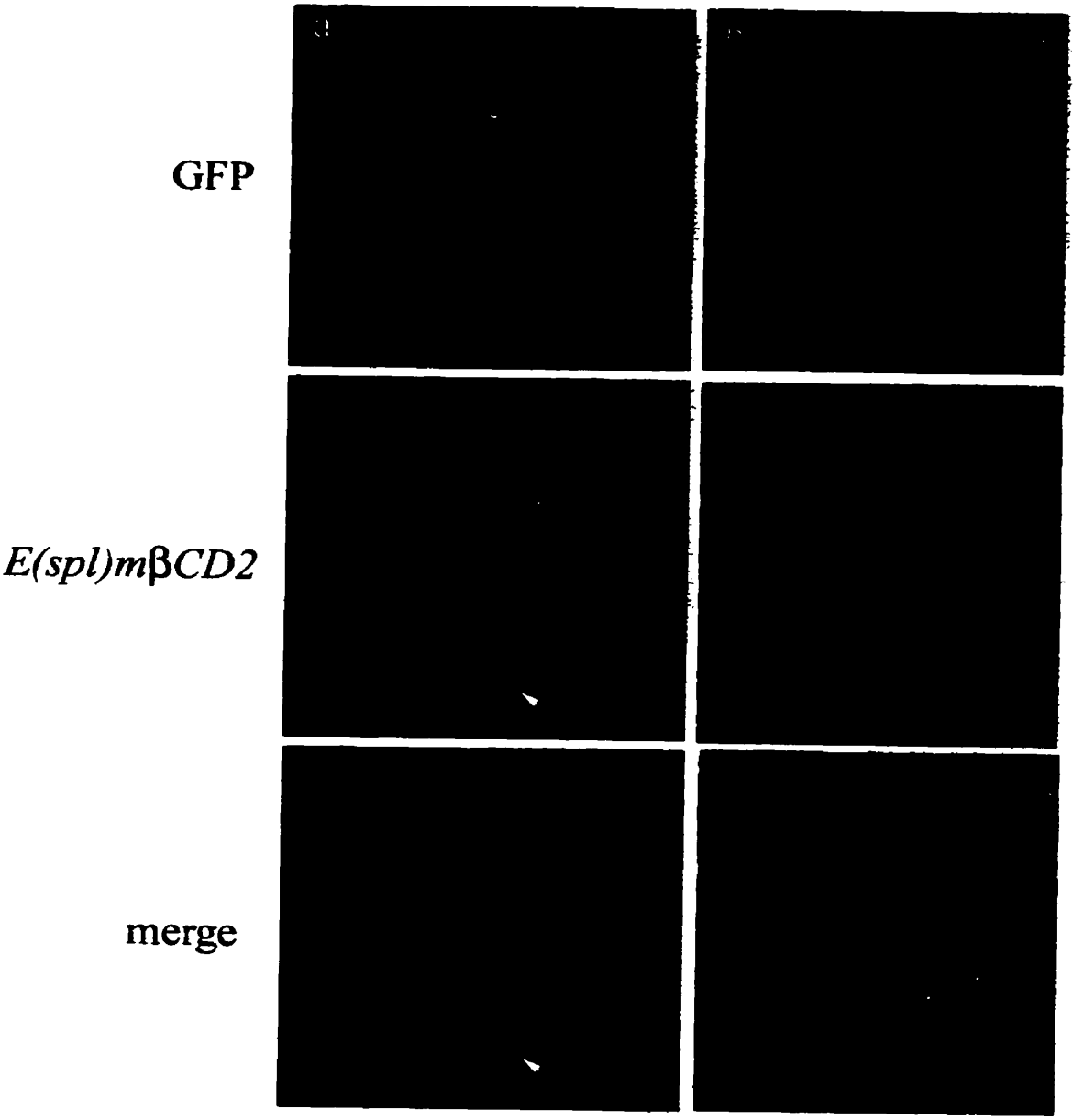
expression of *E(spl)mβ-CD2* and *Ser*. The formation of ectopic joints associated with *dve*⁻ clones suggests the activation of the Notch signalling pathway, while loss of joints when *dve* is ectopically expressed suggests that Notch activation is prevented. I generated *dve*⁻ clones using FLP/FRT system, with GFP as a marker. Clones were induced at 36-60h of development and pupal leg discs were dissected at 2-4h after puparium formation. *E(spl)mβ-CD2* expression was detected using an αCD2 antibody directed against the *E(spl)mβ-CD2* reporter construct. Clones of mutant cells, marked by the absence of GFP, that spanned across an endogenous stripe of *E(spl)mβ-CD2* expression showed a small amount of ectopic *E(spl)mβ-CD2* expression, just inside the borders of the clone. However, strong ectopic *E(spl)mβ-CD2* expression could be detected in the cells surrounding the clone (Fig. 21). The ectopic expression of *E(spl)mβ-CD2* indicates that ectopic activation of Notch has occurred, which in turn contributed to the formation of ectopic joints. It should be noted that only one *dve*⁻ clone was seen in this experiment.

To investigate whether the reverse was true, that inhibition of joint formation in the presence of ectopic *dve* was due to the loss of N activation, I examined the expression of *E(spl)mβ-CD2* in pupal legs that were expressing *UAS-dve* under the control of *omb-GAL4*. Everting leg imaginal discs were dissected from pupae and stained for *E(spl)mβ-CD2* expression. *omb-GAL4* directed the expression of *UAS-dve* as well as *UAS-GFP*, thus marking the region in which ectopic *dve* was expressed. *omb-GAL4* is expressed in the dorsal half of the leg, perpendicular to the endogenous *E(spl)mβ-CD2*

Figure 21. Expression of *E(spl)mβ–CD2* in leg discs containing dve⁻ clones.

a. Disc from a *yw hsFLP; FRT42D dve^{01738} lac Z / FRT 42D GFP; dAP-2^{15} FRT 80 E(spl)mβ–CD2 /+* pupa (2-4h after puparium formation) stained with a mouse α rat CD2 antibody and goat α mouse AlexaFluor 488 secondary conjugate. A large *dve^{01738}* clone is seen spanning the length of all tarsal segments, marked by the loss of GFP expression. Ectopic *E(spl)mβ-CD2* expression can be seen in segment ta5, outside the borders of the clone. Arrow points to ectopic *E(spl)mβ-CD2* expression outside clone borders.

b. Higher magnification image of disc in a. showing segment ta5. Some ectopic *E(spl)mβ-CD2* expression is seen just inside the borders of the clone. Higher levels of ectopic *E(spl)mβ–CD2* expression is seen outside the clone.


GFP
E(spl)mβCD2
merge

stripes. Ectopic *dve* expression turned *E(spl)mβ-CD2* off whenever the two domains intersected (Fig. 23). However, comparison with the expression of GFP revealed that the inhibition of *E(spl)mβ-CD2* was not autonomous, as the GFP expression domain was broader than the resulting domain in which *E(spl)mβ-CD2* was lost. Together with the clonal analysis, these results show that DVE antagonizes the effects of Notch activation, helping to promote correct joint formation. However, the non-autonomous manner with which this occurs suggests that the interaction between *dve* and N target genes is indirect.

An explanation for the non-autonomous induction of N activation could be that DVE also controls the expression of one of the Notch ligands. To investigate this possibility, I performed a genetic mosaic analysis of *Ser* expression in *dve*$^-$ clones. As in previous experiments, this was done using the *FLP/FRT* system and GFP as a clone marker. Clones were induced at 36-60h of development, pupal imaginal discs were dissected at 2-4h after puparium formation and *Ser* expression was detected using a SER specific antibody. Indeed, ectopic *Ser* expression was detected within the clones (Fig. 22). Ectopic *Ser* was only detected as a proximal expansion of the endogenous *Ser* stripe. That is ectopic *Ser* was not seen in smaller clones which did not encompass the region of *Ser* expression. Additionally, in larger clones, ectopic *Ser* expression did not fill the area of the entire clone, as was observed in *dAP-2*$^-$ clones, but rather spread up to 5 cell widths. Ectopic *Ser* expression was never detected outside of the clone borders. The induction of ectopic *Ser* expression in *dve* mutant clones, suggests that DVE has a role in

Figure 22. Expression of SER in leg discs containing *dve*⁻ clones.

a. Leg disc from a *yw hsFLP; FRT42D dve*^{01738} *lac Z / FRT 42D GFP* pupa (2-4h after puparium formation) stained with a rat α SER antibody and a goat α rat Cy3 secondary conjugate. A large clone spanning segments ta1-ta3 is seen, marked by the loss of GFP expression. Ectopic SER expression is visible just proximal of the ta2 and ta3 rings of endogenous SER expression. Ectopic SER expression does not extend throughout the clone, but is visible only several cell diameters form the endogenous stripes. No ectopic SER expression can be detected outside the clone. Arrows point to ectopic SER expression.

b. Leg disc from a *yw hsFLP; FRT42D dve*^{01738} *lac Z / FRT 42D GFP* pupa (2-4h after puparium formation) stained with a rat α SER antibody and a goat α rat Cy3 secondary conjugate. A large clone spanning the length of the tarsal segments ta1-ta4 is seen, marked by the loss of GFP expression. Ectopic SER expression can be seen extending proximally from the endogenous ring of SER expression in segment ta1. Arrow points to ectopic SER expression.

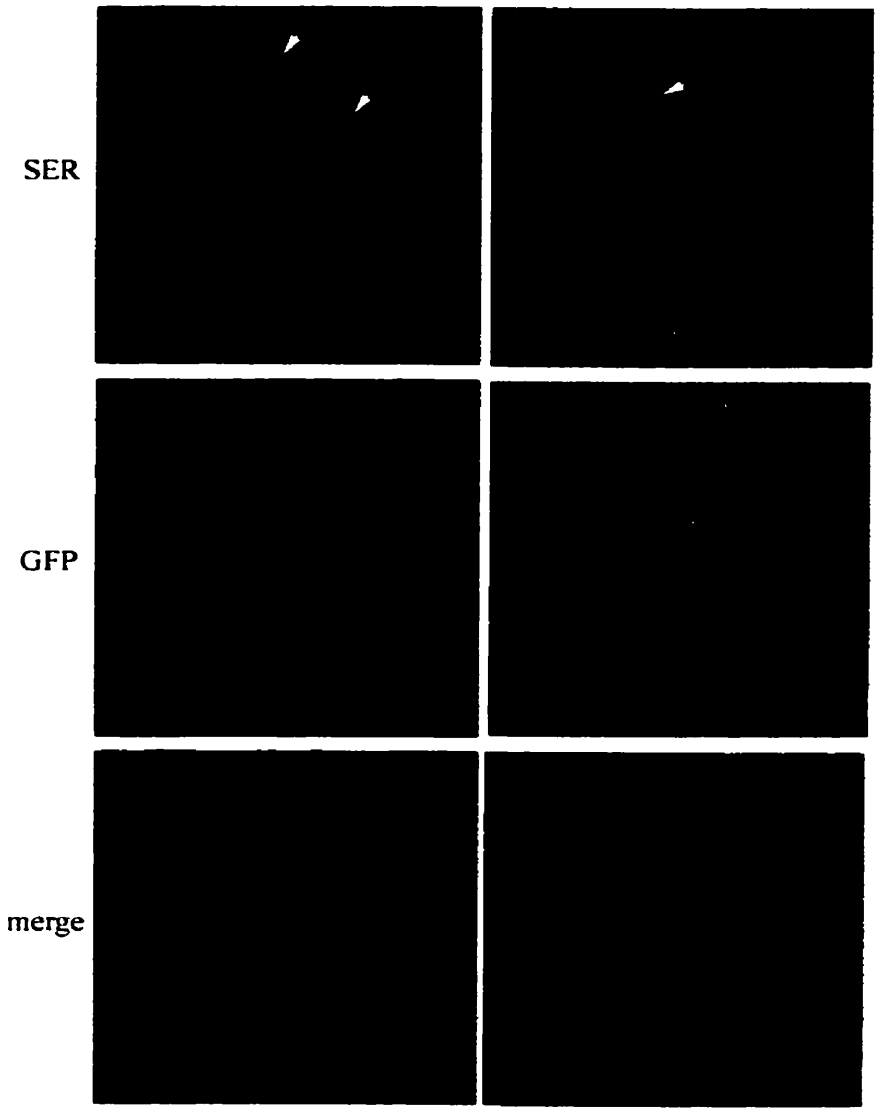
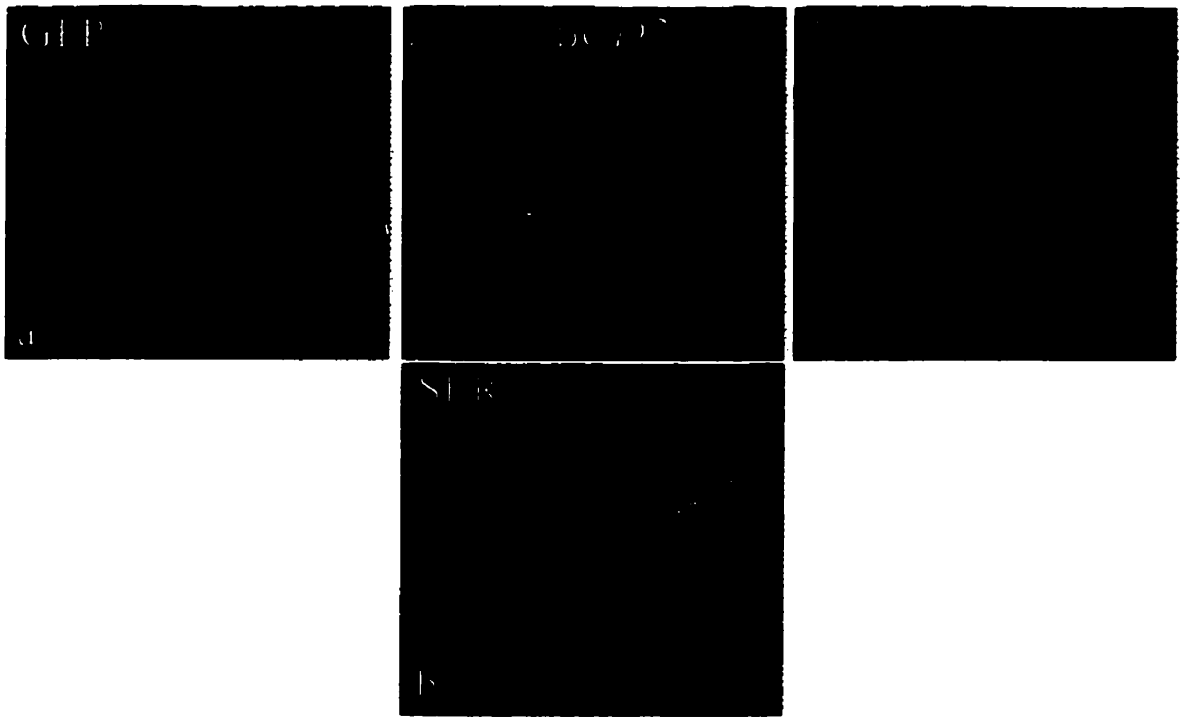

SER
GFP
merge

Figure 23. Expression of *E(spl)mβ- CD2* and SER in leg discs expressing ectopic dve.

a. Leg disc from a *omb Gal4; UAS-GFP/+; UAS-dve/ dAP-2^{15} FRT 80 E(spl)mβ-CD2* pupa (2-4h after puparium formation) stained with a mouse α rat CD2 antibody and goat α mouse AlexaFluor 488 secondary conjugate. The *omb* domain of expression, where DVE is ectopically expressed is visualized by the expression of GFP. *E(spl)mβ–CD2* expression is repressed by the ectopic presence of DVE.

b. Leg disc from a *omb-Gal4; UAS-GFP/+; UAS-dve/+* pupa (2-4h after puparium formation) stained with a rat α SER antibody and goat α rat Cy3 secondary conjugate. SER expression is inhibited in the *omb* domain of expression, where DVE is expressed ectopically.



inhibiting *Ser* expression. To test this directly, I examined *Ser* expression in the presence of ectopic *dve*, using *omb-GAL4*to drive *UAS-dve*. *Ser* expression was again examined in developing pupal legs. Ectopic *dve* expression was capable of inhibiting *Ser* expression whenever the two domains intersected (Fig. 23). The autonomy of this interaction could not be determined, as no marker for the *omb* expression domain was present. However, these results clearly indicate that one function of DVE is to inhibit *Ser* expression.

Chapter 4: Discussion

In this study I have explored the roles of *dAP-2* and *dve* in the genetic regulation of joint formation in *Drosophila melanogaster*. Previous work has demonstrated that the activation of the Notch signalling pathway is necessary and sufficient to induce proper joint formation (Bishop et al., 1999; de Celis et al., 1998; Rauskolb and Irvine, 1999). The correct spatial expression of *Dl*, *Ser* and *fng* results in the localized activation of the N receptor required to position segment boundaries and establish joints. I have shown that *dAP-2* and *dve* are key participants in this pathway. *dAP-2* and *dve* appear to have opposite effects on segmentation, however, both have a common function in regulating the expression of the gene for the Notch ligand *Ser*.

4.1 Roles of the dAP-2 and DVE transcription factors in leg joint development

The work performed during this study began as an analysis of *l(3)1215*, a mutation that blocks joint formation and was therefore a candidate to act in Notch signalling in the leg. During the course of this work, I determined that *l(3)1215* is an allele of the *Drosophila* homologue of the gene for the AP-2 transcription factor. Genetic screening for novel alleles of *dAP-2* has also revealed that the original *dAP-2* $^{l(3)1215}$ mutation as well as the new alleles isolated in this screen are strong hypomorphic or null mutations. This can be concluded based on the similarity of phenotypes among different

alleles as well as when compared in *trans* to the deficiency uncovering this region. This similarity also indicates that the major role of dAP-2 is in limb development.

Previous studies of dAP-2 function have shown that it is required downstream of Notch signalling, where it functions to promote joint formation (Kerber et al., 2001, Monge et al., 2001). An examination of molecular marker expression a *dAP-2*$^{-}$ background was performed to identify potential targets of the dAP-2 transcription factor. The expression of several known Notch downstream targets and joint markers, *nub*, *disco lacZ* and *E(spl)mβ–CD2* was not affected by a *dAP-2* mutation, indicating that Notch activation is still occurring in these mutants (Fig. 9). This result was not unexpected as work by Kerber et al., (2001) has shown that the expression of certain genes downstream of Notch such as *E(spl)* and *big brain (bib)* was unchanged in *dAP-2* mutant clones. In addition, two known joint markers, *fj-lacZ* and *odd-lacZ* were found to be affected in *dAP-2* mutants, however their role in joint formation was not pursued in great depth (Fig 10). The expression of *fj-lacZ* was technically difficult to examine as I was unable to reproduce the previously published tarsal expression pattern. The involvement of *odd* was also not pursued as only a minor change in its expression was seen. The expression patterns of *Ser* and *dve-lacZ* exhibited very strong changes and so their expression pattern and function was examined in more detail (Fig. 11, Fig 13). It was discovered that dAP-2 functions in the medial tarsus, ta2-ta4, to control the expression of these two genes. Genetic mosaic analysis and misexpression of *dAP-2* showed that dAP-2 acts cell

autonomously to block the expression of *Ser* (Fig. 18). dAP-2 function is also required for the proper expression of the joint marker *dve* in tarsal segments ta2-ta4.

The homeobox gene *dve* was shown to also function in the process of leg patterning. Genetic mosaic analysis and ectopic expression indicate that DVE function is required to block the formation of ectopic joints. In addition these studies revealed that DVE has a role in regulating the expression of *Ser*. *Ser* expression is inactivated by the action of DVE (Fig 21, Fig. 22.). This in turn has an indirect effect on Notch activation and Notch downstream gene expression in the tarsus as demonstrated by ectopic *E(spl)mβ–CD2* expression.

These molecular epistasis experiments indicate that both DVE and dAP-2 are required to repress *Ser* expression. Thus both of these transcription factors play a key role in the evolution of the segmental pattern by positioning stripes of *SER* expression necessary for Notch activation leading to joint formation. However, loss or gain-of-function of each of these genes results in opposite phenotypes in adult fly legs. Loss of *dAP-2* function results in loss of joints, while loss of *dve* function results in ectopic joints (Kerber et al., 2001; Monge et al., 2001; this study). Conversely, ectopic expression of *dAP-2* results in the formation of ectopic joints, while ectopic *dve* expression results in inhibition of joint formation (Kerber et al., 2001; this study). dAP-2 function is necessary to block *Ser* expression, and the loss of *dAP-2* function results in *Ser* activation in all cells that lack *dAP-2*. DVE function is also sufficient to autonomously block *Ser* expression, although in the absence of DVE, *Ser* activation extends only several cells

from the endogenous stripe of expression. Some possible explanations for these differences will be discussed below.

4.2 dAP-2 and DVE define a new repeating domain of gene expression in the P-D axis

The repeating domains of cells expressing the Notch ligands and *fng* adjacent to domains of Notch activation are critical for proper leg segmentation. In this study I have shown that the expression of *dAP-2* and *dve* defines new domains of gene expression in the P-D axis. From this pattern of expression, four distinct, repeating zones could be identified along the proximal-distal axis of the tarsus of pupal legs at 2-4h after puparium formation; Ser expression, *E(spl)mβ–CD2* expression, *E(spl)mβ–CD2* together with strong *dAP-2* and strong *dve-lacZ* expression and finally weak *dAP-2* and *dve-lacZ* expression.

One problem, that stems from these studies is the degree of overlap between *E(spl)mβ–CD2* and *dAP-2* expression. Kerber et al., (2001) report that *dAP-2* expression and *E(spl)mβ–CD2* expression correspond exactly. In this study, I found that dAP-2 and *E(spl)mβ–CD2* expression overlap by approximately 5 cell diameters (Fig. 15). Regions, approximately 3 cells in length, which express only *E(spl)mβ–CD2* exist proximally to the region of overlap, while regions expressing only *dAP-2* exist distally to the overlap. This discrepancy could not be attributed to different materials used as both this study and

the work performed by Kerber et al., (2001) used the same α-dAP-2 antibody and *E(spl)mβ–CD2* reporter construct. Several reasons for this difference in reported pattern are possible. It may be that Kerber et al., (2001) examined the expression pattern of *dAP-2* in legs of slightly younger pupae. At the point in time in which this was examined dAP-2 could be present entirely within the *E(spl)mβ–CD2* domain and the staggered pattern develops later as the disc everts. Alternatively, the earlier complex structure of the disc may have interfered with visualization of the expression pattern. Imaginal discs of third instar larvae are highly folded. As the discs evert throughout pupal development, leg segments extend and unfold. The expression pattern of *dAP-2* and *E(spl)mβ–CD2* would have to be examined at different points of imaginal disc development to determine if this possibility exists. A second explanation for this difference may be that Kerber et al., (2001) overlooked the staggering of the two domains because expression of *dAP-2* proximal to the *E(spl)mβ–CD2* domain is weaker than that present within the domain.

4.3 The roles of Dl and Ser in tarsal segmentation

The wild-type expression patterns as well as molecular epistasis experiments begin to suggest a mechanism of how these genes function together to regulate joint formation. The expression of genes shown to be involved in joint development such as *Ser, Dl, fng, dAP-2* and *dve* is present in a striped pattern. This striped expression of all these genes is required for proper joint formation. Alteration to this pattern results in either loss of endogenous joints or the formation of ectopic joints. However, the loss-of-

function phenotypes of these genes suggest a hierarchy to their action. Legs obtained from *Dl* hypomorphs are shortened and show an overall loss of joint formation (Bishop et al., 1999). *dAP-2* mutants, also have shortened legs and lack joints, however, the reduction in overall length is not as severe as in *Dl* hypomorphs. *fng* also affects joint formation and segment length (Rauskolb and Irvine, 1999). *Ser* null mutants on the other hand, only show loss of joint formation, while the length of segments is unaffected (Bishop et al., 1999). These phenotypes indicate that while both SER and DL are ligands for Notch and lead to its activation, they have varying effects on leg development.

Further evidence for SER and DL having different roles in leg patterning come from different effects on the expression of the joint marker *disco-lacZ* (Bishop et al., 1999). In *Dl* mutant leg discs *disco-lacZ* stripes do not form properly; fewer stripes are found and they are wider. In *Ser* mutant legs, however, *disco-lacZ* stripes form properly but their expression is eventually lost.

Evidence from this study also suggests that *Dl* and *Ser* are regulated differently. *Ser* is subject to regulation by dAP-2, while *Dl* is apparently independent of it. One rationale for this difference is that the function of each of these ligands is required at different times in the course of leg development. It may be that DL function is required to establish the initial stripes cells where Notch is activated, while SER acts to maintain or increase this activation. Such roles would be consistent with what is observed in *dAP-2* mutants. *Dl* expression remains unchanged in *dAP-2* mutant legs and it is therefore able to signal to adjacent cells and activate Notch, which in turn allows for *E(spl)mβ–CD2* to

be expressed in the striped pattern. It can be argued that part of the cause of shortened legs in *Dl* mutant flies is due to the loss of dAP-2 function, which has been shown to lead to increased cell death (Kerber et al., 2001). *dAP-2* has been shown to depend on Notch activation, thus in the absence of DL, N activation would not occur and *dAP-2* would not be expressed, leading to cell death. This possibility has not been tested directly. One method of showing that *dAP-2* expression is dependent on DL function, but not SER would be to test the expression of *dAP-2* in the absence of DL or SER function. The prediction for this experiment would then be that in *Dl* mutants, *dAP-2* would not be expressed, while its expression would remain in *Ser* mutants.

A possible reason for two differing mechanisms for the action of the ligands could be the presence of the glycosylase FNG. In the wing disc, FNG functions to make Notch a better receptor for DL than SER and the evidence suggests that FNG-modified Notch cannot transduce the SER signal (Bruckner, et al., 2001; de Celis et al., 1996). Evidence that FNG plays a similar role in the leg comes from the ectopic expression of *fng*. Misexpression of *fng* throughout the tarsus, using *Dll-GAL4/UAS-fng* results in loss of tarsal joints. This is a strikingly similar phenotype to that observed in *Ser* mutants, suggesting that in the presence of FNG, SER is unable to signal through Notch (Bishop et al., 1999). However, in this study, the expression pattern of *fng* was not investigated. This was technically difficult as no FNG-specific antibodies are available. A *lacZ* enhancer trap was available, however, due to the tight linkage of *fng* and *dAP-2* on the meiotic map, it was difficult to generate a *fng-lacZ dAP-2*$^{-}$ recombinant chromosome. Rauskolb

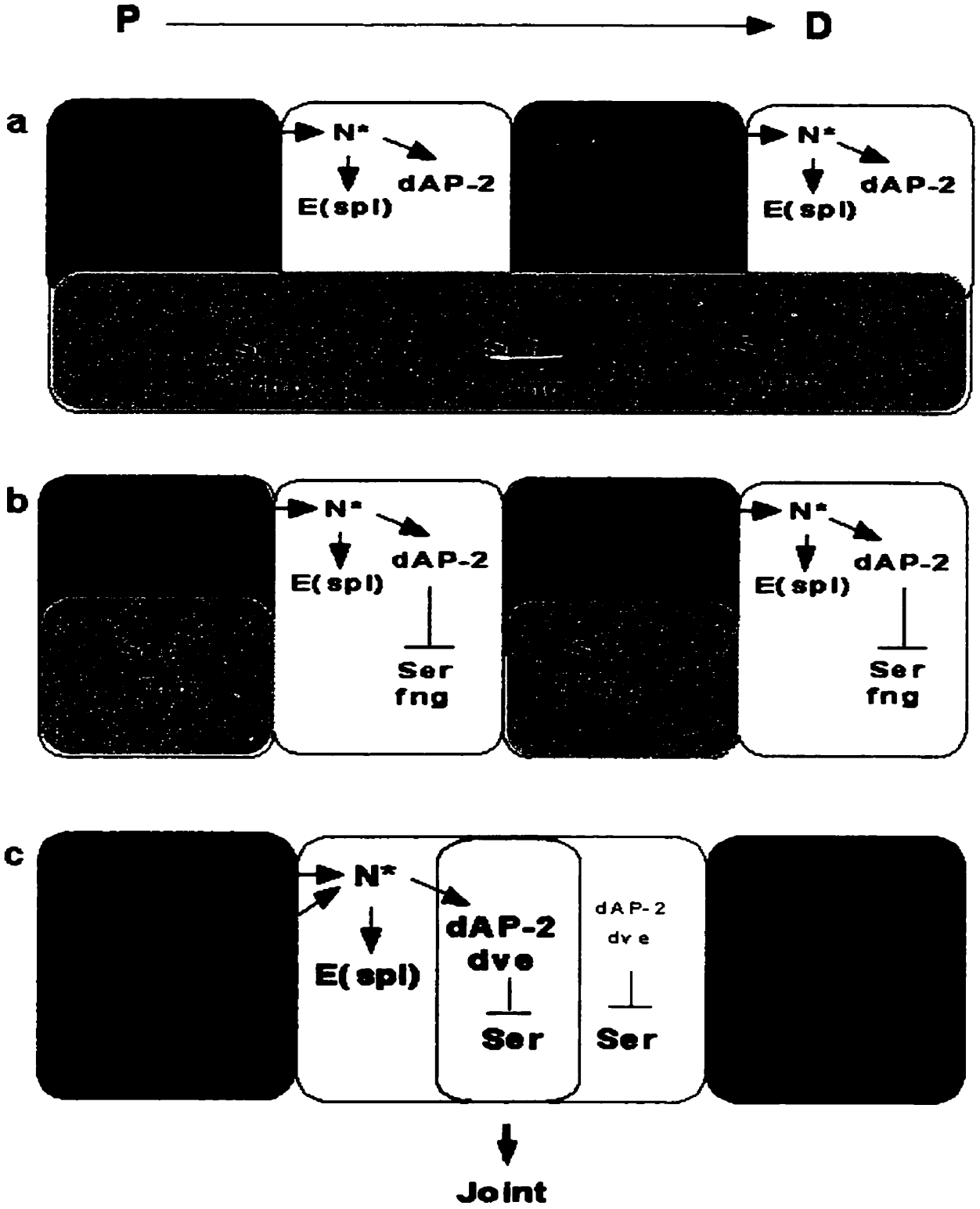
and Irvine (1998) report that SER and *fng* mRNA are expressed in the same cells, suggesting that the two may be regulated in the same manner. It would also be necessary to determine whether *fng* expression is influenced by dAP-2. It is possible that *fng*, like *Ser* becomes ectopically expressed in the absence of dAP-2. In the presence of FNG throughout the tarsus, SER would be unable to signal and influence Notch activation.

Figure 24. Model for the establishment of SER expression domains.

a. Initially, DL expression is present in a striped pattern, while SER and FNG expression is present throughout tarsal segments. The presence of FNG prevents SER from signalling. DL signals to adjacent cells to activate N (N*). N-activation induces expression of downstream target genes such as *E(spl)* and *dAP-2*.

b. dAP-2 expression in the N-activation domain autonomously represses *Ser* and *fng*. SER and FNG are now expressed in the same domain as DL.

c. DL signals to adjacent cells to activate N. N-activation induced downstream genes such as *E(spl)* and dAP-2 and dve. dAP-2 and DVE autonomously repress *Ser*, helping to establish the proper pattern of SER expression. SER is now able to signal to adjacent cell to increase N activation. As a result of the action of these genes, four domains of expression are established; DL and SER domain, N-activation domain, N-activation and dAP-2 + DVE domain, and a dAP-2 +DVE domain. The N-activation and dAP-2 domains responsible for initiating joint formation.


P
D
a
N*
E(spl)
dAP-2
N*
E(spl)
dAP-2
b
N*
E(spl)
dAP-2
Ser
fng
N*
E(spl)
dAP-2
Ser
fng
c
N*
E(spl)
dAP-2
dve
Ser
dAP-2
dve
Ser
Joint

The mechanism of how the *Ser* and *Dl* expression domains are established is also not yet understood. A possible model of how this process occurs could be that initially *Dl* expression is set up in a striped pattern, while *Ser* and *fng* expression is present uniformly throughout the tarsus (Fig 24). The presence of FNG would inhibit signalling by SER, but DL would be able to signal to adjacent cells and activate Notch (Fig. 24). Activation of Notch would induce *dAP-2* expression, which together with DVE, would autonomously block *Ser* and *fng*. This would then generate regions where *Ser* and *fng* are not expressed, allowing SER as well as DL, to signal to these cells and increase Notch activation. This model needs to be tested by following the expression patterns of *Dl*, *Ser* and *fng* throughout development until their final pattern is established in everting pupal legs. This model predicts a dynamic pattern of *Ser* and *fng* expression as well as dAP-2 being capable of inhibiting *fng* expression. In this model, the ectopic expression of *Ser* seen in *dAP-2* mutants would be the result of a lack of repression, rather than the induction of novel ectopic expression.

4.4 A model for Notch-mediated joint formation.

A general model of how these genes interact in the process of joint formation can also be suggested (Fig. 24). Delta signalling to adjacent cells would lead to Notch activation and the expression of downstream targets such as *E(spl)mβ–CD2* and *dAP-2*. *dAP-2* expression could lead to the expression of *dve*, or be required for its maintenance. Their combined action would lead to the inhibition of *Ser* expression. Establishment of a

striped expression pattern of *Ser* would then allow for the enhancement of Notch activation because of the combined signalling of SER and DL, which would be required for proper joint formation (Bishop et al., 1999). The subsequent increase in levels of Notch activation could be responsible for inhibiting *dAP-2* expression, generating the offset pattern of *E(spl)mβ–CD2* and *dAP-2* expression. Since both Notch activation and dAP-2 have been shown to be required cell autonomously for joint formation, it can be argued that the cells which contribute to the formation of the adult joint are cells in which both *E(spl)* and *dAP-2* are expressed. Furthermore, it can also be argued that the interaction between the domain of only Notch activation and the domain of both Notch activation and *dAP-2* is necessary to initiate joint formation.

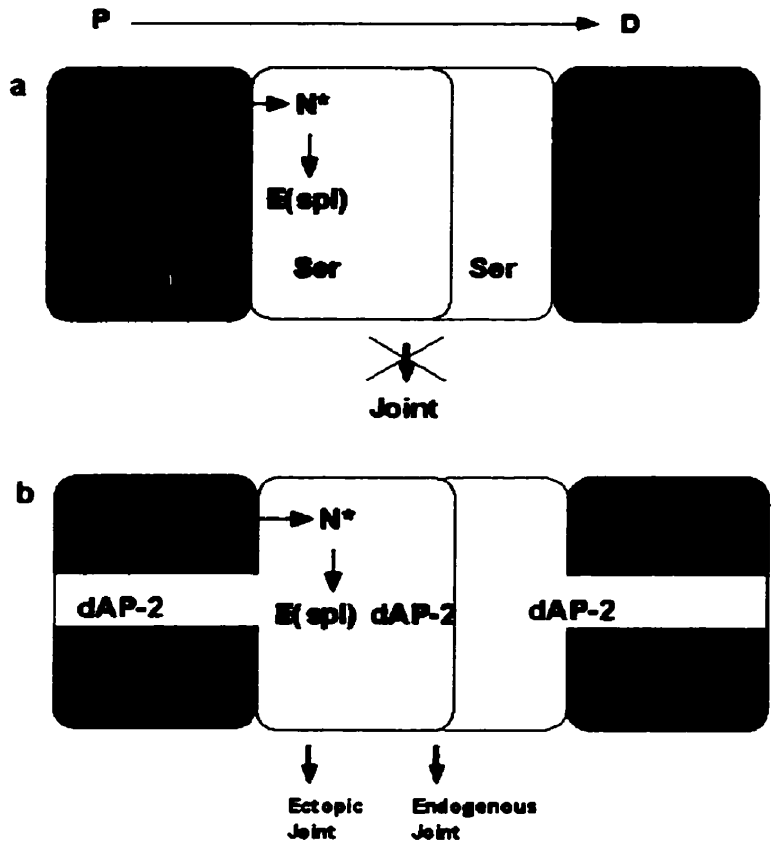
This model would explain the phenotypic effects observed in *dAP-2* loss and gain-of-function flies. In *dAP-2* mutant flies (Fig. 25) *Dl* expression would occur as normal, leading to the activation of Notch and the wild type expression of *E(spl)mβ–CD2.* However, in the absence of dAP-2, *Ser* and *fng* would not be turned off properly and thus their pattern would fail to resolve. Lack of SER signalling would result in Notch activation not being properly enhanced. In this model, ectopic *Ser* expression would not be the direct cause of the *dAP-2* phenotype. The region of overlap between *E(spl)mβ–CD2* and *dAP-2* would also not exist, leading to a failure of joint formation, but the shortening of legs due to cell death would be due to another function of dAP-2. The phenotype observed when *dAP-2* is ectopically expressed can also be explained using

this model. In the presence of ectopic dAP-2, *Ser* is turned off in its endogenous domain. Ectopic dAP-2 in the zone where normally only

Figure 25. Model explaining *dAP-2*⁻ and ectopic *dAP-2* phenotypes.

a. In the absence of dAP-2, DL is able to signal to adjacent cells to induce N-activation (N*), which allows for *E(spl)* expression. However, in the absence of dAP-2, SER expression is not repressed and is therefore present throughout the tarsus. Lack of dAP-2 may also prevent DVE from being properly expressed. No joints are formed as dAP-2 is not present in the N-activation domain and lack of SER signalling may cause the level of N-activation to be insufficient to induce joint formation.

b. In the presence of ectopic dAP-2, *Ser* is autonomously repressed throughout its endogenous domain. The domain of overlap between N-activation and dAP-2 is also increased, causing ectopic joints to be induced.


P
D
a
N*
E(spl)
Ser
Ser
Joint
b
N*
dAP-2
E(spl)
dAP-2
dAP-2
Ectopic
Joint
Endogenous
Joint

*E(spl)mβ–CD2*would normally be found would lead to an expansion of the region of overlap between *E(spl)mβ–CD2* and *dAP-2*. As a result ectopic joints form in the now expanded region. Ectopic joints form only as far as the region of overlap extends, providingan explanation of why ectopic joints form only in close proximity to the endogenous joint. The *E(spl)mβ–CD2* domain does not expand into the region where *Ser* expression has been turned off by the action of dAP-2, because of the presence of DL in this region. The effects of ectopic *Dl* expression suggest that it can act in the leg to autonomously antagonize the effects of N activation (de Celis et al., 1998; Bishop et al. 1999)

Several inconsistencies and assumptions exist in this model that need to be explained and tested. First, the assumption that *Dl* is not inhibited by the ectopic expression of *dAP-2* is based on the observation that loss of dAP-2 function has no effect on *Dl* expression, however it has not been tested directly. This could be tested using the *GAL4/UAS* system to misexpress *dAP-2* and examine *Dl* expression.

A second problem, which also stems from the incomplete overlap between *dAP-2* and *E(spl)mβ–CD2* is the question of how *Ser* is repressed in cells where only *E(spl)mβ–CD2* is expressed. One possibility may be that high levels of Notch activation are able to repress *Ser* expression even in the absence of dAP-2. This could occur either directly through Su(H), the transcription factor that mediates Notch signalling, or through another target downstream of Notch.

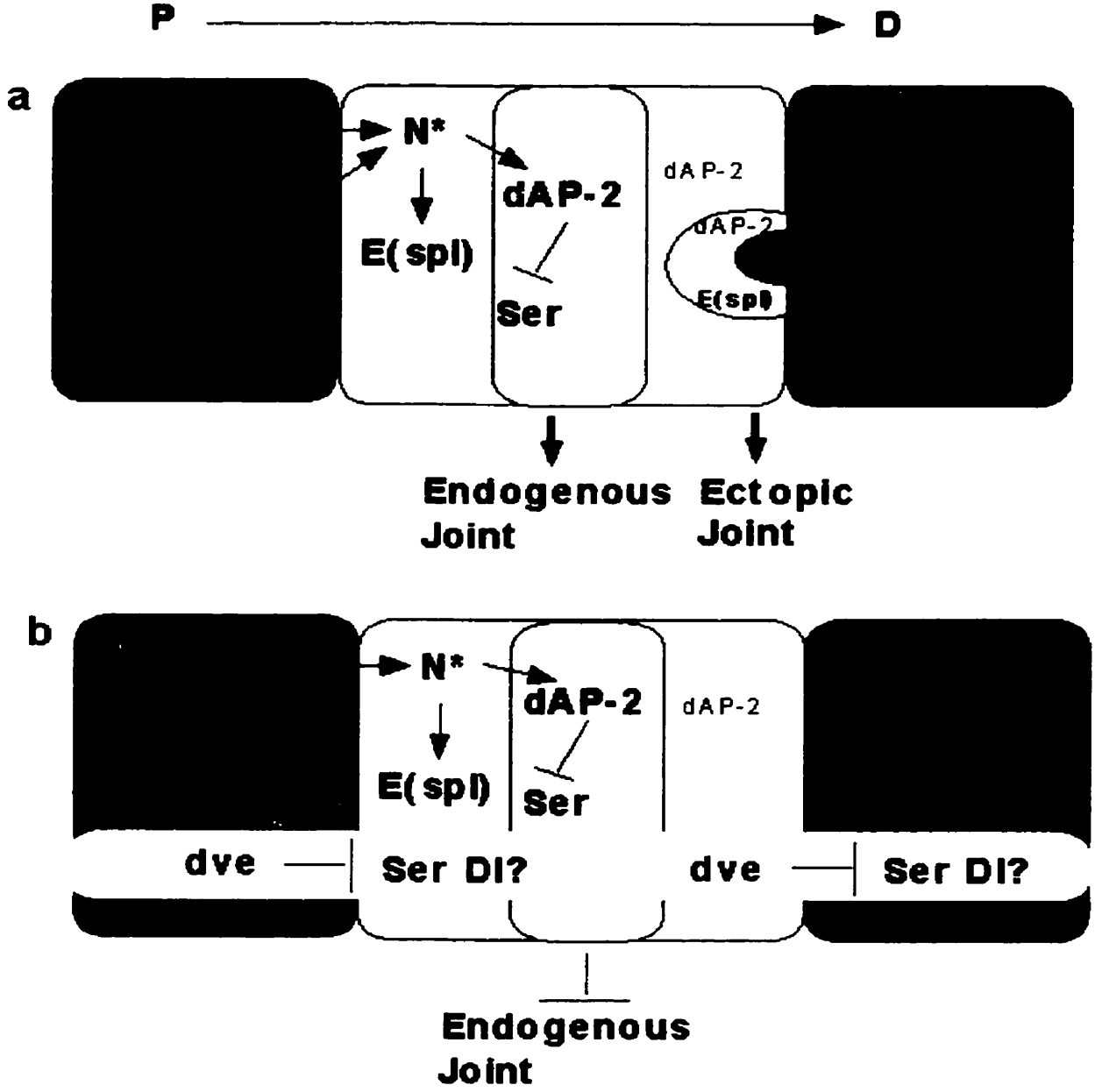
An additional problem that needs to be addressed in this model is the relationship between *dAP-2* and dve. As was described above, both of these genes are found in the same cells and both function to repress *Ser*. However they are required in different regions outside the endogenous *Ser* expression domain. In *dve*⁻ clones, *Ser* expression is only activated in mutant cells just proximal to the endogenous stripe. However, if dAP-2 is found in the same cells as *dve-lacZ*, then some dAP-2 should be present in the *dve*⁻ clone and should therefore repress *Ser* expression. Several explanations can be offered as to why this is not observed. The first explanation for this phenomenon could be that the *dve* allele used in these experiments is a hypomorph. Currently no information is available on the strength of this mutation. It may be possible that some DVE activity remains in the clonal tissue. This residual activity may be sufficient to inhibit some of the *Ser* expression. A second possibility is that high dAP-2 levels are able to inhibit *Ser* expression while lower levels are not. *dAP-2* is expressed strongly at its proximal edge, overlapping with *E(spl)mβ–CD2* and weakly at its distal edge, bordering on *Ser* expression. In this case dve⁻ clones located in the zone where only *dve-lacZ* and dAP-2 are found, would contain insufficient levels of dAP-2 to inhibit *Ser* and would therefore express the ligand. However, mutant tissue located more proximally would contain higher levels of dAP-2, which would be capable of repressing *Ser*. As a result ectopic *Ser* expression would only be found at the distal edge of the dAP-2/*dve LacZ* domain. Alternatively, the dAP-2 and *dve LacZ* domains may not overlap exactly, with no dAP-2 present at the distal edge. This possibility is consistent with what is observed in *dve*⁻

clones, where ectopic *Ser* is only observed in cells where low levels of dAP-2 are normally found. Support for this can also be offered by the fact that *dve*$^-$ clones located in tissue surrounding the endogenous joint have no effect on the joint, in adult cuticle.

Figure 26. Model explaining loss of *dve* and ectopic *dve* phenotypes.

a. In *dve*$^-$ clone, SER expression expands proximally from its endogenous domain, until levels of dAP-2 are sufficient to inhibit its expression. SER then signals to adjacent cells to activate N. Since dAP-2 is also present in this domain, this is sufficient to induce ectopic joint formation.

b. In the presence of ectopic DVE, SER expression is repressed in its endogenous domain. This is sufficient to prevent adequate levels of N-activation and endogenous joint formation is disrupted.


P
D
a
N*
E(spl)
dAP-2
Ser
dAP-2
dAP-2
E(spl)
Endogenous Joint
Ectopic Joint
b
N*
E(spl)
dAP-2
Ser
dAP-2
dve
Ser Dl?
dve
Ser Dl?
Endogenous Joint

The model proposed here, together with the above explanation could also be used to explain the adult phenotype of *dve*$^-$ flies as well as flies expressing ectopic dve (Fig. 26). In flies containing *dve*$^-$ clones, *Ser* expression extends from its normal region,due to a lack of repression by DVE and dAP-2. Ectopic *Ser* is then able to induce ectopic N activation surrounding the clone. This then generates a region where both N activation and *dAP-2* expression is present, which is sufficient for the formation of an ectopic joint. In the presence of ectopic *dve*, *Ser* is blocked in its endogenous domain. Due to the lack of SER, Notch activation is not maintained properly and joint formation is disrupted.

This model also predicts that small *dAP-2*$^-$ clones located only in the endogenous dAP-2 domain would result in ectopic *Ser* activation. *Ser* expression could then induce Notch activation in cells surrounding the clone. These cells would express *dAP-2* in addition to activated Notch and should induce ectopic joints, similar to what is observed in *dve*$^-$ clones. However, this phenotype has not been reported in adult legs in the study performed by Kerber et al. (2001). It may be that small clones were not generated in their study. It may also be that additional factors are present that prevent SER from signalling to adjacent cells and inducing ectopic joint formation. One possibility that also must be tested is that DVE may regulate the expression of both Notch ligands, *Ser* and *Dl*. This may help to explain the differences between *dve* and *dAP-2* mutant phenotypes. In *dAP-2*$^-$ clones, only *Ser* expression is induced. SER signalling may be insufficient to induce high levels of Notch activation required to induce joint formation. In *dve*$^-$ clones, however,

both *Ser* and *Dl* would become expressed and their combined signalling may be required to induce joint formation.

The model proposed as a result of this study suggests roles for dAP-2 and DVE as transcriptional repressors, acting on *Ser* expression. Together, the action of these genes regulates the proper formation of joints in the tarsus. Although, much of the way in which tarsal joints are established has been clarified, additional work must be done to further understand this process. The relationship and epistasis between *dAP-2* and *dve* must be examined more closely. This can be done by genetic mosaic and misexpression experiments, looking at both dAP-2 regulation of *dve* and the possibility of DVE regulating *dAP-2*. The regulation of *dve* and *dAP-2* by SER and DL should also be examined in the same manner.

The phenotype observed in adult flies carrying dve^- clones is similar to what is observed in planar cell polarity mutants, such as dsh^w and pk^{sple}. This group of genes also involved joint formation and segmentation, the planar cell polarity genes, appear to have an opposite effects on joint formation. Mutants of either the pk^{sple} or the *dsh* locus display ectopic joints in each of the tarsal segments (Gubb et al., 1999; Held et al., 1986). This results in flies that have twice as many joints as normal. The planar cell polarity genes are involved in signalling that polarizes epithelial cells in the horizontal plane of the epithelium (Reviewed in Mlodzik, 2000). This signalling may be responsible for the unidirectional activation of Notch in the leg. However, the interaction of these two pathways remains unclear. It may be possible that these genes perhaps together with

DVE and dAP-2 cause cells on one side of the *Ser* domain to be different from cells on the opposite side, allowing for N activation to occur only in one direction. This is an interesting aspect of joint development which has not yet been examined.

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