Development 125, 4483-4493 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
DEV7610

The roles of the homeobox genes *aristaless* and *Distal-less* in patterning the legs and wings of *Drosophila*

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Accepted 1 September; published on WWW 20 October 1998

SUMMARY

In the leg and wing imaginal discs of *Drosophila*, the expression domains of the homeobox genes *aristaless* (*al*) and *Distal-less* (*Dll*) are defined by the secreted signaling molecules Wingless (Wg) and Decapentaplegic (Dpp). Here, the roles played by *al* and *Dll* in patterning the legs and wings have been investigated through loss of function studies. In the developing leg, *al* is expressed at the presumptive tip and a molecularly defined null allele of *al* reveals that its only function in patterning the leg appears to be to direct the growth and differentiation of the structures at the tip. In contrast, *Dll* has previously been shown to be required for the development of all of the leg more distal than the coxa. Dll protein can be detected in a central domain in leg discs throughout most of larval development, and in mature discs this domain corresponds to the distal-most region of the leg, the tarsus and the distal tibia. Clonal analysis reveals that late in development these are the only regions in which *Dll* function is required. However, earlier in development *Dll* is required in more proximal regions of the leg suggesting it is expressed at high levels in these cells early in development but not later. This reveals a correlation between a temporal requirement for *Dll* and position along the proximodistal axis; how this may relate to the generation of the P/D axis is discussed. *Dll* is required in the distal regions of the leg for the expression of tarsal-specific genes including *al* and *bric-a-brac*. *Dll* mutant cells in the leg sort out from wild-type cells suggesting one function of *Dll* here is to control adhesive properties of cells. *Dll* is also required for the normal development of the wing, primarily for the differentiation of the wing margin.

Key words: *aristaless* (*al*), *Distal-less* (*Dll*), Pattern formation, Proximodistal axis, *Drosophila melanogaster*, Leg, Wing

INTRODUCTION

Secreted polypeptide signaling molecules belonging to several different families, such as Wnts, TGFβs and Hedgehogs, have been shown to direct cell fate choices made during development, in a variety of animal systems. In *Drosophila*, patterning of the legs and wings is governed in large part by the products of the *wingless* gene (Wg, a Wnt) and of the *decapentaplegic* gene (Dpp, a TGFβ; reviewed by Lawrence and Struhl, 1996) which are expressed in ventral and dorsal regions of the developing leg, respectively, so that there is only a single site where *wg*- and *dpp*-expressing cells are juxtaposed: the presumptive distal tip (Campbell et al., 1993). The combination of Wg and Dpp signals directs the formation of the proximodistal axis (P/D; base to tip) in the leg (Campbell and Tomlinson, 1995; Campbell et al., 1993; Diaz-Benjumea et al., 1994) and several targets of this signal combination have been identified including the homeobox genes *aristaless* (*al*) and *Distal-less* (*Dll*; Campbell et al., 1993; Diaz-Benjumea et al., 1994). The aims of this paper are, firstly, to determine the role played by *al* and *Dll* in the formation of the P/D axis and, secondly, to use these data to address the question of how Wg and Dpp direct the formation of this axis.

At least two models have been proposed to explain how the Wg/Dpp combination controls the formation of the P/D axis. Originally, it was suggested that an organizer may be established at the site where Wg and Dpp expressing cells were juxtaposed; this site corresponds to the presumptive distal tip of the leg, which is characterized by the expression of *al* (Campbell et al., 1993; Fig. 1A,B). The putative distal organizer would act as the source of a secondary signal which would direct outgrowth and define cell identity along the P/D axis. It was also suggested that *al* may play a role in the activity of this organizer because misexpression of *al* can result in secondary P/D axes in the wing. This could not be tested genetically because a null allele of *al* was not available.

More recently, it has been proposed that Wg and Dpp may define cell fates along the P/D axis directly rather than by inducing a secondary signal (Lecuit and Cohen, 1997). It was shown that expression of downstream targets of the Wg/Dpp combination were activated or lost cell autonomously following activation or loss of the Wg or Dpp signaling pathways. One of

these targets was *Dll*. Genetic studies have shown that *Dll* is required for the normal development of the legs and other serially homologous structures such as the antennae. Null mutants are embryonic lethal and show a loss of the Keilin's organs, sensory structures thought to represent larval legs (Sunkel and Whittle, 1987). Leg imaginal discs develop in these mutant embryos but do not have the potential to form leg tissue more distal than the most proximal segment, the coxa (Cohen et al., 1993). This supports the results of clonal analysis with null mutants which demonstrated an autonomous requirement for *Dll* activity in all of the leg more distal than the coxa (Cohen and Jurgens, 1989a); it was proposed that *Dll* is required in the legs and antennae to elevate development above the 'ground state' of body wall/coxa and that the homeobox gene *extradenticle* may define this state (Gonzalez-Crespo and Morata, 1996). More recently, it has been suggested that *Dll* may be a 'master control' gene for defining ventral appendages because misexpression in the dorsal appendages, the wings, can result in transformations to legs (Gorfinkiel et al., 1997).

In addition to this absolute requirement for leg development, analysis of hypomorphic mutants suggested that *Dll* may also play an active role in specifying cell fates along the P/D axis. Hypomorphic mutants can result in the deletion of distal segments and a compression of the P/D axis in the leg; most of the hypomorphs fall into an allelic series with the weaker alleles showing only mild effects whilst in the strongest, patterning of all the leg segments, apart from the coxa, is disrupted (Cohen et al., 1989; Cohen and Jurgens, 1989b; Sunkel and Whittle, 1987). Consequently it was suggested that there is a graded requirement for *Dll* activity along the P/D axis with the maximal requirement being distal and that this may correspond to a graded distribution of Dll protein (Cohen and Jurgens, 1989b). However, subsequent cloning and expression studies revealed that *Dll* expression is not graded in the leg disc, but Dll protein can be detected at uniform levels in the presumptive distal region of the mature third instar leg disc corresponding to the tarsus and the distal tibia and also in a proximal ring (Diaz-Benjumea et al., 1994; Panganiban et al., 1994; Fig. 1E,F). This presents a paradox: late in development Dll can be detected only in the tarsus and distal tibia, but the genetic data reveals that *Dll* function is also required more proximally, in the femur and all of the tibia. This is complicated by a more recent study which questioned where *Dll* is actually expressed because, when analysed in adults, a *Dll*-Gal4 line appears to be expressed throughout the tibia and weakly in the femur, i.e. in regions where no Dll protein can be detected in imaginal discs (Gorfinkiel et al., 1997). In addition this study showed that misexpression of *Dll* can induce the development of secondary P/D axes in the leg (Gorfinkiel et al., 1997), but the effect appears to be indirect because the ectopic *Dll* appears to induce ectopic *wg* expression which in turn induces ectopic expression of the endogenous *Dll* gene. The reason why ectopic *Dll* might induce ectopic *wg* expression is not clear, but is strikingly similar to the situation following ectopic *al* expression in the wing (Campbell et al., 1993). This study also demonstrated a role for *Dll* in patterning the wing where it appears to have a nonautonomous role in the differentiation of the wing margin.

Here, the role of *al* and *Dll* in patterning the appendages is investigated by clonal analysis with null alleles. A molecularly characterized null *al* allele is described and clonal analysis shows that *al* is only required for the growth and differentiation of the tip of the leg and not for the formation of the P/D axis. A detailed clonal analysis with a null *Dll* allele is described and reveals a more complex spatial and temporal requirement for *Dll* along the P/D axis of the leg than was previously realised. The role of *Dll* in establishing the P/D axis of the leg is discussed in the light of these results, along with what this data may reveal about how Wg and Dpp specify positional identity along this axis.

MATERIALS AND METHODS

al and *Dll* fly stocks

Dll^{SA1} is a null allele (Cohen et al., 1989). Dll^{3} is the strongest hypomorph (Cohen and Jurgens, 1989b; Sunkel and Whittle, 1987). al^{ice} is the strongest allele that survives to adult (Campbell et al., 1993). al^{130} is associated with the inversion In(2L)al^{130} (Schneitz et

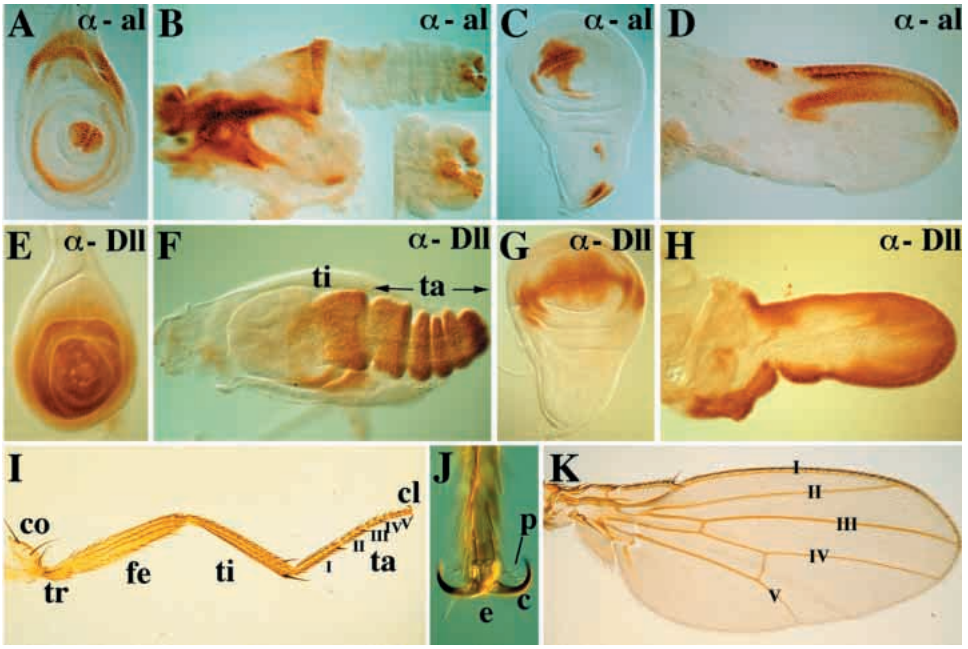


Fig. 1. Wild-type legs and wings. (A,E) Late, third instar leg discs, (B,F) partially everted legs, (C,G) late, third instar wing discs, (D,H) partially everted wings. Upper panels showing Al expression (antibody), middle panels Dll expression (antibody). In the legs, Al is restricted to the tip; Dll is expressed in the presumptive tarsus (ta) and distal tibia (ti; it is also expressed in a more proximal domain that is out of focus on these figures). In the wing Al is expressed in an anterior domain extending proximally from the tip, whilst Dll shows graded expression centred on the wing margin. (I) Adult leg. (J) Tip of leg. (K) Adult wing. c, claw; cl, claw organ; co, coxa; e, empodium; fe, femur; p, pulvillus; ta, tarsus; ti, tibia, tr, trochanter

al., 1993); homozygotes survive to adulthood. em212-Gal4 is an insert in the *Dll* gene and was used here because in combination with a UAS-Dll line it can rescue *Dll* mutants, suggesting its expression closely resembles that of the endogenous gene (Gorfinkiel et al., 1997). UAS-lacZ was from Bloomington (4-1-2), Act5C>Draf^{+}>nuc-lacZ was from Struhl and Basler (1993), UAS-Flp was from G. Struhl (unpublished).

Generation of *al* null allele

l(2)k16513 is a P-element line from the Berkeley Drosophila Genome Project (BDGP) mapped to the same region as *al* (Spradling et al., 1995). The precise location of the P-element was determined by plasmid rescue of flanking DNA to be just 5′ to the *RpI135* gene (Kontermann et al., 1989; accounting for the lethality of this line) and to be about 17 kb 3′ to *al*. Mobilization of this P-element with Δ2-3 flies (Robertson et al., 1988) produced a male, X21, with an *al* phenotype over al^{1} that still carried the *w+* marker of the P-element. Plasmid rescue of flanking DNA showed the ends of the P-element of X21 to be intact and that the 3′ end was identical to that of *l(2)k16513*. However, the 5′ end was now located 5′ to the *al* transcription unit revealing X21 to have a deletion of about 26 kb including all of the *al* transcription unit (Fig. 2B). X21 is still lethal over *l(2)k16513*, but mobilization of the P-element resulted in several revertants viable over *l(2)k16513*. Southern analysis of one, al^{ex}, showed that, at this level of resolution, it was associated with a precise excision of the P-element of X21.

Clonal analysis

This was done using the FRT/flp technique (Xu and Rubin, 1993) with and without Minutes (Morata and Ripoll, 1975) using the following stocks.

(1) al^{ex} Minute+ cuticle clones: *y*; al^{ex}, *stc*, *FRT39E*/Dp(1:2)sc^{19}, *M(2)201*, *FRT39E*; *hs flp*. Clones are *y* (*y+* is carried in the *sc* duplication), which marks all the bristles, and are mutant for *stc* which marks the hairs on the wing (Jiang and Struhl, 1995).

(2) Wild-type and *Dll*

Dll cuticle clones (non-Minute): *y, hs flp; FRT42, sha,* Dll^{SA1}*/FRT42, CD2.2R.1*

Dll disc clones (non-Minute): *y, hs flp; FRT42 sha* Dll^{SA1}*/FRT42 arm-lacZ*

Wild-type clones: as above, without the Dll^{SA1}.

Minute clones: with M(2)60E on the *arm-lacZ* or the *CD2.2R.1* chromosome.

Cuticle clones are *y* (*y+* is carried in the *CD2.2R.1* transgene) and are mutant for *sha* which marks all the hairs on the wing, but does not affect the wing margin bristles and hairs (Phillips et al., 1990). Clones in the disc lose the ubiquitously expressed *arm-lacZ* marker (Vincent et al., 1994). *FRT*, *hs flp* and *y+* transgenes: described by Chou and Perrimon (1992); Jiang and Struhl (1995); Xu and Rubin (1993). Other markers and chromosomal rearrangements are described by Lindsley and Zimm (1992).

To generate clones in larvae, vials were given a 1-hour heat shock at 35°C once during development. For the *Dll* study, this was done at the following times after egg laying: 40-48 hours (corresponding to 1st instar), 48-60 (early second), 60-72 (late second), 72-84 (early third) and 84-96 (mid third). Embryos were given a 1-hour heat shock at 37°C between 3-6 hours after egg laying. For each time period at least 75 legs were examined. The number of segments bearing clones was calculated as a percentage of the total number of segments scored (the numbers do not represent the total number of clones because more than one clone may be present in a single segment and a single clone may contribute to more than one segment).

Immunolabelling

The basic technique and Al antibody: Campbell et al. (1993). Other antibodies: Dll (Vachon et al., 1992); Cut (Blochlinger et al., 1988); Achaete (Skeath and Carroll, 1991); Bab, raised to BabII (gift of D. Godt and F. Laski, personal communication); β-gal, (Cappell). Immunofluoresence was viewed on Bio-Rad 600 and 2410 confocal microscopes.

RESULTS

Generation and phenotype of al^{ex}, a null *al* allele

A null allele of *al* was generated by deleting a small region of DNA, including all of the *al* transcription unit but no other genes, through the imprecise excision of a closely linked P-element inserted just 5′ to the *RpI135* gene (encoding a subunit of RNA polymerase III; Kontermann et al., 1989) and 17 kb 3′ to *al* (Fig. 2A). One excision line, X21, corresponded to a 26 kb deletion of genomic DNA removing all of the *al* transcription unit; no others have been identified in this region (Fig. 2B). The P-element remains intact in X21 and, although the original line has no detectable *lacZ* expression, X21 has an expression pattern corresponding to a subset of that of *al* (Figs 1A,C, 2D,E). X21 is a double mutant for *al* and *RpI135*, but precise excision of the P-element reverted the mutation in *RpI135*, resulting in a line mutant only for *al*: al^{ex}.

al^{ex} homozygotes die as embryos with no obvious phenotype. The phenotype of al^{1} over al^{ex} is similar to that over a deficiency, including the loss of the arista. The embryonic lethality and some of the adult phenotypes of al^{ex} are rescued by a genomic fragment containing the *al* transcription unit (Campbell et al., 1993). To characterize *al* function in the development of the leg and wing, large homozygous clones of al^{ex} were generated early in larval development using the

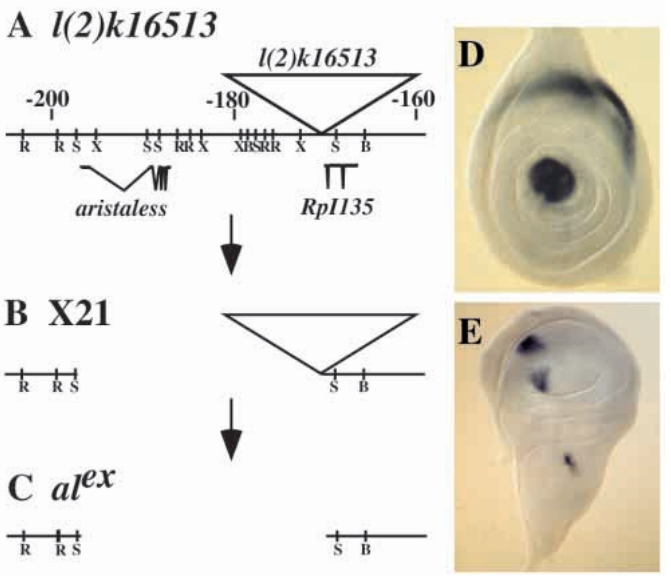


Fig 2. Generation of al^{ex}, a null allele of *al*. (A) Molecular map of region 21C1,2; coordinates correspond to Schneitz et al. (1993). (B) Mobilization of the *l(2)k16513* P-element resulted in an excision event, X21, where the P-element remained intact, but there was a complete deletion of the *al* transcription unit. X21 is mutant for *al* and is still lethal over *l(2)k16513*. (C) Precise excision of the P-element of X21 leads to reversion of the lethality over *l(2)k16513*. This chromosome, al^{ex}, is only mutant for *al*. (D,E) Leg and wing discs from X21 showing β-gal expression (antibody); these are a subset of *al* (see Fig. 1A,C). B, *Bam*HI; R, *Eco*RI; S, *Sal*I; X, *Xba*I.

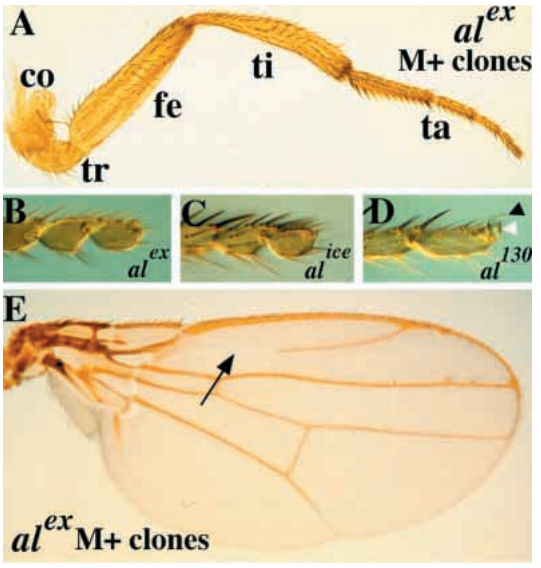


Fig. 3. Phenotype of *al* mutations. (A) Adult leg consisting largely of al^{ex} clones (Minute technique). The P/D axis of the leg is normal apart from the tip. (B) Magnification of the tip in A; the claw organ is completely absent (compare with Fig. 1J) and the last two tarsal segments are reduced in size (compare with D). (C) Tip of leg from al^{ice} homozygote; this is identical to that in B. (D) Tip of leg from al^{130} homozygote; this phenotype is weaker than that in B and C: a rudimentary claw (white arrowhead) and the pulvillus (black arrowhead) are present. The tarsal segments are of normal size. (E) Adult wing consisting almost entirely of al^{ex} clones. This wing is patterned normally apart from a gap in the proximal part of vein II (arrow) and widening of vein III particularly near the margin.

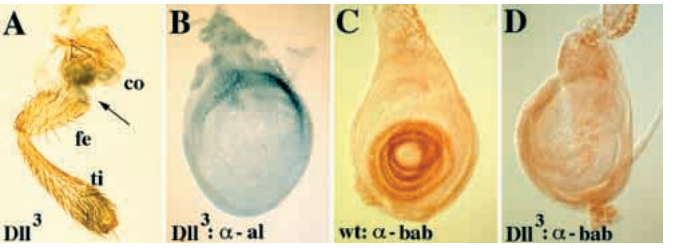


Fig. 4. Al and Bab expression in Dll^3, a hypomorphic mutant. (A) Adult leg from Dll^3 homozygote. The leg is truncated at the end of the tibia (ti) so the tarsus is absent; the tibia and femur (fe) are reduced in size. (B) Al expression in Dll^3 leg disc. The central domain of Al is absent (compare to Fig. 1A). (C) Bab expression (antibody) in a wild-type leg disc. Bab is expressed in the presumptive tarsus in the central region of the disc (but is absent from the very centre). (D) Bab expression in Dll^3 homozygote. Bab expression is lost.

Minute technique. In the wing the only clear phenotype associated with these clones is a deletion of part of vein II (Fig. 3E). In the leg the only region affected by the clones is the tip of the leg where the claw organ is completely deleted (Fig. 3A,B). To delete both claws, clones have to be present in both anterior and posterior compartments. These phenotypes are identical to those produced by the strong al^{ice} allele, homozygotes of which survive to adults (Fig. 3C), but are more severe than those of another molecularly defined allele, al^{130}, in which an inversion breaks in the 3′ end of the gene (Schneitz et al., 1993; Fig. 3D). Outside the appendages al^{ex} clones show extreme *al* phenotypes in the sternopleurum and scutellum.

al and *bab* expression in a *Dll* hypomorph

Dll^3 is the strongest hypomorph in which all of the tarsus is deleted and the tibia and femur are reduced in size (Fig. 4A; Cohen and Jurgens, 1989a; Sunkel and Whittle, 1987). The expression of two genes required for the patterning of the tarsus, *al* and *bric a brac* (*bab*; Godt et al., 1993) was examined in Dll^3 leg discs. In wild-type discs, *al* is expressed in the centre of the disc (Fig. 1A) and *bab* in the rest of the presumptive tarsus (Godt et al., 1993; Fig. 4C). In Dll^3 leg discs no *al* or *bab* expression can be detected in the centre of the discs (Fig. 4B,D).

Dll clonal analysis in the legs

Clonal analysis was performed with a null allele, Dll^{SA1} (Cohen et al., 1989). Clones were generated in a non-Minute

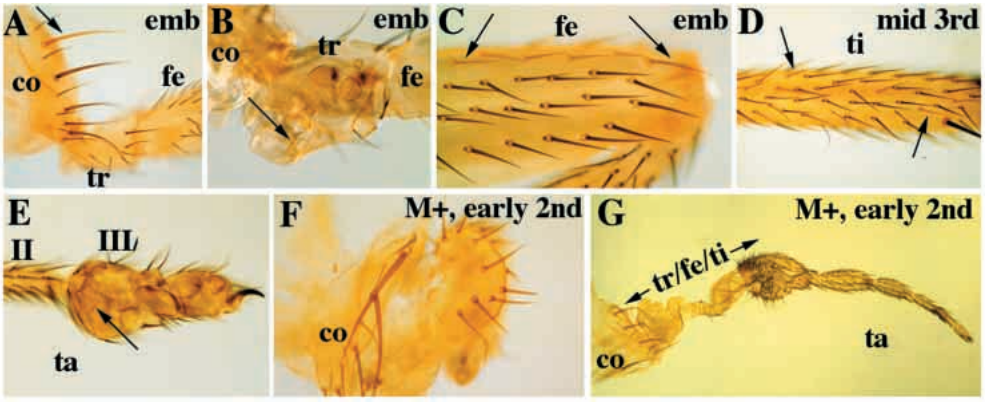


Fig. 5. Phenotype of Dll^{SA1} mutant clones in adult legs. The clones are *yellow* and were generated at different times during development (indicated in the top right). Those shown in F and G were generated in a Minute background. (A) Clone in coxa (arrow) has developed normally. (B) Patterning of the trochanter has been disrupted: there is some outgrowth ventrally and although no *y* bristles can be detected externally (not shown), internal cuticular vesicles can be identified containing at least one *y* bristle (arrow). (C) Large dorsal clone in femur (between arrows) has developed normally, although none of the mutant bristles has a bract. (D) Numerous clones in the tibia have developed normally, but again none of the mutant bristles (two arrowed) have bracts. (E) Tip of the leg containing numerous cuticular vesicles, a single *y* bristle inside the leg is arrowed. (F) Severe leg truncation consisting entirely of mutant tissue. Only the coxa has developed normally. (G) The tarsus (ta) has developed normally and is genotypically wild-type, but the trochanter (tr), femur (fe) and tibia (ti) have not. The tissue between the arrows is composed distally of wild-type tissue and proximally of mutant tissue.

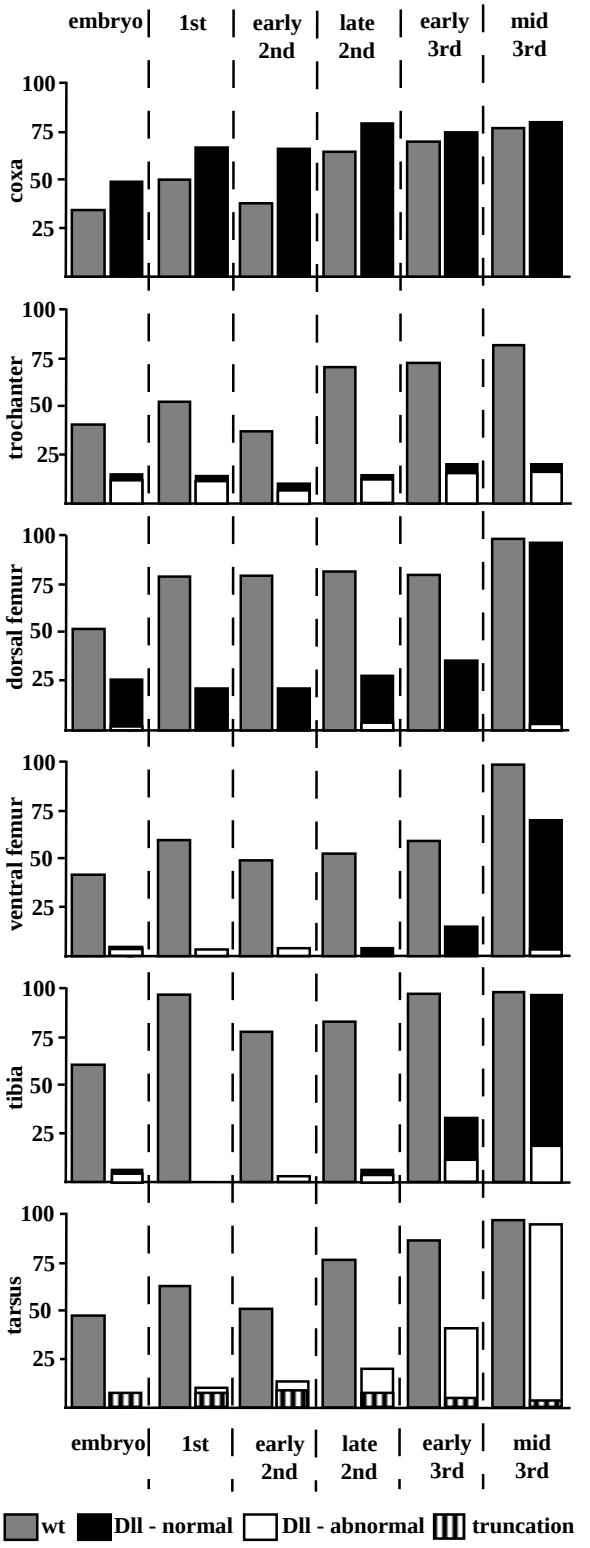
background at various times during development and the resulting adult legs were compared to legs containing wild-type clones generated at the same time. Legs were scored for the presence of a clone in each of the leg segments, whether the clonal tissue had developed normally and whether the leg was truncated. When abnormally patterned tissue failed to differentiate bristles that could be scored (see below), the abnormality was assumed to be due to the presence of a Dll^{SA1} clone and scored as such. The results are summarised in Table 1. Previously it was shown that *Dll* clones generated early in development failed to be recovered in the region more distal than the coxa, whilst later in development phenotypically wild-type *Dll* clones (but lacking bracts) could be recovered in the proximal tibia and femur but not in more distal regions where they segregate out as cuticular vesicles (Cohen and Jurgens, 1989; Gorfinkiel et al, 1997). These results are generally supported by the present study (Fig. 5) which provides more detail as to the temporal requirement for *Dll* in the different leg segments (Table 1) showing that the requirement for *Dll* in the femur and most of the tibia is lost by about the early third instar. Additional observations include a clear difference in the time at which normally patterned Dll^{SA1} clones can be recovered in the dorsal compared to the ventral femur (here 'ventral' corresponds only to the ventral third): Dll^{SA1} clones can be recovered in the dorsal femur when they are generated at any stage in development (Table 1; Fig. 5C), although early in development their frequency is reduced compared to wild-type. In the trochanter, almost no wild-type *Dll* clones are recovered at any stage in development (Fig. 5B); there is a proximal ring of *Dll* expression in the third instar leg disc that probably corresponds to the trochanter.

When a leg is composed almost entirely of Dll^{SA1} mutant tissue (using the Minute technique) then the region more distal to the coxa is represented only by a small stump of tissue (Fig. 5F). A marked reduction in the P/D axis can be identified in leg discs consisting almost entirely of Dll^{SA1} tissue (Fig. 6C,D), showing that the leg truncations produced by loss of *Dll* are not caused simply by cell death late in development but may be caused by disruption of normal patterning and growth or cell survival during development. In discs containing larger regions of wild-type tissue, this tissue is generally found in the centre of the disc surrounded by Dll^{SA1} tissue (Fig. 6E,F) and contrasts to wild-type clones which form irregular patterns contributing to any region of the leg. Legs derived from these type of discs develop normal distal regions, but the leg between this region and the coxa is aberrant: there is a marked reduction in growth, the division into segments is disrupted and the size and density of bristles is reduced (Fig 5G). Leg discs containing *Dll* clones were also stained for Vestigial expression to determine if the mutant tissue might be transformed into wing. No ectopic Vestigial expression could be detected in these discs (not shown).

Dll expression in legs

Previous studies of *Dll* expression in developing legs suggested that the protein is restricted to the presumptive tarsus and distal tibia. However, the mosaic analysis presented here indicates a requirement for *Dll* gene function in the presumptive femur and proximal tibia early in development. If *Dll* is expressed in the more proximal parts of the leg early but then lost from this region then this would account for these observations. To show that *Dll* is expressed in more proximal regions earlier in development, the following line was generated: UAS-Flp/Dll-Gal4; Act5C>cassette>nuc-lacZ. Flippase (Flp) will be expressed only in cells expressing *Dll* and this in turn will

Table 1. Percentage of leg segments containing wt or Dll clones generated at different times during development



remove the Flp-out cassette, thus switching on expression of *lacZ* in these cells under the constitutive Actin promoter and providing an inherited marker for *Dll* expression. The probability of the Flp-out event happening in any given cell will depend upon the strength of Gal4 expression and the length of time it is expressed. Analysis of third instar leg discs from this line reveals that clones expressing β-gal can be detected anywhere in the leg disc (i.e. including regions outside of the central Dll protein-positive domain), but at much higher frequency in the centre of the disc, suggesting *Dll* is expressed early in development in all the leg disc cells (Fig. 7A).

As an alternative approach we utilized the phenomenon of perdurance of protein products to investigate whether evidence of earlier *Dll* transcriptional activity could be detected in the more proximal regions of the leg in which *Dll* transcription and Dll protein are absent later in development. To do this we used the Dll-Gal4 line to drive expression of UAS-GFP or UAS-lacZ, to test the possibility that Gal4, GFP or β-gal may perdure for longer than Dll protein. In late third instar leg discs, the domain of high level GFP expression is generally coincident with Dll protein revealed by antibody staining, but much weaker staining can also be detected in the presumptive proximal tibia/femur (Fig. 7B). At earlier stages the level of GFP expression outside the Dll protein domain is much stronger (Fig. 7C) suggesting the weak staining later (and the stronger staining earlier) is due

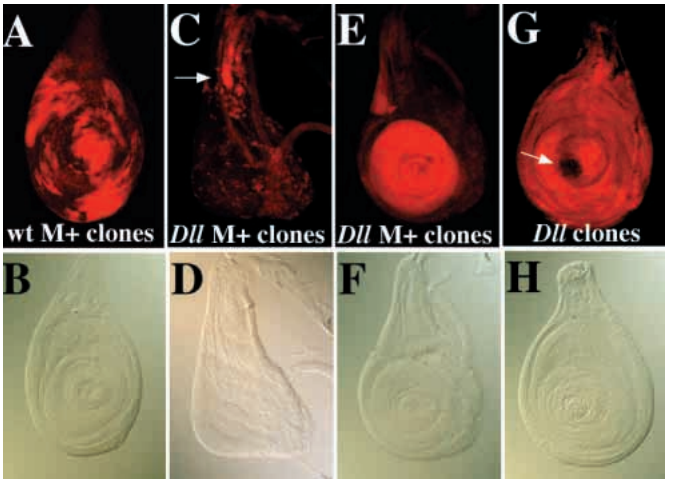


Fig. 6. Phenotype of Dll^{SA1} mutant clones in leg discs. The upper panels reveal clones by the loss of the red marker; the lower panels show Nomarski images of the discs above. (A,B) Wild-type clones generated in a Minute background. Clones can be identified anywhere along the P/D axis in the disc and have an irregular outline. The disc has the characteristic set of concentric, circular folds that loosely correspond to segments along the P/D axis. (C,D) Dll^{SA1} clones generated in a Minute background. This disc consists almost entirely of mutant tissue apart from a small region of wild-type tissue (arrowed). Although the outline of the disc is similar to a wild-type, the folds are absent revealing a much reduced P/D axis. This would probably develop into a leg similar to that in Fig. 5H. (E,F) Dll^{SA1} clones generated in a Minute background. The wild-type tissue has sorted out from the Dll mutant tissue forming a central domain with a smooth boundary. This would probably develop into a leg similar to that in Fig. 5I. (G,H) Dll^{SA1} clones generated in a wild-type background. A *Dll* mutant clone can be identified in the distal region (arrow) and appears to have sorted out from the wild-type tissue.

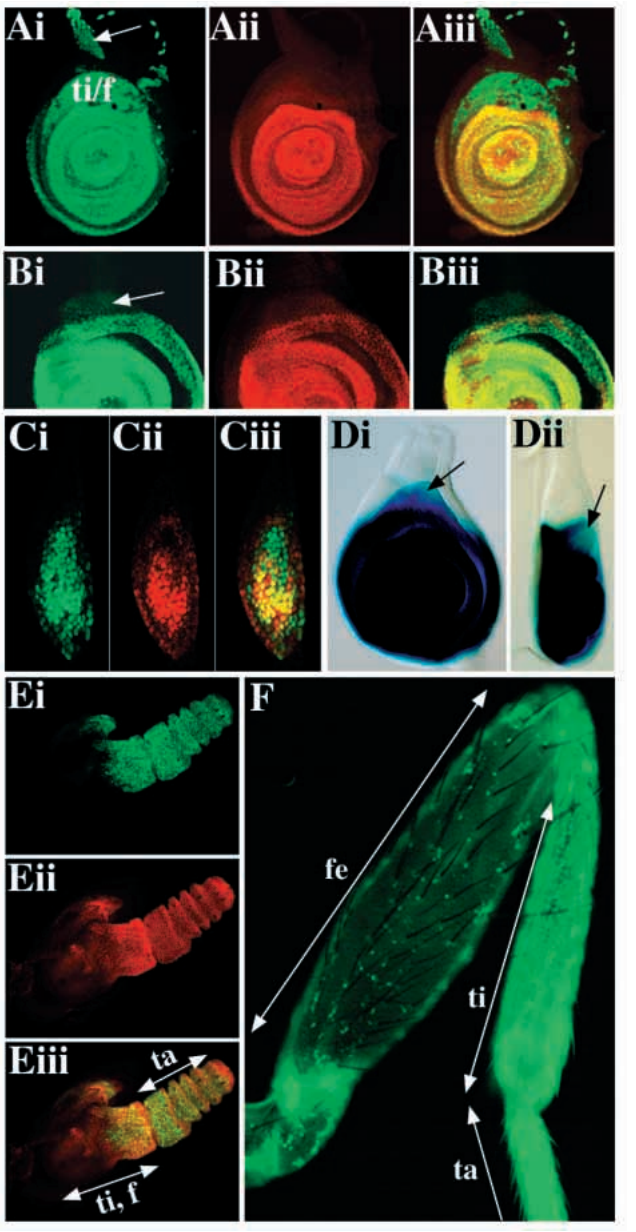


Fig. 7. *Dll* expression in legs. (A) Mature leg disc from line UAS flp/Dll-Gal4; Act5C>cassette>nuc-lacZ. (i) β-gal (antibody), (ii) Dll (antibody), (iii) merged image of i and ii. β-gal can be detected in a much wider domain than Dll protein including the proximal tibia/femur (ti/f) and regions giving rise to the body wall (arrow). (B) Dorsal half of mature leg disc from line Dll-Gal4; UAS-GFP (i) GFP; (ii) Dll (antibody). Weak GFP expression can be detected in the femur (arrow), but no Dll protein can be detected here. (C) Same as in B, but a much younger disc (late second instar). Strong GFP expression can be detected outside of the Dll protein domain. (D) Mature leg disc from line Dll-Gal4; UAS-lac Z; X-gal stained. Again weak staining is found in the femur (arrow). (E) Same as B and C, partially everted leg. The GFP expression is largely coincident with Dll protein staining and is confined to the tarsus (ta) and distal tibia (ti); no weak GFP expression outside of this domain can be detected on this preparation. (F) Adult leg from line Dll-Gal4; UAS-GFP showing GFP expression. Again strong expression is found in the tarsus (ta) and distal tibia (ti), but now it is also expressed in most of the cells of the tibia (ti) and in a single cell associated with each bristle in the femur (fe).

to perdurance of GFP or Gal4. Weak *lacZ* expression can also be detected in the presumptive proximal tibia/femur and again is likely due to perdurance (Fig. 7D).

By the time leg eversion occurs, the low level expression of GFP is generally no longer evident in the femur and proximal tibia (Fig. 7E). However, in the adult, GFP expression is clearly expressed in most of the cells in the proximal tibia and at high levels in single cells associated with each bristle in the femur (Fig. 7F; this expression appears to be initiated early in pupal life). There is a variable amount of expression elsewhere in the femur (not shown). These observations are also supported by analysis of Dll-G4 driving *yellow* (*y*) expression: although most bristles in the femur show little or no *y* expression, there is clear expression in all of the bracts (not shown).

Dll clonal analysis in the wing

Unlike the leg, Dll^{SA1} clones can be recovered anywhere in the wing even when they are generated early in development. As also shown by Gorfinkiel et al. (1997), the primary effect of these clones is on the differentiation of the wing margin. The characteristic hairs or bristles found at the margin are deleted or rudimentary in Dll^{SA1} clones located at the margin (Fig. 8A). In contrast to Gorfinkiel et al (1997) we find that the effect of these clones is autonomous so that, for example, a clone situated only on the ventral side of the margin will not affect the adjacent margin bristles or hairs in the dorsal region (Fig. 8A). The expression of three genes normally found at the margin, *wg*, *cut* and *ac* (Couso et al., 1994; Jack et al., 1991; Phillips and Whittle, 1993) was examined in wing discs containing Dll^{SA1} clones. Both *wg* and *cut* are expressed normally in Dll^{SA1} clones (Fig. 8B,C), but *ac* expression at the margin is lost autonomously in these clones (Fig. 8E).

DISCUSSION

al and *Dll* and specification of the proximodistal axis of the *Drosophila* leg by Wg and Dpp

The combination of the two secreted signaling molecules Wg and Dpp induces the formation of the P/D axis in the leg of *Drosophila* (Campbell et al., 1993; Campbell and Tomlinson, 1995; Diaz-Benjumea et a., 1994). It was originally suggested that the Wg/Dpp combination may establish an organizer at the distal tip that controlled patterning along the P/D axis and that this organizer is characterized by expression of the *aristaless* (*al*), homeobox gene (Campbell et al., 1993; Fig. 9Bii). Here, we show that even if such an organizer does exist then *al* is not absolutely required for its activity because removing *al* at the tip using a null allele does not prevent formation of the P/D axis, although it does prevent the formation of the structures normally found at the tip of the leg (Fig. 3). Ectopic *al* can induce outgrowths in the wing and these are associated with ectopic Wg expression, but there is no clear explanation for this phenomenon; it is possible that *al* may have some redundant function in maintaining Wg expression at the tip.

The existence of a distal organizer has also been questioned by a study that suggests Wg and Dpp may control patterning along the P/D axis directly rather than by inducing a secondary signal (Lecuit and Cohen, 1997). Analysis of downstream targets of the Wg/Dpp combination, including *Dll*, revealed these to be direct targets of the Wg and Dpp signaling pathways. A fuller understanding of this process requires a detailed analysis of the role of these downstream targets in the specification of the P/D axis. It has been shown previously (see Introduction) and here (Fig. 5F) that there is an absolute requirement for *Dll* activity for the formation of the P/D axis in the leg more distal than the most proximal segment, the coxa. However, most of these studies do not discriminate between a passive requirement of *Dll* for the development of this region of the leg and an active involvement in the specification of cell fates along the P/D axis in this region. Support for the latter view comes from analysis of hypomorphic alleles which are consistent with higher levels of *Dll* activity being required distally than proximally (Cohen and Jurgens, 1989b). However, Dll protein does not show a graded distribution (Diaz-Benjumea et al., 1994; Panganiban et al., 1994) and, as pointed out in the Introduction, there is a paradox between where *Dll* is expressed and where its activity is required: late in development Dll protein can be detected only in the tarsus and distal tibia, but the genetic data reveal that *Dll* function is also required cell autonomously in more proximal regions, the femur and all of the tibia (Table 1, Fig. 9). The results presented here can provide an explanation for both this paradox and for the phenotype of hypomorphic alleles and, further, may shed more light upon the mechanism by which Wg and Dpp control patterning along the P/D axis in the leg.

Spatial and temporal requirement for *Dll* in the leg

The results of the detailed clonal analysis with a *Dll* null allele presented here can be summarised as follows and are in general agreement with previous studies (Cohen and Jurgens, 1989a; Gorfinkiel et al., 1997). Before about the early third instar there is an autonomous requirement for *Dll* in cells of the leg more distal than the coxa, apart from the dorsal femur, but later in development this requirement is limited to a distal domain corresponding to the tarsus and distal tibia. This domain corresponds to the region where Dll protein can be detected at these stages with antibodies. Its requirement in more proximal regions earlier in development suggests it must be expressed in these regions at these stages. Either it is transiently expressed in these regions during early larval life or it is continually expressed at low levels (too low to be detected with antibody) but it only functions transiently in early stages.

Direct evidence that *Dll* is in fact expressed in the femur and proximal tibia at some stage in development is provided by generating inheritable marker gene expression in cells expressing *Dll* using the *Dll*-Gal 4/UAS-flp; Act5C>cassette>nuc-lacZ line (Fig. 7A). This suggests that *Dll* is expressed in all cells of the presumptive leg, but does not distinguish between expression in the embryo or later during larval life. Although the low level expression of GFP or β-gal driven by Dll-Gal4 in the proximal tibia/femur in third instar discs (Fig. 7B,D) may result from low level expression of *Dll* (below the level of antibody detection), we favour the model that it results from perdurance from earlier expression in these cells because this expression appears to decay throughout development (Fig. 7). It should be noted that at late larval stages the domain showing high levels of *Dll* expression corresponds to the region where *Dll* function is required so it is not unreasonable to suggest that similar high levels of expression will correspond to the regions where *Dll* function is required at earlier stages.

Additional support for this comes from a comparison of Dll

and Extradenticle (Exd), expression in early and late leg discs (Gonzalez-Crespo and Morata, 1996). Exd, or more correctly nuclear localized Exd (Mann and Abu-Shaar, 1996), is restricted to the periphery of the disc and defines the body wall and proximal extreme of the leg. In leg discs from second instars this nuclear Exd domain is directly adjacent the central Dll domain (detected by antibody), but later a clear gap develops between the distal Dll domain and the Exd domain. This suggests that during development the cells in this gap have lost either Dll or nuclear Exd expression (or both) and we would suggest that it is Dll.

If the above view is correct, the leg may be loosely divided into three regions along the P/D axis: (1) proximal – no *Dll* expression; (2) intermediate – expressed and required early, but not later, and (3) distal – continuous expression and requirement. This would then provide a clear explanation for the paradox of the apparent sites of *Dll* expression not explaining all the mutant phenotypes. Additionally, it is possible that one source of positional information along the P/D axis is the length of time a cell expresses *Dll*. The phenotype of *Dll* hypomorphs would then be explained if the lowered *Dll* activity in the centre of the leg discs from these mutants (which, for example, only allows the formation of intermediate leg segments in the strongest hypomorph; Fig. 4A) was equivalent to the transient expression of *Dll* in normal legs (which, according to the present proposal, also corresponds to intermediate leg segments).

Two exceptions to this simple hypothesis are the femur and the trochanter. Firstly, there is a differential requirement for *Dll* in the dorsal and the ventral femur (i.e. at the same P/D level) and at present there is no explanation for this, although it is possible that it is related to the situation in the embryo where dorsal Dpp appears to act as a repressor rather than activator of *Dll* expression (Goto and Hayashi, 1997). Secondly, the trochanter which is proximal, appears to require *Dll* activity even late in development. This late requirement may correspond to the proximal ring of *Dll* expression in the leg discs. It should also be noted that expression in this domain appears to be independent of Wg and Dpp (Diaz-Benjumea et al., 1994).

An additional peculiarity is that although *Dll* mutant tissue can differentiate normally sized bristles, it fails to form bracts at the base of these bristles even when the clones are generated late in development in proximal locations. This probably reveals a later function for *Dll* during pupal development because *Dll* expression extends more proximally during pupal life, and in the adult *Dll* appears to be expressed in a support cell of each bristle (Fig. 7F). Not all of these develop bracts, but *Dll* may be required in these cells to allow the development of this structure.

Static and dynamic models for Wg and Dpp controlling the formation of the P/D axis

If the model suggesting cell fate along the P/D axis is specified by Wg and Dpp directly (Lecuit and Cohen, 1997) proves to be correct, then one static, simplified version of this model could hold that different cell fates are established above strict concentration thresholds of Wg/Dpp which in turn would correspond to precise distances from the sources of these molecules (Fig. 9Biii). However, one possible problem with this simplified model is that of growth: as the imaginal disc grows in size the distance of any one cell from the source will vary so that for such a strict model to produce precise patterning, all cell fates may have to be established simultaneously. Relevant data suggest this not to be the case (Cohen, 1993). To account for growth, Lecuit and Cohen (1997) proposed that different target genes may require Wg and Dpp for different periods of time before expression becomes independent of these signals. Following growth, this could result in overlapping domains of target genes, and these domains could be established at different times in development. However, an alternative way of viewing this model is to propose that different cell fates are established not simply on the basis of how much Wg and Dpp they receive but by how long they receive it (Fig. 9Biv). Early in development most of the presumptive leg cells will receive a specific level of Wg and Dpp, but as the disc increases in size then presumptive proximal cells at the edge of the disc will begin to receive less Wg and Dpp as they become situated further from the sources, so that they will experience this specific level of Wg and Dpp for a shorter period of time than more centrally located, presumptive distal cells. Consequently, the length of time a cell receives this specific level of Wg and Dpp may provide positional information along the P/D axis: the longer

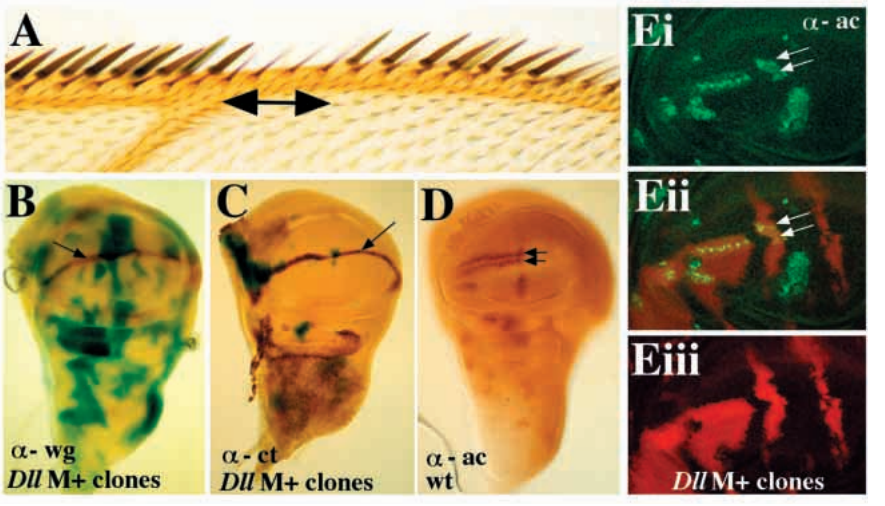


Fig. 8. Phenotype of Dll^{SA1} mutant clones in adult wings and discs. (A) Small clone (extent denoted by arrows) in adult wing marked by *y* and *sha*, present only in the ventral compartment. The ventral margin bristles are absent, but the dorsal hairs are still present showing the effect is autonomous. (B) Wg expression (brown; antibody) in a disc containing numerous *Dll* clones (wild-type tissue is green). Wg expression along the wing margin is normal in *Dll* mutant cells (arrow). (C) Cut (Ct) expression (brown; antibody) in a disc consisting almost entirely of *Dll* mutant tissue. Loss of *Dll* has no effect on Ct expression at the wing margin (arrow). (D) Achaete (Ac) expression (brown; antibody) in a wild-type disc. Ac is expressed in two lines (arrows) either side of the wing margin in the anterior compartment. (E) Ac expression (green) in a disc containing *Dll* mutant clones (wild-type tissue is red). The arrows are positioned as in (D). Ac expression at the margin is lost in *Dll* mutant cells; this effect is autonomous even across the D/V boundary.

it receives it the more distal it becomes. We would like to propose that the present results on *Dll* may provide some evidence for such a dynamic version of this model. These results suggest presumptive intermediate level cells express *Dll* early in development but it is lost later, whilst presumptive distal cells show continuous expression. If we assume that a cell expresses *Dll* above a certain threshold of Wg/Dpp, then its expression may be lost during development at the edge of its expression domain when these cells become situated further from the sources of Wg and Dpp as the disc grows in size.

One problem with this model is that maintenance of *Dll* expression does not appear to require continuous Wg and Dpp signaling (Lecuit and Cohen, 1997). *Dll* expression is not lost in clones of a Dpp receptor, *thick veins*, or a Wg signal transducer, *dishevelled*, even when these are made during the second instar, i.e. at a time when the present results suggest that *Dll* is still transiently expressed in some cells. There are at least two possible explanations for this. Firstly, the above model is correct but that it is impossible to determine timing of gene function by making clones because this ignores the possibility of perdurance of gene products. Secondly, the model is incorrect, but this may be because it assumes that Wg and Dpp are the only limiting factors controlling *Dll* expression (and patterning along the P/D axis): there may be an additional signal, possibly derived from the presumptive tip, which is also required for *Dll* expression.

A temporal mechanism for axis formation is more evident in vertebrate appendages where positional identity along the P/D axis appears to be determined by such a mechanism: the longer a cell spends in the 'progress zone', the region behind the tip of the developing limb, the more distal it becomes (Summerbell and Lewis, 1975; Wolpert et al., 1975). Consequently, the P/D axis is determined in a proximal to distal

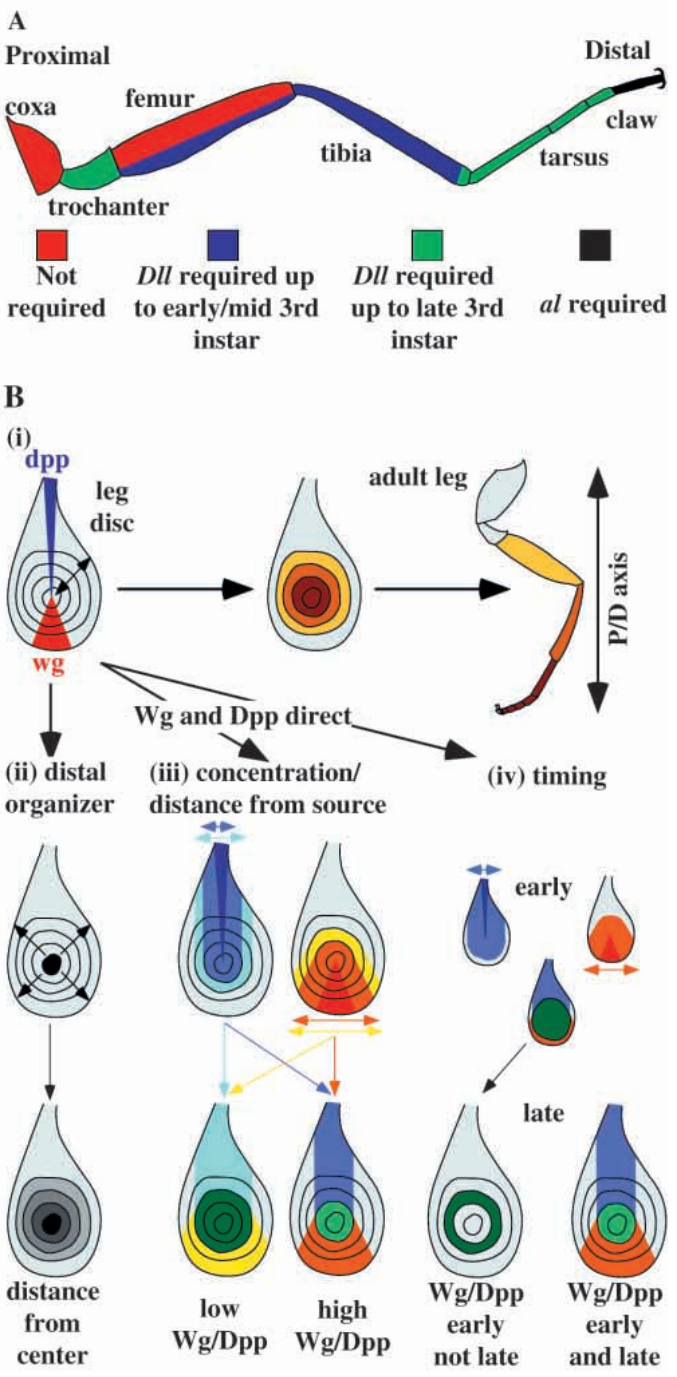


Fig. 9. (A) Summary of the requirements for *Dll* and *al* in the formation of the P/D axis of the leg. In the leg, *al* is only required for the normal growth and differentiation of the tip. Early in development *Dll* is required in all of the presumptive leg cells apart from the coxa and the dorsal femur. The requirement in the rest of the femur and the tibia is lost in the early to mid third instar, whilst there is a continuous requirement for *Dll* in the trochanter, distal tibia and tarsus up to the late third instar. (B) Specification of the P/D axis by Wg and Dpp. (i) The P/D axis of the adult leg corresponds to the radius of the disc so the question being addressed is how are concentric circular domains established by the dorsal Dpp and ventral Wg stripes. (ii) The distal organizer model proposed that a signaling centre is established at the site where Wg- and Dpp-expressing cells are juxtaposed in the centre of the disc, the presumptive distal tip. This would be the source of a secondary signal that would establish cell fate along the P/D axis according to distance from the source. *al* is expressed at the site of this proposed organizer, but the present results suggest that, even if such an organizer exists, *al* does not play an essential role in its activity. An alternative model suggests that Wg and Dpp specify different cell fates along the P/D axis directly. There are at least two possible mechanisms by which this could occur. (iii) Different target genes are activated at different threshold concentrations of Wg and Dpp which correspond to different distances from their site of synthesis: Wg and Dpp are diffusible and the dark and light colours represent high and low concentrations surrounding their sources. The overlap of low Wg/low Dpp will establish a wider circular domain than high Wg/high Dpp. (iv) One possible problem with the latter model is that the distance a cell is from the sources of Wg and Dpp will vary as the disc grows in size. An alternative model can be based upon the length of time a cell receives the Wg and Dpp signals. Early in development much of the disc will be within the overlap of a given Wg concentration and Dpp concentration. As the disc grows in size the cells at the edge of the original overlap-zone will fall below this Wg/Dpp threshold as they become situated further from the sources. Later in development the same Wg and Dpp concentrations will define a proportionately smaller central domain surrounded by the cells that were above these threshold concentrations earlier in development but not at this late stage. The present results on the requirement for *Dll* within the leg, outlined in A, suggest such a mechanism may be operating in this system because it appears that *Dll* is expressed over a greater length of the P/D axis early in development than later.

sequence. In *Drosophila*, there is contradictory evidence as to the order in which segments are specified along the P/D axis (reviewed by Cohen, 1993) and further studies are required to resolve this question.

Dll is required for tarsal gene expression and to modify the adhesive properties of cells

The leg trunctations associated with *Dll* hypomorphs correlate with the loss of expression of genes required for the development of the tarsus, including *al* and *bric à brac* (*bab*; Fig. 4). However, further positive and/or negative signals are required to establish the expression domains of *al* and *bab* because *Dll* is expressed uniformly throughout the tarsus (Diaz-Benjumea et al., 1994; Panganiban et al., 1994; Fig. 1E,F), whereas *al* is expressed only at the tip and *bab* is expressed in the rest of the tarsus but is absent from the tip (Godt et al., 1993; Figs 1A,B, 4C). Thus, it is possible that *Dll* may not directly control cell identity along the P/D axis in the leg, but may be required to permit the expression of other genes that do this.

Another function of *Dll* in the leg may be to control the adhesive properties of cells because *Dll* clones clearly sort out from surrounding wild-type cells (Figs 5, 6). In adults, *Dll* clones can often be identified as internal cuticular vesicles, particularly in distal regions late in development, suggesting that the mutant cells have sorted out from the surrounding wild-type cells (Fig. 5). This phenomenon can be dramatically demonstrated in leg discs using the Minute technique, where the majority of wild-type tissue is confined to a central domain surrounded by mutant tissue (Fig. 6E,F). The modification of adhesive properties of cells destined to generate a leg may be very important, firstly to define a homogenous population of presumptive leg cells and secondly to induce this group of cells to form an outgrowth perpendicular to the body wall.

Dll is required for the differentiation of the wing margin

In the mature wing disc, *Dll* is expressed in the wing pouch in a graded fashion centred on the wing margin and appears to be downstream of Wg in this appendage (Neumann and Cohen, 1997; Zecca et al., 1996). Its function in the wing is quite distinct from that in the leg. Firstly, it is not required for growth and axis formation in the wing, because a wing disc in which *Dll* has been almost completely removed by clones is morphologically normal (Fig. 8). Additionally, it does not appear to affect cell adhesion in the wing because *Dll* clones generated early in development can be recovered anywhere in the adult wing. However, these clones do have distinct phenotypes, the most striking being an autonomous deletion of the bristles and hairs normally found at the wing margin (Fig. 5). The margin is characterized by the expression of a number of genes including *wg*, *cut* and *achaete* (*ac*; Couso et al., 1994; Jack et al., 1991; Phillips and Whittle, 1993): *wg* and *cut* are expressed normally in *Dll* clones in the wing (Fig. 8B,C); this is not surprising because Wg appears to be upstream of *Dll* and the phenotype of *wg* and *cut* mutations in the wing is more severe than *Dll* (Couso et al., 1994; Diaz-Benjumea and Cohen, 1995; Jack et al., 1991). However, *ac* expression at the margin is absent in cells lacking *Dll* (Fig. 8E) showing that *Dll* is required for the normal differentiation of the wing margin.

Evolutionary implications

Homologues of *Dll* have been identified in a wide variety of animals and these are often expressed in their developing appendages (Carroll et al., 1994; Dolle et al., 1992; Panganiban et al., 1994; Panganiban et al., 1995). *Dll* is expressed in both the wing and the leg of *Drosophila*, but the present results suggest that it has distinct functions in these two appendages. Thus, although the expression patterns in other species are quite striking, the present results suggest that it may not be possible to assign a single function to *Dll* in all these types of appendages. However, we suggest that if *Dll* does have a common function in many of these appendages it is probably closer to that of *Dll* in the *Drosophila* leg (because these are probably more ancient than wings in evolutionary terms) and this may be to control the adhesive properties of cells to allow the formation of outgrowths from the body wall.

For materials and stocks used in the course of this study we would like to thank Marcel Wehrli, Gary Struhl, Yu Chen, Isabel Guerrero, Dorothea Godt, Frank Laski, Karen Blochlinger, Larry Ackerman, Yuh-Nung Jan, Steve Cohen, Xiwei Wang, Roel Nusse, Kathy Matthews and the Bloomington stock center, Phyllis Oster and the Bowling Green stock centre, Pat O'Farrell, Joseph Jack, Grace Panganiban, Kathy Vorwerk, Sean Carroll. For useful comments on the manuscript we would like to thank Richard Mann, Gary Struhl and anonymous reviewers.

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