The homeobox gene *Distal-less* induces ventral appendage development in *Drosophila*

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This study investigates the role of the homeobox gene *Distal-less* (*Dll*) in the development of the legs, antennae, and wings of *Drosophila.* Lack of *Dll* function causes a change in the identity of ventral appendage cells (legs and antennae) that often results in the loss of the appendage. Ectopic Dll expression in the proximal region of ventral appendages induces nonautonomous duplication of legs and antennae by the activation of *wingless* and *decapentaplegic.* Ectopic *Dll* expression in dorsal appendages produces transformation into corresponding ventral appendages; wings and halteres develop ectopic legs and the head–eye region develops ectopic antennae. In the wing, the exogenous Dll product induces this transformation by activating the endogenous *Dll* gene and repressing the wing determinant gene *vestigial.* It is proposed that *Dll* induces the development of ventral appendages and also participates in a genetic address that specifies the identity of ventral appendages and discriminates the dorsal versus the ventral appendages in the adult. However, unlike other homeotic genes, *Dll* expression and function is not defined by a cell lineage border. Dll also performs a secondary and late function required for the normal patterning of the wing.

[*Key Words: Drosophila* distal appendages; dorsal-to-ventral limb transformation; *Distal-less; vestigial*]

Received February 24, 1997; accepted in revised form July 4, 1997.

The adult structures of *Drosophila* are constituted by a main body or ''trunk'', and a number of outgrowths or appendages such as wings, legs, antennae, etc. All these structures are differentiated by imaginal cells that are grouped in specific imaginal discs in the head and thorax (for review, see Cohen 1993). In the thorax, each adult segment is formed by the derivatives of two types of discs—one contributing to the dorsal and the other to the ventral part of the segment. The humeral, wing, and haltere discs form the dorsal prothoracic, mesothoracic, and metathoracic regions, respectively. Ventrally, there is a pair of leg discs per thoracic segment. In the head, most of the cephalic structures are differentiated by the eye–antennal disc, with the exception of the clypeous and the proboscis. These structures originate from other discs (Gehring and Seippel 1967). The eye–antennal disc is more complex than the thoracic discs because it is formed by precursors from more than one embryonic segment (Cohen and Jürgens 1991; González-Crespo and Morata 1995). Moreover, unlike the thoracic discs, it contains dorsal and ventral derivatives. The antennal part can be transformed into a complete leg in homeotic *Antennapedia* (*Antp*) mutations (Gehring 1966), whereas the eye part can be transformed into a wing by *ophtalmoptera* mutations. This suggests that the antenna is a ventral derivative and the eye a dorsal derivative (see Morata and Lawrence 1979).

Several developmental characteristics are common to dorsal and ventral appendages. For example, the role of *engrailed* (*en*), *hedgehog* (*hh*), and *decapentaplegic* (*dpp*) in the signalling mechanism responsible for morphogenesis (Basler and Struhl 1994). However, other genes such as *wingless* (*wg*), *apterous* (*ap*), *vestigial* (*vg*), and *Distal-less* (*Dll*) are expressed very differently in dorsal and ventral discs (Cohen 1993). Of these genes, *Dll* appears to have a critical role in the development of ventral appendages, legs, and antennae (Sunkel and Whittle 1987; Cohen and Jürgens 1987a,b). It is expressed in the central part of the leg and antennal discs, a region that contains the precursor cells of the more distal regions of both appendages (Cohen 1993). Activation of *Dll* expression in the leg and antennal discs is triggered by localized expression of *hh* (Díaz-Benjumea et al. 1994; Campbell and Tomlinson 1995) in the posterior compartment, which directs the expression of *wingless* (*wg*) in ventral–anterior cells and *dpp* in dorsal–anterior cells close to the anterior–posterior (A/P) compartment boundary (Basler and Struhl 1994; Díaz-Benjumea et al. 1994). The juxtaposition of *wg*- and *dpp*-expressing cells in the central region of the disc activates *Dll* (Díaz-Benjumea et al. 1994; Campbell and Tomlinson 1995). It has been proposed that the proximo-distal (P/D) axis of the limb is established by cell–cell interactions that maintain *Dll*

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expression (Díaz-Benjumea et al. 1994; Held et al. 1994, 1995; Campbell and Tomlinson 1995). These Wg and Dpp signals confer dorsalizing and ventralizing properties to the cells close to their respective expression domains (Peifer et al. 1991; Couso et al. 1993; Struhl and Basler 1993; Díaz-Bemjumea and Cohen 1994; Held and Heup 1996). Mutual repression by Wg and Dpp signalling systems generates a stable regulatory circuit by which each gene maintains its own expression in a spatially restricted domain (Brook and Cohen 1996; Jiang and Struhl 1996; Johnston and Schubiger 1996; Penton and Hoffman 1996; Theisen et al. 1996; Heslip et al. 1997). Ectopic expression of *wg* or *dpp* in the leg imaginal disc can induce ectopic expression of Dll and therefore duplication of the P/D axis (Díaz-Benjumea et al. 1994). However, it is not known whether *Dll* activity is able to induce the formation of the appendage.

Genetic and mosaic analyses have shown that *Dll* is required specifically in the areas defined by its expression pattern. The removal of *Dll* activity gives rise to a phenotype interpreted as the loss of most of the leg, from the trochanter to the tarsus (Cohen and Jürgens 1989a,b). A similar effect is found in the antennal cells that fail to develop in the absence of *Dll* function (Cohen and Jürgens 1989a,b). It has been argued (Cohen and Jürgens 1989b; Cohen 1993; González-Crespo and Morata 1996) that the region of the leg corresponding to *Dll* expression is the true appendage and that the proximal leg structures, coxa and pleurae, are formed by an expansion of the trunk. According to this theory, *Dll* expression would define the true appendage.

Although it is clear that *Dll* has an important role in appendage development, its specific function in the determination of leg and antennal patterns is uncertain. Dll^- cells fail to develop in these appendages and consequently, it is not known whether its function is connected with a developmental switch as in other homeobox genes such as *en, Ultrabithorax* (*Ubx*), or *ap* (Morata and Lawrence 1975; Morata and García-Bellido 1976; Blair 1993; Díaz-Benjumea and Cohen 1993; Guillén et al. 1995; Tabata et al. 1995; for review, see Lawrence and Morata 1994). Moreover, *Dll* is also expressed in the wing imaginal disc (Díaz-Benjumea and Cohen 1995), although the functional significance of this expression is unknown.

To further investigate the developmental role of *Dll*, we have re-examined the phenotype of Dll^- cells in the ventral and dorsal appendages and also expressed the Dll product ectopically in distinct locations of different imaginal discs. *Dll* was shown to have two separate functions: a primary function to induce the formation of ventral appendages and their identity and a secondary function involved in the differentiation of the wing margin pattern.

Results

Expression and requirements for Dll *in the dorsal and ventral appendages*

The eye–antennal, leg, and wing discs are of primary concern in this study, although *Dll* is also expressed in the genitalia (N. Gorfinkiel, G. Morata, and I. Guerrero, unpubl.). The Dll product accumulates in the central part of the leg and antennal discs. This region corresponds to the distal elements of the appendages (Fig. 1A,B) (Díaz-Benjumea et al. 1994). A more proximal ring of expression exists in the leg disc and is separated from the main body by an area of little or no expression (see also Cohen 1993). The wing disc has a very different expression pattern. The product is first detected in the early third instar in a few cells of the distal region of the wing pouch at both sides of the D/V border (Fig. 1C), long after full expression is established in the leg (Díaz-Benjumea et al. 1994). By the second half of the third instar the Dll product accumulates along the D/V border as described previously (Díaz-Benjumea and Cohen 1995), extending to the wing pouch (Fig. 1D). Therefore the activity of *Dll* in the wing not only differs from that of the antenna and leg in its topography of expression but also appears later. *Dll* expression in the third-instar haltere disc was also examined and was found to differ from that in the wing disc at the same stage; the Dll product accumulates in two regions in the anterior and posterior compartments, respectively, but there is no detectable expression along the D/V border (Fig. 1E). Because *Ubx* mutations transform the haltere into a wing disc, it is suggested that *Ubx* acts as a negative regulator of *Dll* in adult cells as reported for the embryo (Cohen et al. 1989).

These expression patterns can be visualized directly in the adult structures using the GAL4/UAS–*yellow*$^+$ (y^+) method (Calleja et al. 1996). Several GAL4 insertions were found in the *Dll* locus allowing distinction of the adult regions where *Dll* is expressed according to the y^+ rescue observed. These results are schematized in Figure 1F. In the adult leg, the coxa and pleurae do not show signs of y^+ rescue, although there is clear rescue in part of the trochanter where some bristles are y^+. There is weak rescue in the femur that appears to be restricted to the bristles that show intermediate pigmentation between y^- and y^+ and finally there is strong rescue in the region from the tibia to the tarsus. In the antenna, the Dll product is present in the aII and aIII antennal segments and the arista. The wings of *Dll*-GAL4/UAS–y^+ flies show y^+ rescue in nearly all the bristles and hairs along the anterior and posterior compartments of the wing margin. The y^+ rescue also extends into some cells of the inner region of the wing blade, but the precise limit is difficult to estimate. The description of the adult Dll expression pattern is in accordance with that observed in imaginal discs.

According to the expression studies described above, *Dll* subdivides the appendages into two clearly defined regions; one containing and the other not containing the Dll product. Because homeotic genes expression is often defined by cell lineage (compartment) borders (for review, see Lawrence 1992), cell lineage analysis was performed to ascertain whether the border of Dll expression corresponds to a cell lineage restriction. Previous work (Steiner 1976) has already shown that there is no restriction. Using the FRT/FLP method (Golic 1991), y^- clones

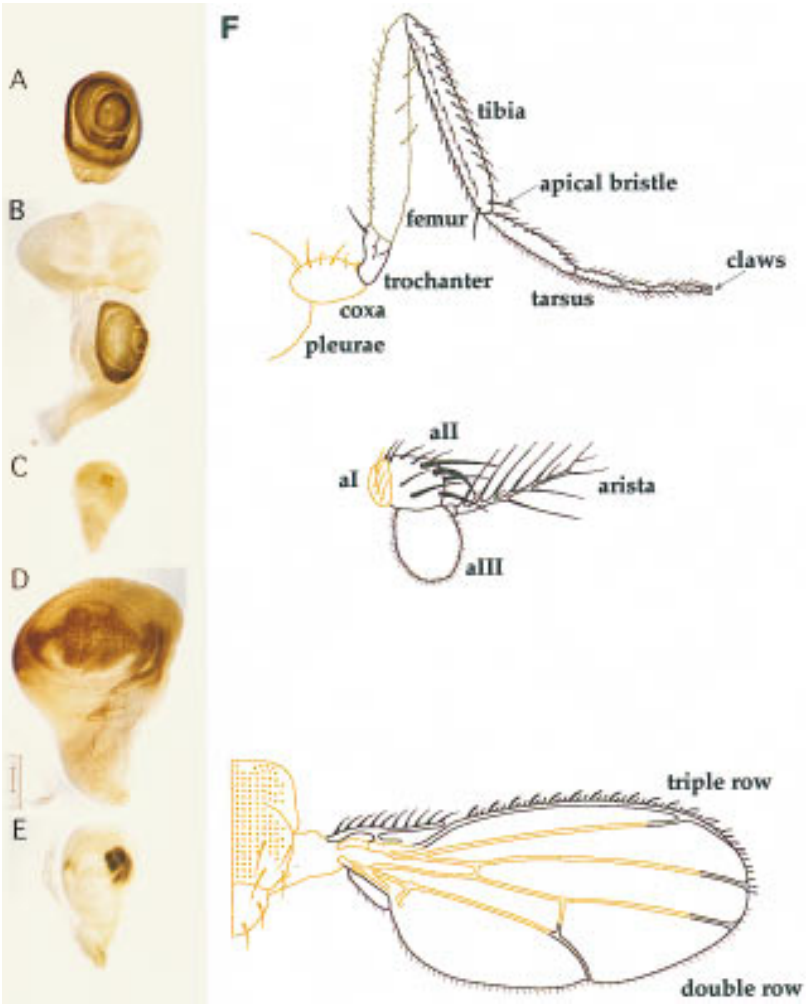

Figure 1. Dll expression domains. (*A–E*) Wild-type Dll expression patterns visualized with Dll antibody in late third instar imaginal discs (except for *C*). (*A*) Leg disc; (*B*) Eye–antennal disc; (*C*) early third instar wing disc; (*D*) wing disc; (*E*) haltere disc; (*F*) schematic representation of Dll expression domains in leg, antenna, and wing as visualized in *Dll*–GAL4/UAS–y^+ flies. The brown color stands for the y^+ rescue. Note the paler brown color indicating weak y^+ rescue in the femur. See main text for a detailed description.

were induced at different periods during larval development (see Materials and Methods). Special attention was paid to the leg clones in the proximity of the trochanter and to the antennae in the border between aI and aII. It was observed that even clones initiated at early third instar [72–96 hr after egg laying (AEL)] may extend to *Dll* expressing and nonexpressing cells. The same result is obtained by analysis of the behavior of *armadillo* (*arm*)–*lacZ* clones in the leg imaginal disc. Clones (marked by the lack of β-gal staining) induced after 72 hr of development can extend to both Dll-expressing and not expressing domains (Fig. 2A). Consequently, *Dll* expression is not maintained by cell lineage.

Expression patterns suggest that *Dll* is required for the development of both ventral and dorsal appendages until late in development, although the distinct expression patterns in the antennal and leg discs with respect to the wing discs suggest different functions. Early requirements for *Dll* in the antennal and leg discs have been reported already (Cohen and Jürgens 1989b) and can be summarized as follows: Dll^- cells cannot proliferate in these appendages with the exception of the more proxi-

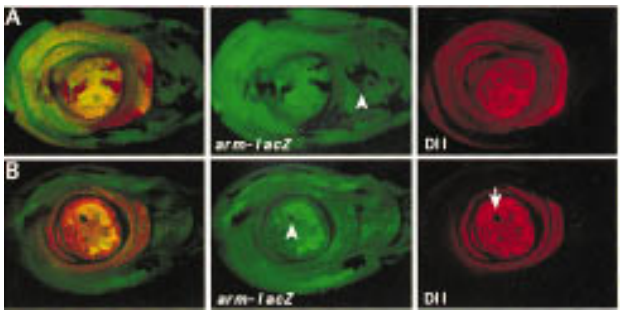

Figure 2. Mitotic recombinant clones in leg discs induced by the FRT–FLP system at 72–96 hr AEL (early third instar). Leg discs were stained with anti-β-gal (green) and anti-Dll (red). (*A*) Wild-type clones marked by the absence of β-gal expression. The arrowhead indicates a clone that extends to the boundary between Dll expressing and Dll nonexpressing cells. (*B*) Dll^{SA1} clones marked by the absence of β-gal and Dll expression and their twin spots by the elevated level of β-gal. Note that the Dll^{SA1} clones do not proliferate further. Here and in all remaining images of leg discs, dorsal is to the *right*.

mal structures, the pleurae and coxa of the leg and the first segment of the antenna. It is noteworthy that the coxa and aI antennal segment are considered homologous structures (Posthlewait and Schneidermann 1971). Therefore the leg and antennal discs exhibit homologous expression and requirement for *Dll*.

Using the FLP/FRT method, *Dll*$^-$ clones were induced during different developmental periods of the leg, eye–antennal, and wing discs (Fig. 3). The results of lack of Dll function in the legs are illustrated in Figure 3A–C. Early clones, induced during the first and second instar (24–72 hr AEL), behave as reported by Cohen and Jürgens (1989b)—they only appear in the pleural and coxa regions and produce no morphological alteration. Very few small and abnormal clones were found in the femur to tarsus region. Although clones were undoubtedly produced in these regions, they appear to be eliminated from the region (see also Cohen and Jürgens 1989b). In the antennae, first-instar *Dll*$^-$ clones (Fig. 3D–F) are detected because they are able to differentiate aI antennal and a small part of the aII segment but fail to form the rest of aII and aIII segments and the arista. This is in agreement with previous observations (Cohen and Jürgens 1989b).

In contrast with early clones, those induced during the third-larval (72–120 hr AEL) periods are recovered frequently in the distal regions of both legs and antennae. In the trochanter and the tibia–tarsus region of the leg, the majority of *Dll*$^-$ clones form vesicles that invaginate inside the appendage. These often differentiate y^- bristles and trichomes that do not resemble those in the vicinity of the clone, indicating that lack of *Dll* function produces a change in the cell type (Fig. 3B,C). Interestingly, the clones in the intervening region, the femur and proximal tibia, behave differently. These clones differentiate bristles of the corresponding type, but are often unable to induce a neighbor cell to differentiate a bract, an accompanying structure of many of the leg bristles (Fig. 3A). It is possible that *Dll* is required only in the bristle mother cells of this region and explains why this requirement has not been visualized by antibody or *lacZ* staining of the disc. Late clones in the antennae are able to differentiate, but in the aII and aIII segments they tend to segregate, forming vesicles that separate from the surrounding wild-type tissue. It is difficult to establish the identity of the patterns formed by these clones but these often differentiate bracted bristles in the base of the arista, suggesting an antenna-to-leg transformation (Fig. 3E,F). A similar transformation has been observed in hypomorphic *Dll* mutations (Sunkel and Whittle 1987; Cohen and Jürgens 1989a).

The loss of early *Dll*$^-$ clones in legs and antennae may suggest a *Dll* requirement for cell proliferation. To test this possibility, the sizes of *Dll*$^-$ and twin *Dll*$^+$ clones in mature discs of genotype FRT *arm–lacZ*/FRT *Dll*^{SA1} were compared. The *Dll*$^-$ clones, marked by lack of β-gal staining, only contain a few cells and are only detected occasionally, but the accompanying twin clone, labeled by the double intensity of β-gal, is much larger in size (Fig. 2B). This effect on proliferation of the leg *Dll*$^-$ cells was not observed in the wing imaginal cells (data not shown).

In contrast with that observed in the leg and antennal

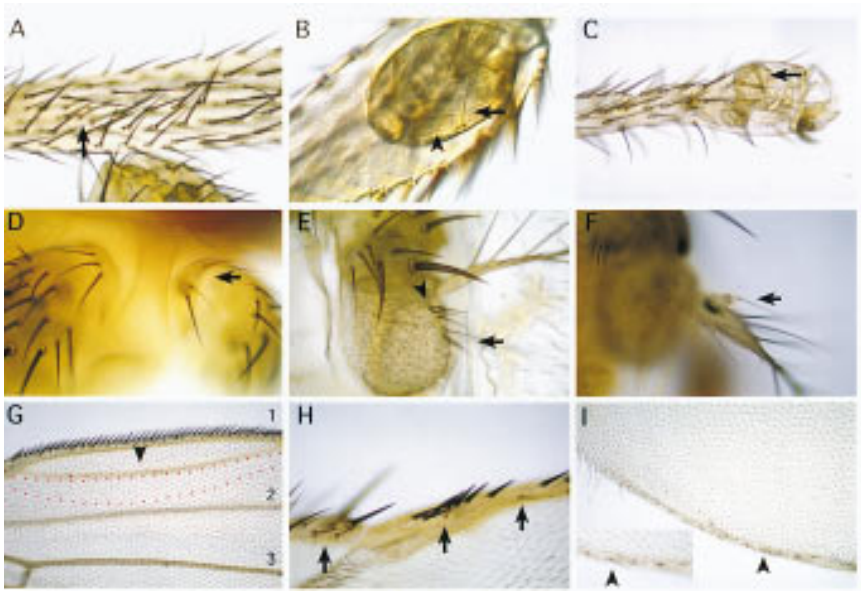

Figure 3. Phenotypic effects of *Dll*^{SA1} clones in the leg (*A–C*), antenna (*D–F*), and wing (*G–I*). The clones were induced during 24–120 hr AEL and marked with *y* except for *G* where clones were marked with *forked*^{36}(*f*^{36}) (see Materials and Methods). (*A–C*) Clones in the leg. Early induced clones (24–48 hr AEL) only appear in the coxa as it has been described previously. Clones induced later (72–120 hr AEL), however, are able to proliferate and differentiate nonbracted bristles in the proximal tibia (*A*) and vesicles of y^- tissue that segregate from the surrounding wild-type tissue in the distal tibia (*B*) and tarsus (*C*). Arrows indicate *y* bristles; arrowheads indicate trichomes that are not present in the distal leg. (*D–F*) Clones in the antenna. An early (24–48 hr AEL) clone in aI (*D*) does not produce a mutant phenotype as aI does not require Dll activity. Late clones (72–120 hr AEL) in the aIII antennal segment (*E*) and arista (*F*) develop bristles sometimes with an associated bract. Arrows indicate y^- bristles; arrowheads indicate bracted bristles. (*G–I*) Clones in the wing. The clones near the D/V margin give rise to extra-vein tissue. The red dashed line indicates a dorsal clone marked with *f* close to vein 1. Normal veins 1, 2, and 3 are indicated. Arrowhead indicates extra-vein (*G*). Clones that abut the D/V boundary also eliminate bristles of the triple row in the A compartment (*H*) and long hairs of the double row in the P compartment (*I*). (*Inset* in *I*) Magnification showing the y^- bristles with socket in the P compartment (arrowhead). Arrows indicate *y* bristles.

discs, both early and late y^- (Dll^-) clones were detected readily in the wing disc (Fig. 3G–I). These clones always affect the wing margin, eliminating the triple row of bristles in the anterior compartment (Fig. 3H) and the double row of long hairs in the posterior compartment (Fig. 3I). These were interpreted as Dll^- clones because the majority of them were able to differentiate a few y^- bristles. An important feature is that they affect both the dorsal and the ventral compartments, even if initiated during the third instar (72–96 hr AEL) after the D/V compartment boundary has been established (Morata and Lawrence 1979) and are therefore supposed to be confined to either compartment. This may indicate a nonautonomous effect or perhaps a transgression of the D/V border by the Dll^- clones. In some experiments, Dll^- clones were marked with $forked^{36}$ (f^{36}) to investigate the behavior of clones away from the margin. It was observed that these internal clones often affected vein differentiation in the vicinity of the wing margin, producing extra veins and sometimes eliminating parts of normal veins. This effect appears at times to be nonautonomous, as wild-type cells near Dll^- cells are often affected (Fig. 3G). Another intriguing feature of Dll^- clones is that they differentiate socketed bristles in the posterior compartment similar to those in the distal part of the anterior compartment (Fig. 3I) and also differentiate a halo of pigment, another feature of the wing margin in the anterior compartment. These observations suggest a late involvement of *Dll* in the maintenance of posterior identity.

Ectopic Dll *expression*

To assay the developmental potential of the Dll product, the GAL4/UAS system (Brand and Perrimon 1993) and a combination of the flip-out and GAL4 activation systems (Pignoni and Zipursky 1997) was used for expression in different body regions. We first checked the activity of the UAS–*Dll* construct by assaying its ability to rescue the *Dll* phenotype when expressed under *Dll* control. The line em212 carries the pGawB transposon inserted in the *Dll* locus and is a null mutant for *Dll*. The em212-GAL4/$Df\ (2R)Dll^{MP}$ combination is lethal, but the lethality is rescued when the UAS–*Dll* construct is added. Consequently, em212-GAL4/$Df\ (2R)Dll^{MP}$;UAS–*Dll* flies survive and are of almost normal phenotype. In similar combinations, the UAS–*Dll* gene also rescues the phenotype of hypomorphic mutations such as Dll^3 or Dll^{IB}.

Ectopic Dll expression in the leg and antennal discs produces duplications of the P/D axis It was found that a general increase of the Dll product in the *Dll* domain, in a wild-type background, affects the more distal segments of the legs and antennal segments that are reduced in size (Fig. 4A) or missing. Therefore an excess of Dll product appears to result in a loss-of-function phenotype. Because the em212–GAL4/+ flies contain a normal dose of *Dll*, the implication is that the excess of Dll product in em212–GAL4/+; UAS–*Dll* flies suppresses the activity of endogenous *Dll* gene. Lower expression levels of the endogenous *Dll* were found in em212–GAL4/*Dll–lacZ*;UAS–*Dll* discs (data not shown).

To assess the effect of ectopic *Dll* in the proximal leg and antennal regions where the gene is not expressed, *Dll* activity was induced in random patches by flip-out, using the same UAS–*Dll* (see Materials and Methods). Leg duplications were obtained when the clones were located in the proximal part of the leg (Fig. 4B). This was also seen in the disc as duplication of the growth cone (Fig. 4C–G). The induction of ectopic legs implicates a nonautonomous process and the marked clone is located in the distal part of the duplicated leg primordia. These ectopic Dll^+ clones repress endogenous *Dll* expression (visualized by Dll–*lacZ* expression) autonomously, but induce *Dll* expression in cells outside of the clone (Fig. 4C). This nonautonomous effect can also be visualized using other ventral disc markers. *bric à brac* (*bab*) is a gene expressed in the leg and antennal discs in the presumptive region of the most distal segments (Godt et al. 1993; Fig. 7D, below) and is required for the proper segmentation of the tarsus. It has been suggested that it is regulated by Dll (Godt et al. 1993). We found that *bab* is activated in the marked Dll^+ clone and also outside of the clone (Fig. 4D). *dachshund* (*dac*) is another gene expressed and required in the leg and antennae. It is expressed in the third antennal disc segment and in the presumptive trochanter, femur, tibia, and proximal tarsal segments of the leg disc (Mardon et al. 1994). *dac* is induced in the duplicated structure outside the labeled Dll^+ clone (Fig. 4G).

Wg and Dpp have a long-range effect and are responsible for ventral and dorsal fates in the leg respectively (Peifer et al. 1991; Couso et al. 1993; Struhl and Basler 1993; Díaz-Benjumea and Cohen 1994; Held and Heup 1996). To explain the long-range effect of Dll^+ clones we analyzed whether *wg* and *dpp* were also activated. As shown in Figure 4, E and F, there is *wg* and *dpp* expression in cells within (probably in complementary domains as in the normal leg) and outside the Dll^+ clone. This is in accordance with the presence of ventral and dorsal structures in the duplicated legs.

Ectopic Dll expression in the wing and haltere discs produces ectopic legs When the Dll product is expressed under the control of certain GAL4 lines that produce uniform *Dll* expression in the wing pouch, such as the C-68a and C-765 lines, it gives rise to rudimentary appendages lacking most structures. However, when GAL4 lines such as E132–GAL4, *optomotor-blind* (*omb*)–GAL4, *apterous* (*ap*)–GAL4, or *patched* (*ptc*)–GAL4 are used to induce localized expression in the wing, this structure is replaced partially by tissue containing bracted bristles and claws typical of the leg (Fig. 5; see legend for frequency). Rudimentary ectopic legs with claws formed at their distal ends are observed (Fig. 5B,C,E). In some cases, these ectopic legs include distal tarsal segments, the tibia, and part of the femur (Fig. 5E). These ectopic legs appear in the proximal part of the wing and at times present apical bristles, a marker of mid-leg identity (Fig. 5E). The halteres undergo very

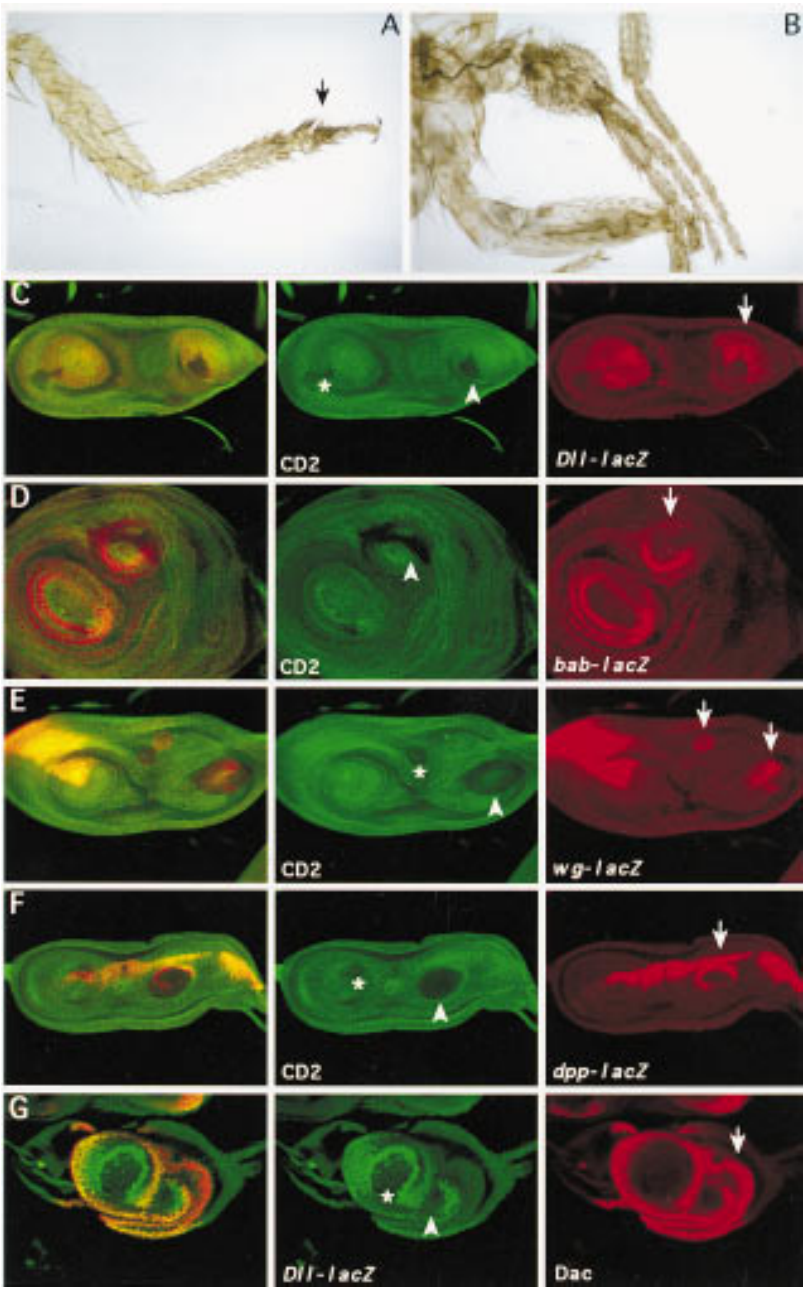

Figure 4. Effect of ectopic expression of Dll in the leg. (*A*) Phenotypic effect of the overexpression of Dll in its own domain. Distal part of the leg of em212–GAL4/UAS–*Dll* flies. The arrow indicates fusion of the tarsi. (*B*) Leg duplication induced by ectopic Dll^+ clones (see Material and Methods). (*C–G*) Dll^+ clones in leg imaginal discs induce duplication of the P/D axis. Clones were marked by the absence of the CD2 marker (except for the last panel) and were scored for the ectopic expression of *Dll–lacZ* (*C*), *bab–lacZ* (*D*), *wg–lacZ* (*E*), *dpp–lacZ* (*F*), and the Daschund (*G*) protein. Note the long-range effect of the Dll^+ clones in the induction of gene expression. Arrowheads indicate clones that induce duplication of the P/D axis; asterisks indicate clones that do not induce duplication; arrows indicate ectopic expression of the different markers.

similar transformations to those described in the wings, presenting also ectopic leg structures (Fig. 5D). It is difficult to ascertain the identity of these legs, although apical bristles were not observed.

The repression of wing development by the ectopic Dll product suggests that wing-forming genes should also be suppressed. The *vg* gene is likely to be affected specifically. This gene is activated in presumptive wing cells at the time of separation of the leg and wing primordia (Cohen et al. 1993) and is selectively required for wing cell proliferation (Williams et al. 1991). It has also been demonstrated that its expression is sufficient to induce outgrowths of wing tissue in other imaginal discs (Kim et al. 1996). The effect of the Dll protein on *vg* expression is illustrated in Figure 6: The Dll product suppresses *vg* expression.

Because *Dll* shows positive autoregulation during embryonic development (Vachon et al. 1992; Castelli-Gair and Akam 1995), the possibility of the GAL4-driven Dll product inducing ectopic activation of the endogenous *Dll* gene during imaginal disc development was investigated. As shown in Figure 6D, exogenous Dll product activates endogenous *Dll*. The area of *Dll* activation corresponds to the part of the wing disc that is morphology

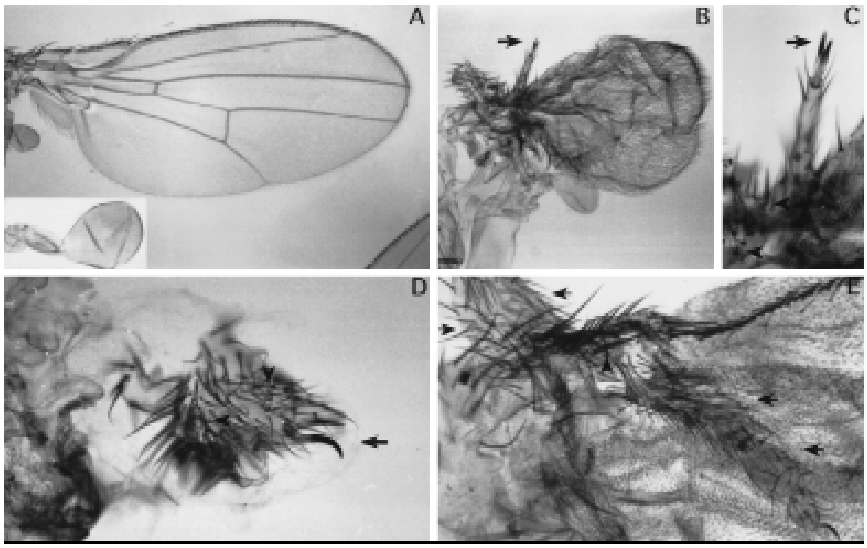

Figure 5. Dorsal to ventral transformation in the thorax induced by ectopic *Dll*. (*A*) Wild-type wing. (*Inset*) Wild-type haltere. (*B*) Ectopic leg tissue emerging from the hinge region of E132-GAL4/UAS–*Dll* flies. (*C*) Magnification of *B*. (*D*) Ectopic leg tissue arising from the haltere of *ap*–GAL4/UAS–*Dll* flies. Arrows indicate the claws; arrowheads indicate bracted bristles. (*E*) Ectopic leg emerging from the proximal region of the wing of E132–GAL4/UAS–*Dll* flies. The arrows indicate the femur, tibia, and tarsi segments. Arrowheads indicate an apical bristle typical of the mesothoracic leg. These phenotypes occur at the following frequencies: 90% of the flies contained bracted bristles and 30% developed claws ($n = 31$) (using the E132–GAL4 line); 50% contained bracted bristles and 30% developed claws ($n = 12$) (using the *ptc*–GAL4); 80% ($n = 12$), and 85% ($n = 34$) contained bracted bristles (using the *omb*–GAL4 and *ap*–GAL4 lines, respectively). These frequencies are higher when looking at leg molecular markers in the imaginal discs.

altered and also corresponds to the area where *vg* activity is suppressed (Fig. 6E,F). Other characteristics of leg development are also reproduced in the ectopic legs. For example, the gene *ap* has restricted expression in the fourth tarsal segment of the leg (Cohen et al. 1992; Fig. 7A) and consequently a ring of *ap* expression is observed where the Dll product induces an ectopic leg (Fig. 7B,C). Another example is the activation of *bab*, a gene specific for ventral discs (Fig. 7D). Ectopic activation of *bab* in the wing and haltere discs as induced by *Dll* ectopic expression was found (Fig. 7E). This activation of *bab* may occur anywhere in the wing disc (Fig. 7F) even in the notal region suggesting that the fate of the leg can be induced anywhere, although adult ectopic rudimentary legs only appear in the hinge region. Figure 7F also shows that the level of ectopic Dll is higher than endogenous Dll as revealed by the Dll antibody. Only high levels of Dll repress Vg. This could explain the coexpression of Dll and Vg in wild-type wing imaginal discs. Dll only represses Vg when its expression is increased.

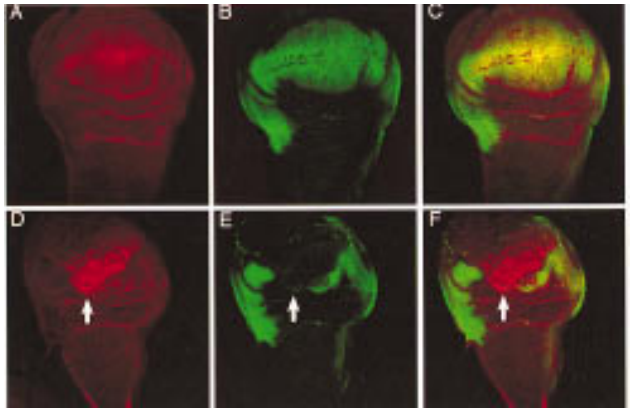

Figure 6. Ectopic expression of *Dll* in the wing disc activates the endogenous gene and represses the wing-specific gene *vg*. (*A*) *Dll–lacZ* expression pattern. (*B*) Vg wild-type expression pattern. (*C*) Merged channels. (*D*) *Dll–lacZ* expression pattern of *omb*–GAL4/UAS–*Dll* wing discs. (*E*) Vg expression pattern in the same disc. (*F*) Merge channels. Arrows indicate the repression of Vg and the activation of endogenous *Dll*. Here and in all remaining images of wing discs, ventral is at the *top* and anterior is to the *left*.

Ectopic Dll expression in the eye and head produces ectopic antennae If the dorsal-to-ventral transformation of the wings and halteres to legs described above reflects an involvement of *Dll* in a general dorsal versus ventral decision concerning appendage organization, homologous transformations in other regions of the body would be expected. The eye–antennal disc contains dorsal and ventral components as suggested by the homeotic transformations described previously (see Morata and Lawrence 1979). *Dll* is expressed and required in the antennal region of the disc but it is not expressed in the eye or head capsule. The latter are considered to be dorsal derivatives (Cohen and Jürgens 1989b).

It was found that the ectopic expression of *Dll* in the eye precursor cells induces the formation of antennal structures. Figure 8, B and C, shows arista, aIII, and aII antennal segments emerging from the eye of a fly of genotype E132–GAL4/UAS–*Dll*. Using the *ptc*–GAL4 and C-68a–GAL4 lines ectopic antennae are observed in different regions of the head, such as the rostral membrane or its most dorsal posterior part (Fig. 8E–G). This may

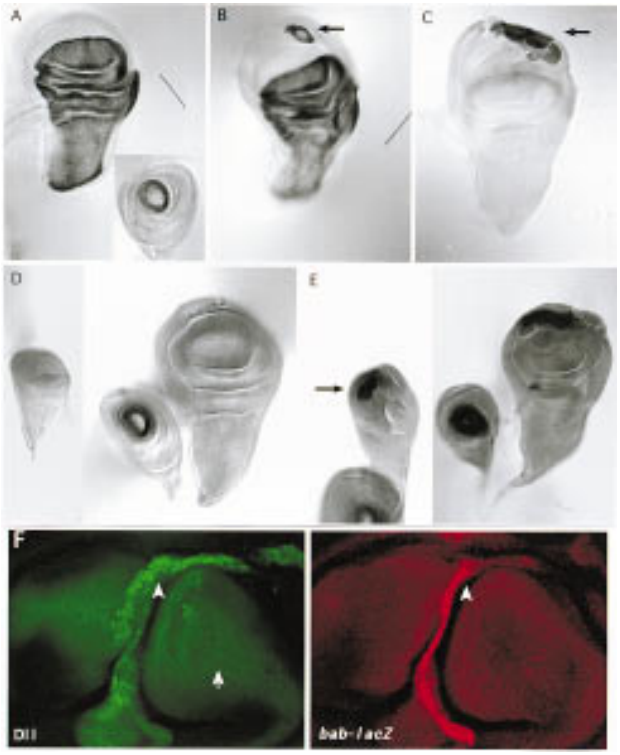

Figure 7. Induction of primordial legs in the wing is accompanied by the activation of the tarsal-specific expression of *ap–lacZ* and *bab–lacZ*. (*A*) *ap–lacZ* expression pattern in wing and leg (*inset*) discs. (*B*) Ectopic *ap–lacZ* expression in the ventral part of the wing disc of E132–GAL4/UAS–*Dll*/*ap–lacZ* flies. Note the ring pattern (arrow) similar to the wild-type *ap–lacZ* expression in the leg disc that corresponds to the fourth tarsus. (*C*) Sibling disc showing ectopic activation of endogenous *Dll–lacZ* (arrow) in the region where ectopic *ap–lacZ* appears. (*D*) Wild-type *bab–lacZ* expression pattern in wing, leg, and haltere discs. (*E*) Ectopic *bab–lacZ* expression (arrows) in the ventral part of the wing and haltere discs of E132–GAL4/UAS–*Dll*/*bab–lacZ* flies. (*F*) Ectopic *bab–lacZ* (red) and Dll (green) expression in a *ptc*–GAL4/UAS–*Dll* wing disc. Note the wild-type pattern of Dll expression at the presumptive wing margin (arrow) and the ectopic Dll and *bab–lacZ* expression driven by the *ptc*–GAL4 line (arrowheads).

reflect the complex organization of the eye–antennal disc (see below) but the significant result is that the eye or head are transformed toward antennae.

As in the wing disc, it was found that the exogenous Dll product induces ectopic activation of endogenous *Dll* in the eye where it is not normally active (Fig. 9A). The *ptc*–GAL4 line (Fig. 9B) was also used to further show ectopic expression of endogenous *Dll* in defined areas of the antennal region of the disc. The induction of ectopic antennae by *Dll* expression in *ptc*–GAL4/UAS–*Dll* flies is also accompanied by the expression of genes such as *en* (Fig. 9D) and *wg* (data not shown) in a subset of cells of the ectopic appendages. In these mutant discs, *en* and *wg* expression appears in several separate patches, probably reflecting the composite nature of the disc.

Discussion

Dll activity induces the formation of ventral appendages

Dll is expressed in the primordia of the larval and adult thoracic and cephalic appendages. In the adult legs, the Dll is domain extends from the trochanter to the tarsus and in the antennae it includes the second and third segments and the arista (see Fig. 1). The *Dll* domain probably represents the original leg appendage (see also Cohen and Jürgens 1989b; González-Crespo and Morata 1996). The proximal part of the leg, the pleura and the coxa, form part of the *extradenticle* (*exd*) domain. This domain is nearly complementary to that of Dll domain (González-Crespo and Morata 1996) and probably represents an expansion of the body trunk, the coxopodite (Snodgrass 1935). Although the argument for the an-

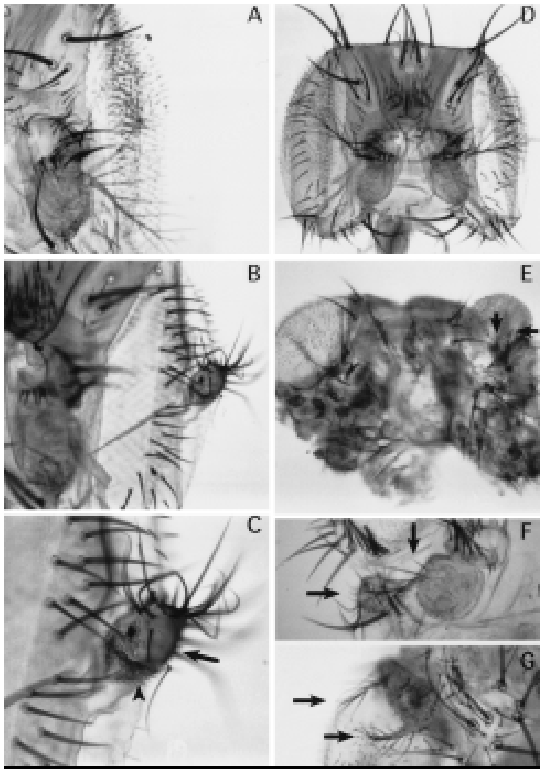

Figure 8. Dorsal to ventral transformation in the head induced by ectopic *Dll*. (*A*) Wild-type eye and antenna. (*B*) Ectopic antenna emerging from the eye of E132–GAL4/UAS–*Dll* flies. (*C*) Detail of the antennal outgrowth shown in *B*. Arrow indicates aII-like tissue emerging from the eye; arrowhead indicates aIII-like tissue. (*D*) Wild-type head. (*E*) Head from *ptc*–GAL4/UAS–*Dll* flies showing ectopic antennae in different locations. (*F*) Detail of a head of C-68a/UAS–*Dll* flies showing ectopic antenna, including aIII and arista. (*G*) Detail of a head of *ptc*–GAL4/UAS–*Dll* flies showing duplicated arista. Arrows indicate original and ectopic antennae. These phenotypes occur at the following frequencies: 46% of the flies developed ectopic antennal tissue in the eye ($n = 26$) (using the E132–GAL4 line); 90% ($n = 10$) and 30% ($n = 27$) developed ectopic antennae in different regions of the head (using the *ptc*–GAL4 and the C-68a lines, respectively).

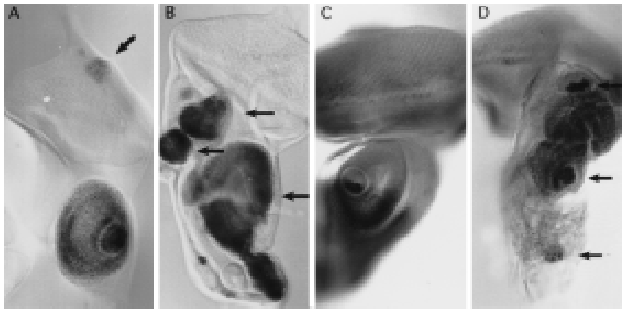

Figure 9. Ectopic expression of the endogenous *Dll* and En in the eye–antennal disc. (*A*) Ectopic activation of the endogenous *Dll* in the eye part (arrow) of the eye–antennal disc of E132–GAL4/UAS–*Dll*/*Dll–lacZ* flies. (*B*) Ectopic activation of the endogenous *Dll* in the eye-antennal disc of *ptc*–GAL4/UAS–*Dll*/*Dll–lacZ* flies. Arrows indicate the three areas with endogenous ectopic *Dll* that may correspond to the ectopic antennae observed in the adult head (Fig. 3). (*C*) En expression pattern in a wild-type disc. (*D*) Ectopic En expression (arrows) in different regions of the eye–antennal disc in those areas that might develop as ectopic antennae.

tenna is not as compelling, the homology relationship between leg and antenna supports the idea of similar organization. For example, the aI segment is considered to be homologous to the coxa (Posthlewait and Schneiderman 1971) and the aII, aIII, and arista similar to the rest of the leg. In concordance to this, the aI segment (like the coxa) does not possess *Dll* function, whereas the rest of the antenna does. Therefore, *Dll* expression domains in legs and antennae are homologous.

These expression patterns reflect a functional requirement as loss of *Dll* function results in a corresponding loss of ventral appendages. In the viable *Dll* mutations the legs and antennae are defective; there is a gradual loss of structures depending on the strength of the mutation (Cohen and Jürgens 1989a). In the strongest viable mutations such as Dll^3, most of the leg is lacking and only the pleura, coxa, trochanter, and part of the femur remain (Sunkel and Whittle 1987; Cohen and Jürgens 1989a). Moreover, clones of cells mutant for null *Dll* alleles generated in early larval development in either legs or antennae are unable to form the Dll domain structures. One reason for this is that Dll^- clones do not proliferate in the Dll domain (Fig. 2B). The lack of growth observations suggests that, in the absence of Dll activity, the normal polarity of the appendage cannot be established and growth of the appendage is prevented. This suggestion is supported strongly by the present finding (Fig. 4B) that ectopic expression of Dll in the proximal regions of leg and antennal discs often results in the generation of a supernumerary appendage.

The induction of these additional appendages is of interest, for they require at least two extracellular signal molecules, Wg and Dpp, that during normal development act on downstream genes to control growth and pattern. The formation of the P/D axis appears to be initiated from the site where cells expressing *wg* are in close association with those expressing *dpp* (Basler and Struhl 1994; Díaz-Benjumea et al. 1994; Campbell and Tomlinson 1995). The combined action of these signals activates *Dll* (Díaz-Benjumea and Cohen 1994; Campbell and Tomlinson 1995). In this work it was demonstrated that *Dll* itself is able to induce this signaling process as shown by the observation that ectopic Dll^+ clones produce a nonautonomous activation of *wg* and *dpp*. This new Wg and Dpp interaction in turn induces *Dll* expression nonautonomously and originates a new P/D axis. A similar positive feedback loop between a homeotic gene and Wg and Dpp also takes place in the embryonic midgut. The expression of Ubx is autoregulatory and requires cell communication involving Wg and Dpp signals (Bienz 1996).

However, these results do not explain the lack of proliferation of the Dll^- cells in the leg and antennal discs, as Wg and Dpp are secreted by the surrounding cells. A possible explanation is that Dll^- cells cannot respond to one or both of these signal molecules required for cell proliferation (Burke and Basler 1996; Penton and Hoffman 1996; Zecca et al. 1997). In this respect, it is worth pointing out that the late requirement of *Dll* in the wing could implicate the reception of Wg and Dpp. The wing margin and wing veins are affected in Dll^- clones and both Wg and Dpp reception are required for the differentiation of these structures late in development (Phillips and Whittle 1993; Couso et al. 1994; de Celis 1997).

Dll is a component of the genetic address determining the identity of ventral appendages

In addition to its role in the induction of the appendage, these results indicate that Dll is also involved in the specification of the identity of ventral appendages. First, it is possible to recover late induced Dll^- clones from legs and antennae, which are able to differentiate adult cuticular structures. These structures are unlike those corresponding to the region of the leg or antenna where the clone is located, indicating a change in the cell type. However, it was not possible to identify the type of structure formed by these clones with the exception of the base of the arista, where they are seen to differentiate leg bristles.

The second and stronger argument comes from the consideration that normal *Dll* activity is required for at least two distinct identities—legs and antennae. Moreover, when expressed ectopically, Dll activity induces the formation of the same two appendages depending on the context of the ectopic expression. In normal development, the genetic context appears to be provided by the activity of the homeotic gene *Antp*. The combination *Dll-on-Antp-off* specifies antennal development whereas *Dll-on-Antp-on* determines leg development. The ectopic expression of *Antp* (Schneuwly et al. 1987) transforms the antenna *(Dll-on-Antp-off)* into a mid-leg *(Dll-on-Antp-on)* and using the same rationale, lack of *Antp* transforms mid-leg into an antenna (Struhl 1981). This suggests that a combinatorial code (Struhl 1982) determines the type of ventral appendage. Induction of

ectopic Dll activity in the eye shows that the combination *Dll-on-Antp-off* (*Antp* is not expressed in the head; Engström et al. 1992) produces antennal development, whereas in the wing disc that contains *Antp* function, especially in the proximal regions (Wirz et al. 1986), the *Dll-on-Antp-on* combination specifies leg development. It is also worth pointing out that ectopic *Dll* expression gives rise to the formation of ectopic leg structures not only in the wing but in the haltere. In the wing they develop with mid-leg identity, as indicated by specific markers. It also seems likely that they develop with hindleg identity in the haltere. The reason for this suggestion is that in the haltere as in the hindleg leg, there is *Ubx* activity that determines third leg identity in normal development. Leg development in the wing lacking Ubx product would result in mid-leg identity.

The results suggest that Dll is a component of a genetic address that determines the identity of ventral appendages. This identity is qualified by properties provided by the selector genes of the ANT-C and the BX-C along the A/P body axis. The role of *Dll* in specifying ventral identity is reflected at the molecular level by the expression of molecular markers in the ectopic primordia like the ringed expression of *ap* in the fourth tarsal segment and *bab,* which is leg specific (Fig. 7). Exogenous *Dll* activity also results in ectopic activation of the endogenous *Dll* gene indicating that the autocatalitic activity of *Dll* found in the embryo (Castelli-Gair and Akam 1995) also operates in the imaginal cells.

However, the mode of action of *Dll* differs significantly from that of other homeotic genes such as *en*, *Ubx,* or *ap* mutations (Morata and Lawrence 1975; Morata and García-Bellido 1976; Díaz-Benjumea and Cohen 1993; Guillén et al. 1995) involved in the specification of the identity of adult structures. The first difference is that the few late Dll^- clones that survive do not produce a clear homeotic transformation. It is, however, possible that *Dll* is not the only contributor to the identity of the appendage and that the elimination of *Dll* results in a ''nonsense codeword'' of active selector genes (Struhl 1982). Examples of this type of situation exist, for example, the effect of *Ubx* or *abd-A* mutations in the posterior abdomen (Lewis 1978; Sánchez-Herrero et al. 1985; Tiong et al. 1985). The second and the more significant difference is that the *Dll* domain is not defined by a compartment border. This indicates that *Dll* activity is not maintained by cell heredity but possibly by cell interactions (Díaz-Benjumea et al. 1994). It is possible that segregation of the ''coxopodite'' and the ''telopodite'' (Snodgrass 1935; González-Crespo and Morata 1996) is achieved through mutual interactions between *Dll* and *exd* and/or *tsh* expressing cells.

The functional interaction of *Dll* with the wing determinant gene *vg* requires further study. Forcing *Dll* expression in the wing or haltere results in suppression of *vg* expression and consequently of dorsal appendage development. In the experiments reported by Kim et al. (1996), targeted *vg* expression produces ectopic wings, and presumably *Dll* suppression in legs and antennae. The rules governing these interactions are not yet understood fully. However, it is possible that the decisive factor involves relative amounts of products. In some of our experiments, targeted *Dll* expression resulted in the loss of the wing, probably as a consequence of *vg* repression. There are may be cases of unbalanced amounts of the two gene products that give rise a developmental conflict that arrests development.

A late Dll function is involved with the differentiation of the wing margin

Our results also indicate that there is a late requirement for *Dll* activity in the wing. The nature of this function is different from that in the leg and antenna; the Dll product appears later in the wing than in the leg discs and also the mutant phenotype is more discrete. Although hypomorphic Dll mutations do not detectably affect wing differentiation (Cohen and Jürgens 1989a), cells mutant for Dll^- null mutations exhibit a phenotype in the wing. These Dll^- clones, unlike those in the legs and antennae, proliferate normally even when induced in the first larval period and may occupy large portions of the wing. Dll^- clones have a phenotype restricted to the wing margin and veins; the triple-row bristles and double-row posterior hairs are lacking or abnormal and the differentiation of the veins is also altered. One interesting aspect of the Dll^- phenotype in the wing is that it is nonautonomous, suggesting that this Dll function involves a signaling mechanism.

Materials and methods

Fly stocks

The following *Dll* alleles were used: Dll^{IB} (Cohen and Jürgens 1989b), Dll^3 (Sunkel and Whittle 1987), Dll^{SA1} (Cohen and Jürgens 1989b) and *Df (2R)*Dll^{MP}(Cohen et al. 1989).

The reporter genes *dpp–lacZ* (Blackman et al. 1991), *wg–lacZ* (Kassis 1990), *ap–lacZ* (ap^{rk568}) (Cohen et al. 1992), *Dll–lacZ* (Dll^{01092}) (Spradling et al. 1995; Zecca et al. 1997), *bab–lacZ* (bab^{A128}) (Godt et al. 1993) are expressed as their respective endogenous RNAs.

The following GAL4 drivers were used: three different insertions in the *Dll* gene (em212–GAL4, MD23–GAL4, MD728–GAL4), an insertion in *ap* (*ap*–GAL4) and another in omb (*omb*–GAL4) as described in Calleja et al. (1996). The MS-1096 line is described in Capdevila and Guerrero (1994) (gift from F. Jiménez and C. Parras). C-765, C-68a GAL4 lines were kindly provided by A. Brand (Brand and Perrimon 1993), *dpp*–GAL4 by M. Hoffman (Morimura et al. 1996), *ptc*–GAL4 by Campos-Ortega and Hinz (Hinz et al. 1994), and E132-GAL4 by W. Gehring (Halder et al. 1995). UAS–y^+ (Calleja et al. 1996) was used to visualize the *Dll* expression pattern in the adult cuticle.

Clones of *Dll* mutant cells were generated by FLP-mediated mitotic recombination as described by Golic (1991) and Xu and Rubin (1993). The hsp70–flipase (FLP122) was obtained from G. Struhl (Struhl and Basler 1993). Males of the genotype *y w* FLP122; FRT42D Dll^{SA1}/*CyO* or *y* f^{36} FLP122; FRT42D Dll^{SA1}/*CyO* were crossed to *y w;* FRT42DP[ry^+; y^+]44B or *y* f^{36}; FRT42DP[$f^{44C}f^{52}$] females (kindly provided by D. Gubb). For lineage restriction analysis, males *y w* FLP122; FRT42D *arm–lacZ* (Chen and Struhl 1996) were crossed to *y w;* FRT42DP[ry^+;y^+]44B females. FLP-mediated recombination was induced by incubat-

ing larvae 24–120 hr AEL at 37°C for 60 min to produce Dll^- clones and by incubating larvae 72–120 hr at 37°C for 10 min to generate *arm–lacZ* clones.

Ectopic expression of Dll *using the GAL4 system*

For the production of UAS–*Dll* transgenic fly lines, a fragment of 1.2 kb of the *Dll* c-DNA (Cohen et al. 1989) containing the entire *Dll* open reading frame (ORF) was cloned in the pUAST plasmid. The recombinant plasmid containing the *Dll* cDNA in the correct orientation was used to transform $y\ w^{118}$ embryos by standard procedures of microinjection. Of the two independent lines that were obtained, only one showed the phenotypes described in this work. The other gave rise to lethal phenotypes when assayed using the different GAL4 lines.

To modify the levels of the UAS construct, we took advantage of the temperature sensitivity of the GAL4 system (Wilder and Perrimon 1993). Using the same GAL4 line, the effects of different levels of the protein at set temperatures were compared.

Generation of random Dll-expressing clones

To generate random clones of ectopic Dll a hybrid of the Flip-out and GAL4 activation systems (Pignoni and Zipursky 1997) was used. Clones expressing GAL4 were induced by flipping out an interruption cassette from an actin > CD2 > GAL4 transgene in a genetic background containing UAS–*Dll*. Females with the genotype FLP 122 [hsp70-flp]; UAS–*Dll* were mated to actin > CD2 > GAL4 males carrying *dpp–lacZ*, *wg–lacZ*, *Dll–lacZ*, or *bab–lacZ* reporters on the second chromosome. After one day of egg laying, adults were removed and the progeny aged for two days, heat-shocked (37°C for 30 min) and dissected three days later. The UAS–y^+ (Calleja et al. 1996) was also introduced to analyze the Dll^+ clones in the adult cuticle.

Whole-mount immunostaining of imaginal discs

X-Gal staining was performed following standard protocols (Ashburner 1989). Peroxidase and immunofluorescence staining were performed as described by Sánchez-Herrero et al. (1996). Anti-Vg (Williams et al. 1991), anti-En (Patel et al. 1989), anti-Dll (Vachon et al. 1992), and anti-Dac (Mardon et al. 1994) antisera were kindly provided by S. Carroll (University of Wisconsin, Madison), T. Kornberg (University of California, San Francisco), S. Cohen (EMBL, Heidelberg, Germany), and G. Mardon (Baylor College of Medicine, Houston, TX), respectively. Imaginal discs were examined under a Zeiss laser scan microscope.

Acknowledgments

We thank E. Sánchez-Herrero for daily discussions and suggestions during this work and also for comments on the manuscript; J.L. Mullor and S. González-Crespo for discussions; J.L. Mullor for unconditional help with the preparation of the photographic work; and R. González for her technical assistance. We also thank A. Brand, M. Calleja, J.A. Campos-Ortega, S. Carroll, S. Cohen, D. Gubb, U. Hinz, M. Hoffman, W. Gehring, F. Jiménez, T. Kornberg, G.F. Lasky, G. Mardon, E. Moreno, C. Parras, G. Struhl, and L. Zipursky for stocks, DNAs, and antibodies. We are supported by grants from the Dirección General Científica y Técnica, the International Human Frontier Science Program (372/94) and an institutional grant from Fundación Areces. N.G. was sponsored by a Mutis fellowship from the Instituto de Cooperación Iberoamericana.

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