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Coexpression of the homeobox genes *Distal-less* and *homothorax* determines *Drosophila* antennal identity

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SUMMARY

The *Distal-less* gene is known for its role in proximodistal patterning of *Drosophila* limbs. However, *Distal-less* has a second critical function during *Drosophila* limb development, that of distinguishing the antenna from the leg. The antenna-specifying activity of *Distal-less* is genetically separable from the proximodistal patterning function in that certain *Distal-less* allelic combinations exhibit antenna-to-leg transformations without proximodistal truncations. Here, we show that *Distal-less* acts in parallel with *homothorax*, a previously identified antennal selector gene, to induce antennal differentiation. While mutations in either *Distal-less* or *homothorax* cause antenna-to-leg transformations, neither gene is required for the others expression, and both genes are required for antennal expression of *spalt*. Coexpression of *Distal-less* and *homothorax* activates ectopic *spalt* expression and can induce the formation of ectopic antennae at novel locations in the body, including the head, the legs, the wings and the genital disc derivatives. Ectopic expression of *homothorax* alone is insufficient to induce antennal differentiation from most limb fields, including that of the wing. *Distal-less* therefore is required for more than induction of a proximodistal axis upon which *homothorax* superimposes antennal identity. Based on their genetic and biochemical properties, we propose that Homothorax and Extradenticle may serve as antenna-specific cofactors for Distal-less.

Key words: *Distal-less*, *homothorax*, *Antennapedia*, *spalt*, Antenna, Leg, Limb, Homeotic transformation

INTRODUCTION

The similar developmental potentials of the *Drosophila* antenna and leg primordia are evidenced by the number of mutations that result in transformation of one tissue into the other. Genes in which loss-of-function mutations lead to partial antenna-to-leg transformations include *homothorax* (*hth*) (Casares and Mann, 1998; Pai et al., 1998), *Distal-less* (*Dll*) (Sunkel and Whittle, 1987), and *spineless* (*ss*) (Balkaschina, 1929; Struhl, 1982). Complete transformations of the antenna into leg are observed with gain-of-function alleles of the *Drosophila* trunk Hox gene *Antennapedia* (*Antp*) (Gehring, 1966). Transformations of leg into antenna are much less common and have been observed with loss of *Antp* function (Struhl, 1981). Analysis of the genetic hierarchies among genes required to distinguish the antenna and the leg is likely to provide insights into both limb development and the generation of morphological diversity. *Dll* and *Antp* previously were found to repress *hth* in the developing leg (Casares and Mann, 1998; Gonzalez-Crespo et al., 1998; Abu-Shaar and Mann, 1998), and *Dll* shown to be required for *ss* expression in both leg and antenna (Duncan et al., 1998). Here, we analyze the relationship between *Dll* and *hth* in the antenna.

Drosophila heterozygous for intermediate and strong loss of function of *Dll* alleles exhibit truncations of the *Drosophila* antenna and leg, indicating that *Dll* plays an essential role in forming the proximodistal (PD) axis in both limb types (Sunkel and Whittle, 1987; Cohen and Jurgens, 1989; this work). Ectopic expression of *Dll* can induce the formation of new PD axes in various positions of the body (Gorfinkiel et al., 1997). These ectopic limbs take on identities appropriate to their anteroposterior position along the main body axis, e.g. antennal elements on the head and leg elements on the wing (Gorfinkiel et al., 1997). This is consistent with the idea that *Dll* plays a single role during limb development, that of inducing the formation of PD axes upon which selector genes, such as the Hox genes, superimpose information regulating limb identity.

However, in *Drosophila* carrying only one functional copy of the *Dll* gene or heterozygous for combinations of hypomorphic *Dll* alleles, the antenna is partially transformed toward leg (Sunkel and Whittle, 1987; Cohen and Jurgens, 1989; this work). The transformation can occur without any concomitant loss of PD information. This indicates that *Dll* has a second function during limb development, specifying antennal cell fates. How *Dll* effects the differentiation of distinct limb types is not immediately apparent since *Dll* is expressed in similar patterns in the presumptive distal and medial cells of both the antenna and the leg (Cohen, 1990; Diaz-Benjumea et al., 1994). One plausible mechanism would be via interaction with other factors whose expression is limb specific.

hth meets three important criteria for a factor that could cooperate with *Dll* to regulate antennal identity: (1) it is expressed throughout the antennal primordium for much of development,

(2) it is required for the differentiation of proximal, medial and distal antennal elements, and (3) it is differentially expressed between the antenna and the leg (Casares and Mann, 1998; Pai et al., 1998). In developing legs, *hth* expression is restricted during embryogenesis to presumptive proximal cells. *hth* and *Dll* expression therefore do not overlap in the leg throughout most of development (Casares and Mann, 1998). *hth* encodes a TALE-class homeodomain protein required for the nuclear localization of a PBC-class homeodomain protein encoded by *extradenticle* (*exd*) (Pai et al., 1998; Rieckhof et al., 1997; Kurant et al., 1998). Exd is a transcriptional cofactor for a variety of homeodomain proteins, including several Hox gene products (reviewed in Mann and Chan, 1996). Genetic studies indicate that both *exd* and *hth* are needed for normal development of the entire antenna and of the proximal leg. Loss of either *exd* or *hth* function in the developing antenna causes cell-autonomous transformation of medial and distal antennal structures into medial and distal leg structures (Casares and Mann, 1998; Pai et al., 1998; Gonzalez-Crespo and Morata, 1995). Ectopic expression of *Meis-1*, a murine homolog of *hth*, can induce the formation of antennae in the genital disc derivatives (Casares and Mann, 1998). However, ectopic expression of either *Meis-1* or *hth* in the developing leg, wing or head does not lead to antennal differentiation (Casares and Mann, 1998; this work), therefore *hth* is insufficient to induce antennal development in most contexts.

In this study, we demonstrate that *Dll* and *hth* are independently regulated and expressed in partially overlapping domains in the developing antenna. Reducing *Dll* activity or eliminating *hth* activity transforms the antenna to leg and results in reduction or loss of *spalt* (*sal*) expression. Ectopic expression of *Dll* in domains of endogenous *hth* expression or coexpression of *Dll* and *hth* using the GAL4/UAS system (Brand and Perrimon, 1993) induces expression of *sal*, and leads to the differentiation of antennal cuticular structures from the eye, leg, wing and anal/genital primordia. Based on these results, we propose that *Dll* and *hth* act at the same level of the genetic hierarchy to coordinately activate the antennal developmental program.

MATERIALS AND METHODS

Immunohistochemistry

Antibody and X-gal stainings were carried out as described (Halder et al., 1998). Antibodies used were: chicken anti-Hth (Casares and Mann, 1998), rabbit anti-Hth (Pai et al., 1998), rabbit anti-Dll (Panganiban et al., 1995), mouse anti-Dll (Vachon et al., 1992), and rabbit anti-Sal (Kuhnlein et al., 1994). Secondary antibodies coupled to Cy2, Cy3 and Cy5 were obtained from Jackson ImmunoResearch. Imaging was carried out on BioRad MRC1024 confocal and Zeiss Axiophot microscopes.

Fly strains

The following fly strains were employed: (1) *dpp-GAL4 (4A.3)/TM6B* (Morimura et al., 1996), (2) *act>CD2>GAL4* (= *actin* promoter-*FRT-CD2-FRT-GAL4*) (Pignoni and Zipursky, 1997), (3) *w; UAS-Dll/ In (2LR) Gla, Gla, Bc, Elp* (Konrad Basler), (4) *w; UAS-GFP-hth8/TM6B, Tb, Hu* (Casares and Mann, 1998), (5) *w; FRT82B* hth^{P1} (Pai et al., 1998), (6) *w; FRT82B* $Antp^{RC3}$ (Gary Struhl), (7) *hth-lacz* (= hth^{05745}; Bloomington), and (8) *sal-lacz* (= $salm^{03602}$; Bloomington).

Stocks constructed by us for these experiments were: (1) Dll^{1}*/CyO wg-lacz*, (2) Dll^{3}*/CyO wg-lacz*, (3) Dll^{7}*/CyO wg-lacz*, (4) Dll^{SA1}*/ CyO wg-lacz*, (5) *y, hs-FLPase; FRT82B, 2piM*, (6) *w; UAS-Dll/In (2LR) Gla, Gla, Bc, Elp; UAS-GFP-hth8/TM6B, Tb, Hu*, (7) *y, hs-FLPase; UAS-Dll/TM6B, Tb, Hu*, (8) *y, hs-FLPase; FRT43D, 2piM*, (9) *w; FRT43D* Dll^{SA1}, and (10) *sal-lacz/CyO; dpp-GAL4/TM6B, Tb, Hu*.

Genetic manipulations

Dll hypomorphic larval imaginal discs were generated by crossing heterozygous *Dll* mutant animals in which each *Dll* mutant chromosome was balanced over *CyO, wg-lacz*. Mutant animals were identified by the absence of X-gal staining in the larval tails. Ectopic expression of *Dll* and *hth* was induced using the GAL4-UAS binary system (Brand and Perrimon, 1993). *dpp-GAL4* was used to activate *UAS-hth* and/or *UAS-Dll* along the anteroposterior compartment boundary of the developing imaginal discs. Clones of cells ectopically expressing *Dll* were generated using a modified GAL4/UAS system (Pignoni and Zipursky, 1997) in which *y, hs-FLPase; UAS-Dll/TM6B, Tb, Hu* flies were crossed to *act>CD2>GAL4* and the resulting larvae heatshocked at 37°C for 10 minutes at 72-96 hours after egg laying (AEL) to induce site-specific recombination between the *FRT* sites, which in turn results in constitutive GAL4 expression in the clones.

Dll, *hth* and *Antp* null clones were generated using the FLP/FRT system (Xu and Rubin, 1993). Animals of the genotypes: (1) *y hs-FLPase; FRT43D, 2piM/FRT43D* Dll^{SA1}, (2) *y, hs-FLPase; FRT82B, 2piM/ FRT82B* hth^{P1}, and (3) *y, hs-FLPase; FRT82B, 2piM/ FRT82B* $Antp^{RC3}$ were heat shocked at 37°C for 1 hour at 48-72 hours AEL and examined in mid- to late- third instar.

RESULTS

Dll is required for antennal identity

Animals heterozygous for *Dll* null alleles exhibit partial antenna-

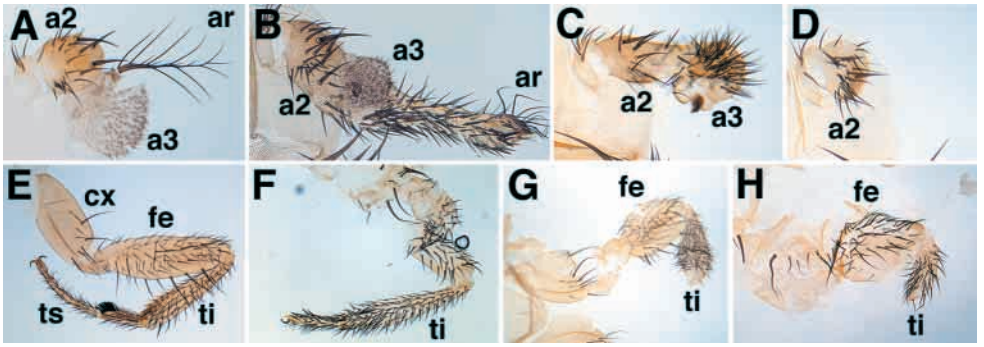

Fig. 1. Antenna and leg phenotypes of *Dll* mutants. (A) Wild-type adult antenna. (B) A weak hypomorphic combination of *Dll* alleles, Dll^{3}/Dll^{7}, results in partial transformation of the antenna into leg. In this case, the distal part of a3 and the proximal part of the arista are transformed toward tibia. (C) An intermediate combination, Dll^{3}/Dll^{1}, results in both transformation and truncation. Here, what remains of a3 resembles leg tissue and the arista is deleted. (D) Strong combinations of *Dll* alleles, such as Dll^{3}/Dll^{SA1} result in antennal truncations. Here, a3 and the arista are deleted. (E) Wild-type adult leg. (F) Leg from a Dll^{3}/Dll^{7} individual. This combination, which causes antenna-to-leg transformations, causes tarsal segment deletions and fusions in the legs. (G) Leg from a Dll^{3}/Dll^{1} individual. This combination, which causes both transformation and mild truncation of the antenna, leads to complete loss of the tarsal segments and shortening of both the femur and the tibia. (H) Leg from a Dll^{3}/Dll^{SA1} individual. *Like* Dll^{3}/Dll^{1}, this allelic combination leads to complete loss of the tarsal segments and shortening of both the femur and the tibia. While Dll^{3}/Dll^{7} flies generally eclose, Dll^{3}/Dll^{SA1} and Dll^{3}/Dll^{1} flies die as pharate adults, and the specimens shown in C, D, G and H were dissected from late stage pupal cases.

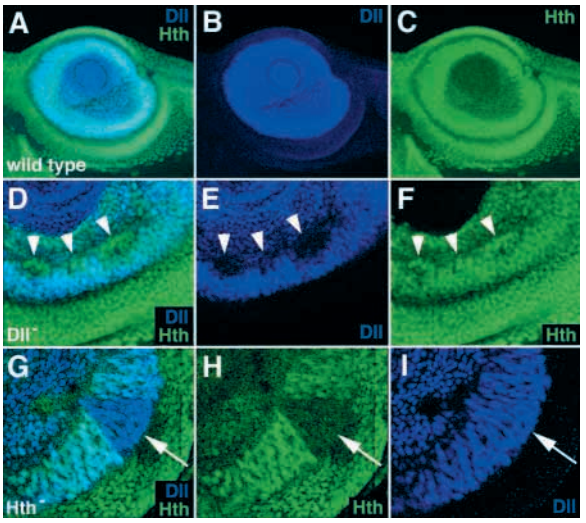

Fig. 2. *Dll* and *hth* are not required for each others expression in the larval antenna. (A-C) Dll (blue) and Hth (green) expression overlap (turquoise) in the presumptive second (a2) and weakly in the third (a3) antennal segments of a wild-type late third instar antennal disc. (D-F) Hth expression (green) is normal in *Dll* null clones (arrowheads). Dll is in blue. (G-I) Dll expression (blue) is normal in an *hth* (green) null clone in a3 (arrow).

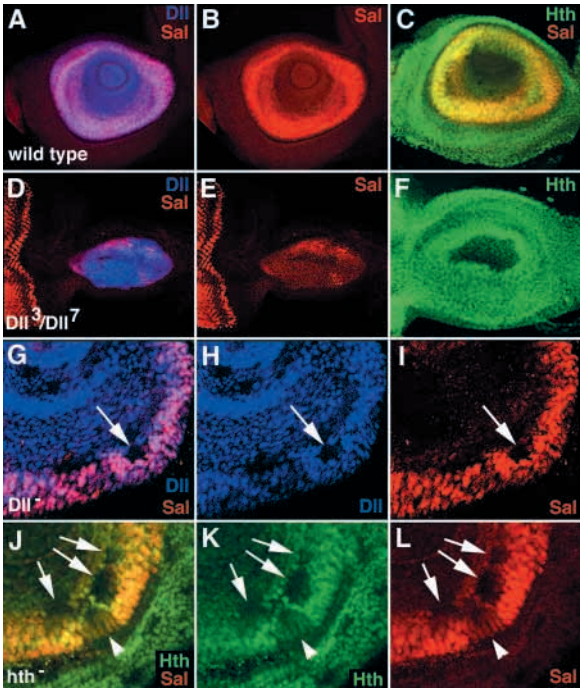

Fig. 3. *sal* expression in the antenna is dependent upon Dll and Hth. (A,B) Dll (blue) and Sal (red) overlap (pink) in the presumptive second (a2) and weakly in the third (a3) antennal segments of a wild-type late third instar antennal disc. The proximal boundaries of both coincide. (C) Hth (green) and Sal (red) overlap (yellow) in presumptive a2 and weakly in a3 of a wild-type antennal disc. The distal boundaries of both coincide. (D,E) In Dll^3/Dll^7 antennal discs, Dll (blue) is expressed at reduced levels in a normal pattern, and Sal expression (red) is greatly reduced. Note that Sal expression is normal in the eye. (F) Hth expression is normal in Dll^3/Dll^7 antennal discs. (G-I) Sal (red) cannot be detected in a *Dll* null clone in a3 (arrow). Dll is in blue. (J-L) *hth* null clones (arrows) in the antenna exhibit loss of detectable Hth (green) and Sal (red). The arrowhead indicates an *hth* null clone positioned on a fold in the disc. Neither Sal nor Hth are detected in the clone, but can be seen as faint staining in the wild-type cells beneath the clone.

to-leg transformations (Sunkel and Whittle, 1987; Cohen and Jurgens, 1989), indicating that Dll levels may be important for antennal determination. Weak hypomorphic combinations of *Dll* alleles also lead to partial transformation of the third antennal segment (a3) and the arista into leg-like structures (Fig. 1A,B). Intermediate hypomorphic combinations of *Dll* alleles transform the medial antenna toward leg and exhibit distal truncations (Fig. 1C). Strong combinations of *Dll* alleles exhibit more severe antennal truncations (Fig. 1D). These same allelic combinations result in progressively more severe truncations of the distal leg (Fig. 1E-H). Notably, the antenna-to-leg transformations are not a property of a specific subset of *Dll* alleles, but are observed with all *Dll* alleles when assayed in appropriate combinations. For the transformation phenotype to be apparent, *Dll* PD function must be largely intact. This is likely due to the necessity of having a PD axis for either antennal or leg identity to be manifested. That we observe transformation without limb truncation indicates that the antennal selector function is more sensitive to *Dll* dosage than its PD function. We emphasize that transformation is a loss-of-function phenotype of *Dll*, not a neomorphic or hypermorphic one. Together, the *Dll* phenotypic

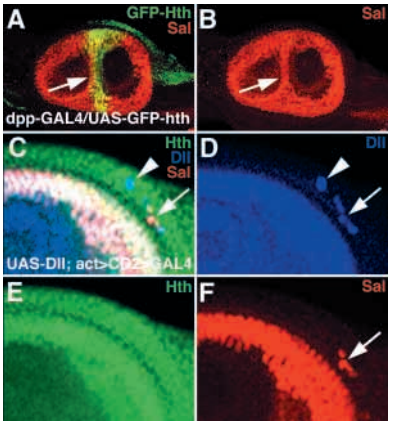

Fig. 4. Ectopic Hth in the Dll domain or ectopic Dll in the Hth domain activates *sal*. (A,B) When Hth (green) is ectopically expressed in the Dll domain of the antenna, *sal* (red) is activated (arrow). In this case, a *dpp-GAL4* driver was used to drive *UAS-GFP-hth* expression along the anterior-posterior compartment boundary. Ectopic Hth is green because it is tagged with GFP (green fluorescent protein). Endogenous Hth is not visible. Note that *sal* expression is not activated by ectopic Hth proximally, i.e. outside of the Dll expression domain, and that there is a low level of ectopic *sal* induced in the area immediately anterior to the ectopic *hth* stripe. This may be because the *dpp* pattern is dynamic and *dpp-GAL4* drove *hth* expression from the UAS element in these cells earlier in development. When Dll (blue, C,D) is expressed proximally in the antennal disc using a flip-out GAL4 cassette and a *UAS-Dll* construct, Sal (red, C,F) is induced in cells expressing Hth (green, C,E). The arrow points to a cluster of cells in which *sal* is activated. The arrowhead indicates a cluster of cells with higher levels of Dll in which *sal* is not activated. We sometimes see downregulation of Hth in such clones.

analysis indicates that *Dll* is required for antennal identity, as well as for limb outgrowth.

Dll and *hth* are not required for each others expression in the larval antenna

Both loss-of-function *Dll* alleles and loss-of-function *hth* alleles lead to antenna-to-leg transformations. It therefore was possible that Dll might activate *hth* expression in the antenna or vice versa. To test whether this is the case, we examined expression of each gene in animals mutant for the other. At late third instar, Dll is expressed in presumptive a2, a3 and arista (Fig. 2A,B), while Hth is expressed in presumptive a1, a2 and, weakly, a3 (Fig. 2A,C). Thus the expression of Dll and Hth overlaps in a2 and a3 (Fig. 2A). In *Dll* hypomorphic antennal discs, both the pattern and level of Hth appear normal (Fig. 3F). In *Dll* null clones in a2 or a3, Hth levels appear normal (Fig. 2D-F). In *hth* null clones in presumptive a2 and a3, Dll levels appear normal (Fig. 2G-I). Thus Dll and Hth are not required during larval stages for each others expression in the antenna.

Dll and *hth* are required together for *sal* expression in the antenna

The experiments described above establish that *Dll* and *hth* act in parallel as antennal selectors. An obvious next question was whether mutations in both genes affect the expression of antennal markers in the same way. To test this, we made use of the antennal marker *spalt* (*sal*). *sal* is one of few genes known to be expressed in the antenna but not in the leg (Wagner-Bernholz et al., 1991). Consistent with the idea that *sal* lies genetically downstream of both *Dll* and *hth*, we found that *sal* expression is restricted to the presumptive medial cells of the antenna (a2 and a3), precisely where the domains of Dll and Hth expression overlap (Fig. 3A-C). To test whether both Dll and Hth are required for *sal* expression in the antenna, the expression of *sal* was examined in *Dll* hypomorphs and in clones null for either *hth* or *Dll*. In *Dll* hypomorphic combinations exhibiting antenna-to-leg transformations, e.g. Dll^3/Dll^7 (Fig. 3D,E) and Dll^1/Dll^3 (not shown), Sal is significantly reduced (Fig. 3E). In *Dll* null clones, Sal is undetectable (Fig. 3G-I). In *hth* null clones, Sal is also undetectable (Fig. 3J-L). Together, these results demonstrate that *sal* expression in the antenna requires both *Dll* and *hth* functions.

Coexpression of Dll and Hth in the antenna activates *sal*

The results described above indicate that Dll and Hth are both necessary for *sal* expression in the antenna. If Dll and Hth were sufficient to activate *sal*, we would predict that ectopic expression of Hth in the Dll domain or ectopic expression of Dll in the Hth domain would induce the expression of *sal*. To test this, *dpp-GAL4* (Morimura et al., 1996) was used to drive expression of a *UAS-GFP-hth* construct (Casares and Mann, 1998) along the anteroposterior compartment boundary of the antennal disc. This resulted in the expression of Hth in a subset of the presumptive distal a3 and aristal cells that normally express Dll, but do not normally express Hth during the third instar (Fig. 4A). Under these conditions, Sal expression is induced (Fig. 4A,B).

To test the consequences of expressing Dll in the Hth domain, a combined 'flip-out' *GAL4*/UAS system was used. In this case, clones of cells expressing Dll were generated in the proximal part of the Hth domain where Dll is normally not expressed (Fig. 4C-F). Only small clones could be recovered (Fig. 4C,D), suggesting that high levels of Dll may be cell lethal. However, cells in these clones frequently express Sal (Fig. 4C,F). We therefore conclude that antennal cells both proximal and distal to the normal Sal domain are competent to express Sal if provided with Dll and Hth, i.e. within the context of the antennal disc, neither Dll nor Hth alone can activate *sal*, but that together they are sufficient to do so.

Ectopic Dll induces *sal* expression and antennal differentiation where there is endogenous Hth

To test whether coexpression of Dll and Hth induces antennal differentiation, we examined the phenotypic consequences of ectopically expressing Dll in Hth domains in areas outside of the antenna. As previously reported (Gorfinkiel et al., 1997), we find that ectopic *Dll* can induce the differentiation of antennal tissue elsewhere in the head (Fig. 5E-G) and ectopic leg tissue on the wing (not shown). However, we also find that ectopic expression of Dll induces the differentiation of antennal structures, primarily arista, in the proximal wing and, less frequently, in the proximal leg (Fig. 6A-D).

The locations where ectopic antennal structures can be induced are correlated with both endogenous Hth expression and ectopic Sal expression. In the eye-antennal imaginal disc, Hth is expressed in the presumptive head capsule and behind the morphogenetic furrow (Fig. 5A,C). Ectopic Dll expression in the Dpp domain of the eye disc creates a region of overlap of Dll with the presumptive head capsule domain of Hth expression (Fig. 5A-C). Ectopic Sal can be detected in the region of overlap, but not in adjacent cells that express Dll but lack endogenous Hth (Fig. 5A,D). Using X-gal stainings of *hth-lacz* pupae, we observe that the sites where differentiation of antennal structures can be induced by ectopic Dll express endogenous *hth* (Fig. 6E,F). Together, these results are consistent with Dll being able to induce antennal structures and *sal* expression only where Hth is present.

Ectopic Dll and Hth together can induce *sal* expression and antennal differentiation in the leg, head and genital discs

hth expression is restricted to presumptive proximal leg cells and *Dll* expression is restricted to presumptive distal leg cells during embryogenesis (Casares and Mann, 1998; Goto and Hayashi, 1997). Thus their expression does not overlap for most of leg development. If Dll and Hth cooperate to induce antennal differentiation, then leg tissue may express *sal* and differentiate antennal structures if provided with Dll and Hth simultaneously. To test this, Dll and Hth were ectopically expressed together using the *dpp-GAL4* driver. Under these conditions, Sal expression was induced in the medial to distal leg disc (Fig. 7A,B) and ectopic antennal structures could be detected on the adult legs (Fig. 7C,D).

Dll and Hth expression also do not normally overlap in the eye, the presumptive head capsule, or the genital disc. When *dpp-GAL4* is used to coexpress Dll and Hth in these tissues, ectopic Sal expression (Figs 8A-C, 9A,C,E) and the formation of ectopic antennal cuticular structures result (Figs 7C,D, 8D, 9B,D). X-gal staining of *UAS-Dll*; *UAS-GFP-hth/dpp-GAL4* pupae harboring a *sal-lacz* enhancer trap, results in specific stainings of the ectopic antennal structures (not shown), indicating that *sal* expression can be correlated directly with antenna formation.

We note that ectopic Dll and Sal are sometimes found where GFP-Hth is very low or not detected (Figs 4A, 5A, 7A, 8A, 9C). This probably reflects our detection methods and not an absence of Hth in these cells. Both Dll and Sal proteins were visualized using antibody reagents that amplified the signals. Hth was visualized by means of the GFP tag in the UAS construct, thus the sensitivity was not as high. The cells in which ectopic Sal can be detected therefore probably contain Hth as well as Dll.

We have compared the frequencies of ectopic antenna formation induced by ectopic coexpression of Dll and Hth in the legs and genitalia with the frequencies of ectopic antenna formation induced by either ectopic Dll or Hth alone. With ectopic Dll alone, we have observed only one recognizable arista on a leg in more than 20 animals, i.e. 120 legs, examined, a frequency of less than 1%. The reason for this low frequency may be due to the fact that there is little overlap of the ectopic Dll with endogenous Hth in the leg disc when *UAS-Dll* is expressed using the *dpp-GAL4* driver. A second possibility is that antennal development is impeded when Antp (or another trunk Hox gene product) is present. As for why ectopic expression of Hth in the Dll domain of the leg does not induce antennal differentiation, when Hth is expressed ectopically there using *dpp-GAL4*, Dll is repressed (not shown). Thus if both Dll and Hth are required for antennal differentiation, these conditions would not lead to ectopic antennae. Indeed, no recognizable antennal structures were found in the legs of the more than 20 animals examined with ectopic Hth alone (not shown). We also have observed no ectopic aristae in the genital disc derivatives of animals ectopically expressing either Dll or Hth alone. In each case, at least 20 animals were examined. In contrast, with ectopic Dll and Hth together, we found 8 aristae on the legs of 7 animals, or 42 legs, a frequency of 19%. These aristae were distributed among T1, T2 and T3 legs, could be found in both males and females, and were often associated with a3-like tissue. We also found aristae and a3-like tissue on both male and female genital disc derivatives of 3 of these 7 animals, a frequency of 43%. These results support the conclusion that Dll and Hth synergize to initiate the antennal developmental program.

Antp may repress *sal* indirectly via *hth*

Antp represses *hth*, thereby restricting *hth* expression to the proximal region of the leg (Casares and Mann, 1998). Antp also represses *sal* in the leg (Wagner-Bernholz et al., 1991). Because antennal *sal* expression is dependent upon both Dll and Hth, we hypothesized that Antp repression of *sal* might be mediated via Antp repression of *hth*, which in turn prevents the overlap of Dll and Hth in the distal leg. Consistent with this possibility, Sal is expressed in *Antp* null clones in the Dll domain where *hth* is derepressed (Fig. 10A-D). We therefore think it likely that Antp may be repressing *sal* expression indirectly by preventing *hth* from being expressed in the Dll domain of the leg. Since both Dll and Hth are required for antennal differentiation, by preventing the coexpression of Dll and Hth, Antp can preclude antennal development.

DISCUSSION

Dll and *hth* act in parallel as antennal selectors

Dll is required for the formation of distal elements in all ventral appendages, including the antenna and the leg. *hth* is required for the formation of proximal elements in the antenna and the leg. For their roles in PD patterning, *Dll* and *hth* act independently. However, *Dll* (Sunkel and Whittle, 1987; Cohen and Jurgens, 1989) and *hth* (Pai et al., 1998; Rieckhof et al., 1997) also function as selector genes in the antenna. Via both loss- and gain-of-function experiments, we have demonstrated here that they cooperate to regulate antennal differentiation (Fig. 11).

Not only are *Dll* and *hth* required in parallel for normal antennal development and antennal *sal* expression, but coexpression of Dll and Hth activates *sal* expression and can induce the formation of antennal structures in many different areas in the body, including the head, wings, legs and anal plates. It has been reported that ectopic expression of either Dll or Hth alone can result in the formation of ectopic antennal structures. This includes ectopic expression of Dll in the head capsule (Gorfinkiel et al., 1997) and ectopic Hth or its vertebrate homolog, Meis-1, in the anal plates (Casares and Mann, 1998). Both cases are consistent with the results and model presented here (Fig. 11), in which coexpression of Dll and Hth is required to determine antennal fates. For instance, we find that the ectopic antennal tissue induced by ectopic Dll alone in the head capsule, proximal leg and proximal wing correlates with sites of endogenous *hth* expression. We also demonstrate that ectopic Dll alone in the presumptive head capsule region of the eye imaginal disc induces *sal* expression only when Dll overlaps endogenous Hth. Together, these results suggest that ectopic expression of Dll alone can only lead to formation of antennal tissue from cells with endogenous Hth.

If Dll and Hth collaborate to regulate antennal development, then the converse should also be true, i.e. ectopic expression of Hth should induce the differentiation of antennal structures wherever Dll is expressed endogenously. Indeed, in the reported instances of arista induction with ectopic Meis-1 or Hth in the anal plates, the ectopic expression was driven by a *Dll-GAL4* line (Casares and Mann, 1998). This produced coincident expression of Dll and Meis-1 or Hth, again consistent with the proposed model (Fig. 11).

However, Hth or Meis-1 alone, when ectopically expressed in Dll domains elsewhere in the body does not lead to ectopic Sal expression (our observations) or to the differentiation of recognizable antennal structures (Casares and Mann, 1998). The explanation for this that we favor is that when Hth is ectopically expressed using the GAL4/UAS system, *Dll* expression is downregulated in the cells producing Hth. These cells would then have Hth, but insufficient Dll. If both are required for antennal differentiation, antennal differentiation would not be possible. Consistent with this idea, we and others (Gonzalez-Crespo et al., 1998) have observed a decrease in *Dll* expression in leg cells ectopically expressing Hth.

Hth and arista formation

Coexpression of Dll and Hth in the leg, the wing, and the genital discs using the *dpp-GAL4* driver frequently leads to the formation of ectopic aristae. However, while Hth is required cell autonomously for arista development, Hth expression is not detected in the presumptive arista of third instar antennal discs, and ectopic expression of either Hth or Meis-1 in the presumptive arista late in development prevents arista formation (P. D. S. D., unpublished results). Since *Dll*

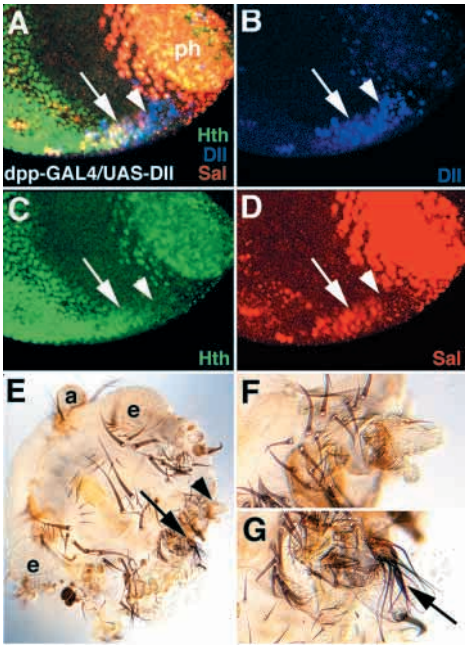

Fig. 5. Ectopic *Dll* in the *hth* domain of the head, induces *sal* expression and antennal differentiation. When Dll (blue, A,B) is expressed in the ventral part of the eye disc using *dpp-GAL4* and a *UAS-Dll* construct, Sal expression (red, A,D) is induced in cells endogenously expressing Hth (green, A,C). The arrow points to a cluster of cells in which *sal* is activated. *sal* is not activated in Dll-expressing cells that lack endogenous Hth (arrowhead). Sal is normally expressed in differentiating photoreceptors (ph). (E) Dorsal view of an adult head in which *Dll* was expressed using *dpp-GAL4* and a *UAS-Dll* construct. Ectopic antennal structures (arrow and arrowhead) are induced on the back of the head. (F) Higher resolution image of the ectopic a3-like structure indicated with the arrowhead in E. (G) Higher resolution image of the ectopic arista and a3-like structure indicated with the arrow in E. Arrowheads in F and G indicate a3 tissue. Arrow in G indicates ectopic arista. a, endogenous antenna; e, what remains of the adult eye following ectopic *Dll* expression.

has functions in PD outgrowth independent of Hth, a plausible explanation is that the ectopic Hth is titrating out the Dll needed for PD outgrowth. If Hth must be lost or reduced to permit arista differentiation, why then does coexpression of Dll and Hth lead to arista development? The explanation that we favor is that it reflects both the ability of *Dll* to autoregulate and the dynamics of *dpp-GAL4* expression. The width of the stripe of *dpp* expression remains fairly constant as the discs grow (Masucci et al., 1990; Weigmann and Cohen, 1999). As a result, the anteriormost cells that express the *dpp-GAL4* driver early, give rise to cells that do not express *dpp-GAL4* later in development. Thus some cells in the anterior compartment will express Hth and Dll from the UAS elements only transiently. Because *Dll* can autoregulate in imaginal discs (Gorfinkiel et al., 1997), transient expression of *Dll* from the UAS element activates the endogenous *Dll* gene. By late third instar, portions of the imaginal discs resemble normal presumptive aristal cells in expressing *Dll* and having expressed, but lost, *hth* expression. It may be that these cells differentiate as arista.

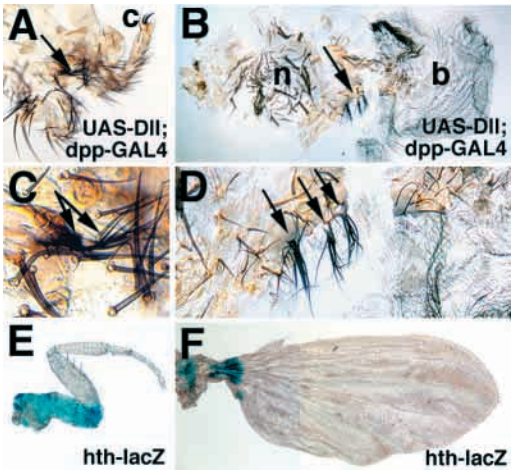

Fig. 6. Ectopic *Dll* in the *hth* domains of the leg and wing induces antennal differentiation. (A,C) Ectopic arista (arrow) induced on the proximal leg by expressing *Dll* with *dpp-GAL4* and a *UAS-Dll* construct. (B,D) Ectopic aristae (arrows) induced on the proximal wing by expressing *Dll* with *dpp-GAL4* and a *UAS-Dll* construct. (E,F) The positions at which aristae can be induced correlate with endogenous *hth* expression detected using an *hth* enhancer trap in the leg (E) and wing (F). c, tarsal claws; n, notum; b, wing blade.

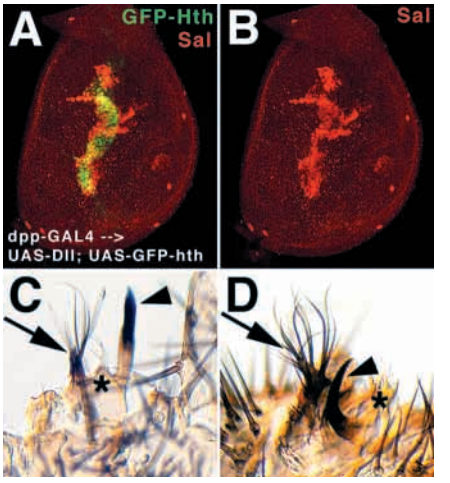

Fig. 7. Ectopic *Dll* and *hth* together induce *sal* expression and antenna formation in the leg. (A,B) A leg disc from an animal of genotype: *UAS-Dll; UAS-GFP-hth/ dpp-GAL4*. Coexpression of Dll (not shown) and Hth (green) can induce Sal expression (red) in the presumptive medial and distal leg. Only the ectopic Hth is visible because it is tagged with GFP. (C,D) Adult legs derived from animals of genotype: *UAS-Dll; UAS-GFP-hth/ dpp-GAL4*. C is a first thoracic segment (T1) leg. D is a third thoracic segment (T3) leg. In each case, transformation of one of the tarsal claws (arrowhead) to arista (arrow) can be seen. The pulvilli are indicated by asterisks. Transformation of only one of the two claws is probably due to the fact that Dpp, and therefore the ectopic Dll and Hth, is expressed only on the anterior side of the anteroposterior compartment boundary, overlapping only the anterior claw primordium.

Could Dll form a functional complex with Hth and Exd in the antenna?

Given that Dll and Hth cooperate to regulate antennal differentiation, it is of interest to elucidate the molecular basis

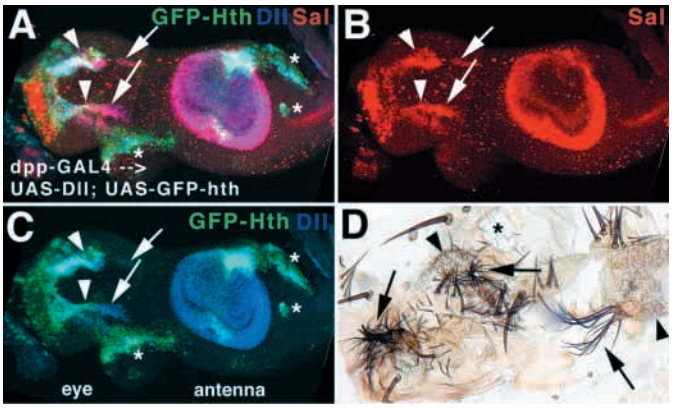

Fig. 8. Ectopic *Dll* and *hth* together induce *sal* expression and antenna formation in the head. (A-C) An eye-antennal disc from an animal of genotype: *UAS-Dll; UAS-GFP-hth/ dpp-GAL4*. Coexpression of Dll (blue) and Hth (green) can induce Sal expression (red) in the eye (arrowheads). Only the ectopic Hth is visible because it is tagged with GFP. Arrows indicate sites where Dll is expressed, but Hth can no longer be detected. This probably reflects *Dll* autoregulation. Sal is expressed in these cells, which probably express endogenous Hth. Asterisks indicate sites in which coexpression of Dll and Hth does not induce Sal. (D) Extra antennal tissue induced on the head of an animal in which Hth and Dll were ectopically expressed. As many as three ectopic antenna are frequently observed on each side. Aristae are indicated with arrows. a3-like tissue is indicated with arrowheads. The eye is marked with an astersk.

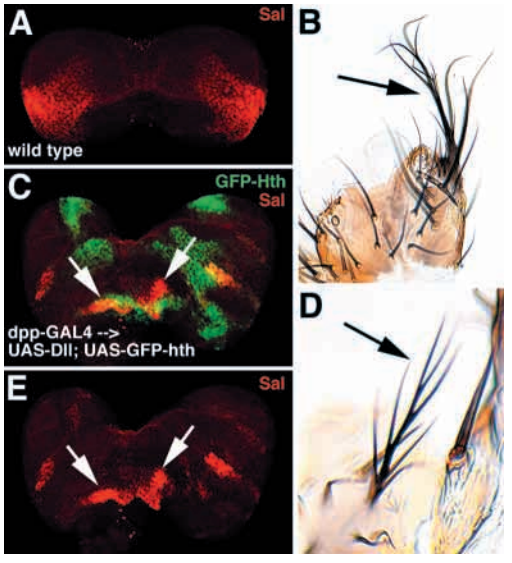

Fig. 9. Ectopic *Dll* and *hth* together induce *sal* expression and antenna formation in the genital disc derivatives. (A) Sal expression (red) in a wild-type male genital disc. (B,D) Aristae induced on female (B) and male (D) genital disc derivatives by ectopically expressing both *hth* and *Dll* with *dpp-GAL4* and *UAS-Dll* and *UAS-GFP-hth* constructs. (C,E) Sal (red) induced in a male genital disc by ectopically expressing both GFP-Hth (green) and Dll (not shown) with *dpp-GAL4* and *UAS-Dll* and *UAS-GFP-hth* constructs. Note that the ectopic *hth* and *Dll* repress endogenous *sal* expression, and that *sal* is not activated everywhere Dll and Hth are expressed.

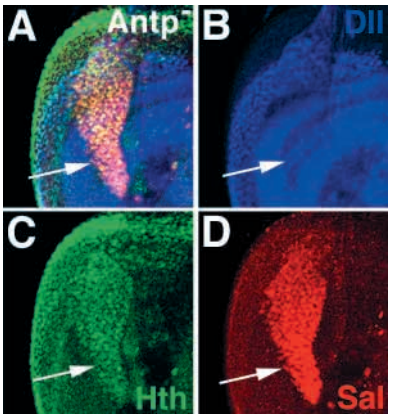

Fig. 10. *Antp* may repress *sal* via *hth*. (A-D) *Antp* null clone in the leg disc in which Dll expression (blue) is normal, and both Hth (green) and Sal (red) are derepressed. Sal is only produced by Hth expressing cells within the Dll domain. The arrow indicates the distalmost portion of the clone.

of this synergy. Exd and its vertebrate counterpart, Pbx, are known cofactors for a variety of homeodomain proteins, including Labial, Engrailed and Ultrabithorax (reviewed in Mann and Chan, 1996). Hth is required for retention of Exd in the nucleus (Pai et al., 1998; Rieckhof et al., 1997; Kurant et al., 1998) and may form part of the functional Exd/Hox complex (Rieckhof et al., 1997). Vertebrate homologs of Hth, the Meis and Prep proteins, have been shown to form trimeric complexes with Hox and Pbx proteins (Berthelsen et al., 1998; Swift et al., 1998; Goudet et al., 1999; Shen et al., 1999). Several lines of evidence now support the idea that Exd and Hth are cofactors for the Dll homeodomain protein in the developing *Drosophila* antenna. These include: (1) the similar antenna-to-leg transformation phenotypes of *Dll*, *hth* and *exd* mutants, (2) the known physical interactions of Exd and Hth with other homeodomain proteins, (3) the fact that *Dll* and *hth* function in parallel to regulate antennal development, and (4)

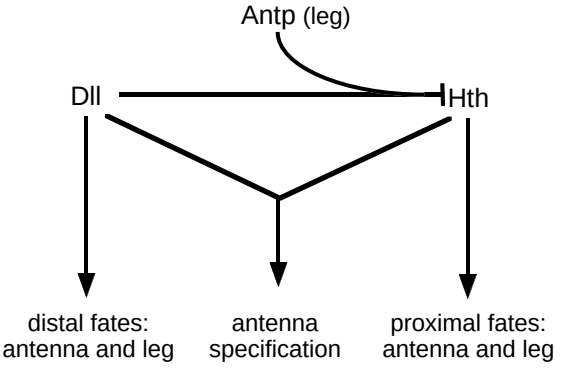

Fig. 11. Genetic hierarchies regulating antenna and leg differentiation and limb outgrowth. *Dll* is required for formation of distal limb elements in both the antenna and the leg. *hth* is required for formation of proximal limb elements in both the antenna and the leg. For their roles in proximodistal patterning, the two genes function independently. However, in the antenna, *Dll* and *hth* cooperate to induce antennal differentiation. *Antp* represses distal expression of *hth* in the leg, precluding the overlap of Dll and Hth and thereby preventing antennal differentiation.

the fact that ectopically expressing Hth can mimic loss of *Dll* function in the antenna. Testing whether Dll, Hth and Exd interact physically and whether such a complex activates antennal enhancers will be important steps toward understanding limb development and tissue-specific gene regulation.

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REFERENCES

Abu-Shaar, M. and Mann, R. S. (1998). Generation of multiple antagonistic domains along the proximodistal axis during *Drosophila* leg development. *Development* **126**, 3821-3830.

Balkaschina, E. J. (1929). Ein Fall der Erbhomoeosis (die Genovariation "Aristapedia") bei Drosophila melanogaster. *Roux's Arch. EntwMech. Org.* **115**, 448-463.

Berthelsen, J., Zappavigna, V., Ferretti, E., Mavilio, F. and Blasi, F. (1998). The novel homeoprotein Prep1 modulates Pbx-Hox protein cooperativity. *EMBO J.* **17**, 1434-1445.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Casares, F. and Mann, R. S. (1998). Control of antennal versus leg development in *Drosophila*. *Nature* **392**, 723-726.

Cohen, S. M. (1990). Specification of limb development in the *Drosophila* embryo by positional cues from segmentation genes. *Nature* **343**, 173-177.

Cohen, S. M. and Jurgens, G. (1989). Proximal-distal pattern formation in *Drosophila*: cell autonomous requirement for *Distal-less* gene activity in limb development. *EMBO J.* **8**, 2045-2055.

Diaz-Benjumea, F., Cohen, B. and Cohen, S. M. (1994). Cell interaction between compartments establishes the proximal-distal axis of *Drosophila* legs. *Nature* **372**, 175-178.

Duncan, D. M., Burgess, E. and Duncan, I. (1998). Control of distal antennal identity and tarsal development in *Drosophila* by *spineless-aristapedia*, a homolog of the mammalian dioxin receptor. *Genes Dev.* **12**, 1290-1303.

Gehring, W. (1966). Bildung eines vollstaendigen Mittelbeines mit Sternopleura in der Antennenregion bei der Mutante *Nasobemia* (*Ns*) von *Drosophila melanogaster*. *Arch. Julius Klaus-Stift. Vererbungsforsch Sozialanthropol, Rassenhyg.* **41**, 44-54.

Gonzalez-Crespo, S., Abu-Shaar, M., Torres, M., C. Martinez-A and Mann, R. S. (1998). Antagonism between *extradenticle* function and Hedgehog signaling in the developing limb. *Nature* **394**, 196-199.

Gonzalez-Crespo, S. and Morata, G. (1995). Control of *Drosophila* adult pattern by *extradenticle*. *Development* **121**, 2117-2125.

Gorfinkiel, N., Morata, G. and Guerrero, I. (1997). The homeobox gene *Distal-less* induces ventral appendage development in *Drosophila*. *Genes Dev.* **11**, 2259-2271.

Goto, S. and Hayashi, S. (1997). Specification of the embryonic limb primordium by graded activity of Decapentaplegic. *Development* **124**, 125-132.

Goudet, G., Delhalle, S., Biemar, F., Martial, J. and Peers, B. (1999). Functional and cooperative interactions between the homeodomain PDX1, Pbx, and Prep1 factors on the somatostatin promoter. *J. Biol. Chem.* **274**, 4067-4063.

Halder, G., Callaerts, P., Flister, S., Walldorf, U., Kloter, U. and Gehring, W. (1998). *Eyeless* initiates the expression of both *sine oculis* and *eyes absent* during *Drosophila* compound eye development. *Development* **125**, 2181-2191.

Kuhnlein, R., Frommer, G., Friedrich, M., Gonzalez-Gaitan, M., Weber, A., Wagner-Bernholz, J., Gehring, W., Jackle, H. and Schuh, R. (1994). *spalt* encodes an evolutionarily conserved zinc finger protein of novel structure which provides homeotic gene function in the head and tail region of the *Drosophila* embryo. *EMBO J.* **13**, 168-179.

Kurant, E., Pai, C. Y., Sharf, R., Halachmi, N., Sun, Y. H. and Salzberg, A. (1998). *Dorsotonals*/*homothorax*, the *Drosophila* homologue of *meis1* interacts with *extradenticle* in patterning of the embryonic PNS. *Development* **125**, 1037-1048.

Mann, R. S. and Chan, S.-K. (1996). Extra specificity from extradenticle: the partnership between HOX and PBX/EXD homeodomain proteins. *Trends in Genetics* **12**, 258-262.

Masucci, J., Miltenberger, R. and Hoffmann, F. M. (1990). Pattern specific expression of the *Drosophila decapentaplegic* gene in imaginal discs is regulated by 3' regulatory elements. *Genes Dev.* **4**, 2011-2023.

Morimura, S., Maves, L., Chen, Y. and Hoffmann, F. M. (1996). *decapentaplegic* overexpression affects *Drosophila* wing and leg imaginal disc development and *wingless* expression. *Dev. Biol.* **10**, 136-151.

Pai, C.-Y., Kuo, T., Jaw, T., Kurant, E., Chen, C., Bessarab, D., Salzberg, A. and Sun, Y. (1998). The Homothorax homeoprotein activates the nuclear localization of another homeoprotein, Extradenticle, and suppresses eye development in *Drosophila*. *Genes Dev.* **12**, 435-436.

Panganiban, G., Sebring, A., Nagy, L. and Carroll, S. B. (1995). The development of crustacean limbs and the evolution of arthropods. *Science* **270**, 1363-1366.

Pignoni, F. and Zipursky, S. (1997). Induction of *Drosophila* eye development by Decapentaplegic. *Development* **124**, 271-278.

Rieckhof, G. E., Casares, F., Ryoo, H. D., Abu-Shaar, M. and Mann, R. S. (1997). Nuclear translocation of Extradenticle requires *homothorax*, which encodes an Extradenticle-related homeodomain protein. *Cell* **91**, 171-183.

Shen, W., Rozenfeld, S., Kwong, A., Kom ves, L., Lawrence, H. and Largman, C. (1999). HOXA9 forms triple complexes with PBX2 and MEIS1 in myeloid cells. *Molec. Cell Biol.* **19**, 3051-3061.

Struhl, G. (1981). A homeotic mutation transforming leg to antenna in *Drosophila*. *Nature* **292**, 635-638.

Struhl, G. (1982). *Spineless aristapedia*: A homeotic gene that does not control the development of specific compartments in *Drosophila*. *Genetics* **102**, 737-749.

Sunkel, C. E. and Whittle, J. R. S. (1987). *Brista*: A gene involved in the specification and differentiation of distal cephalic and thoracic structures in *Drosophila melanogaster*. *Wilhelm Roux Arch. Dev. Biol.* **196**, 124-132.

Swift, G., Liu, Y., Rose, S., Bischof, L., Steelman, S., Buchberg, A., Wright, C. and MacDonald, R. (1998). An endocrine-exocrine switch in the activity of the pancreatic homeodomain protein PDX1 through the formation of a trimeric complex with PBX1b and MRG1 (MEIS2). *Molec. Cell Biol.* **18**, 5109-5120.

Vachon, G., Cohen, B., Pfeifle, C., McGuffin, M. E., Botas, J. and Cohen, S. M. (1992). Homeotic genes of the bithorax complex repress limb development in the abdomen of the *Drosophila* embryo through the target gene *Distal-less*. *Cell* **71**, 437-450.

Wagner-Bernholz, J. T., Wilson, C., Gibson, G., Schuh, R. and Gehring, W. J. (1991). Identification of target genes of the homeotic gene *Antennapedia* by enhancer detection. *Genes Dev.* **5**, 2467-2480.

Weigmann, K. and Cohen, S. M. (1999). Lineage-tracing cells born in different domains along the PD axis of the developing *Drosophila* leg. *Development* **126**, 3823-3830.

Xu, T. and Rubin, G. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-37.