Int. J. Dev. Biol. 42: 431-436 (1998)

Homeotic *proboscipedia* cell identity functions respond to cell signaling pathways along the proximo-distal axis

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ABSTRACT To better understand how the different cell identities composing a segment are attributed and coordinated under the control of a single homeotic selector gene, we examined dose-sensitive homeotic phenotypes associated with gain-of-function and loss-of-function mutations of the homeotic gene *proboscipedia* (*pb*; Hox-A2/-B2). We then employed dose-sensitive segment and cell identity phenotypes resulting from ectopic proboscipedia expression to screen for other interacting loci. We find that *pb*, as well as the homeotic loci *Ultrabithorax*, *Sex combs reduced* and *Antennapedia*, respond to positional information along the proximo-distal axis. This response for *pb* implicates at least two signal transduction pathways, those involving *Ras1* and *Notch*.

KEY WORDS: *cell identity, cell signaling, Drosophila, homeotic, proximal-distal axis*

Introduction

The homeotic selector genes of *Drosophila* specify the identities of the segments composing the embryo and the adult fly. This was interpreted as a consequence of the coordinate regulation of batteries of downstream target genes (García-Bellido, 1975,1977). The prediction that the implementation of these "realizator" genes under "selector" control leads to a differentiated segment has since been widely validated. The remarkably conserved homeotic (HOM) homeodomain proteins are employed to regulate gene transcription in segmental registers. The diverse roles of homeotic proteins in segmental differentiation are seen in the diversity of target genes identified to date, a list including the transcription factors *Distalless* and *spält* (Wagner-Bernholz *et al.,* 1991; Vachon *et al.,* 1992), the chromatin protein modulo (Graba *et al.,* 1994), the centromeric component centrosomin (Heuer *et al.,* 1995; Li and Kaufman, 1996), cell contact molecules encoded by *connectin* and *scabrous* (Gould and White, 1992; Graba *et al.,* 1992), and signaling morphogens *Wnt/wingless* and *TGF-β/decapentaplegic* (Reuter *et al.,* 1990; Capovilla *et al.,* 1994). [For a recent review, see (Graba *et al.,* 1997)].

We are interested in how one HOM selector protein can govern and organize the disposition of the ensemble of cell identities composing a segment. Relative to the embryo, homeotic function in the control of adult pattern formation has been relatively little studied. The attribution of diverse cell types appears to involve a segment-specific interpretation of segmentally-repeated positional information. For example, dominant transformations due to inappropriate Antennapedia expression replace proximal antennal tissue with proximal leg, while distal leg replaces distal antenna. (Postlethwaite and Schneiderman, 1971). Distinctive cell identities such as sex comb teeth have long been used as markers of homeotic function in segmental transformation. Dose-sensitive phenotypes have often proven a useful starting point for seeking specific biological partners, permitting the identification of synergistically-interacting molecules as dominant Modifier mutations of their genes. The complex external structure of an adult segment offers a useful model for studying how cell identity is attributed within a group of cells. We have employed a variety of dose-sensitive dominant HOM-induced phenotypes to examine how the ensemble of cell identities is established within a segment. Our phenotypic evidence indicates that homeotic genes may show a marked response to positional cues in attributing adult cell identities, as a function of proximo-distal position. The identification of dose-sensitive Modifiers leads us to suggest that these HOM functions implicate at least two cell signaling pathways, involving *Ras1* and *Notch*.

Results

Loss- and gain-of-function proboscipedia phenotypes are differentially expressed on the proximal-distal axis

Loss-of-function mutations of the homeotic *proboscipedia* gene lead to recessive, dose-sensitive homeotic transformations of the adult labial palps (Kaufman, 1978; Pultz *et al.,* 1988). The normal adult labium derives from the labial imaginal discs. Uniform Pb

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0214-6282/98/$10.00

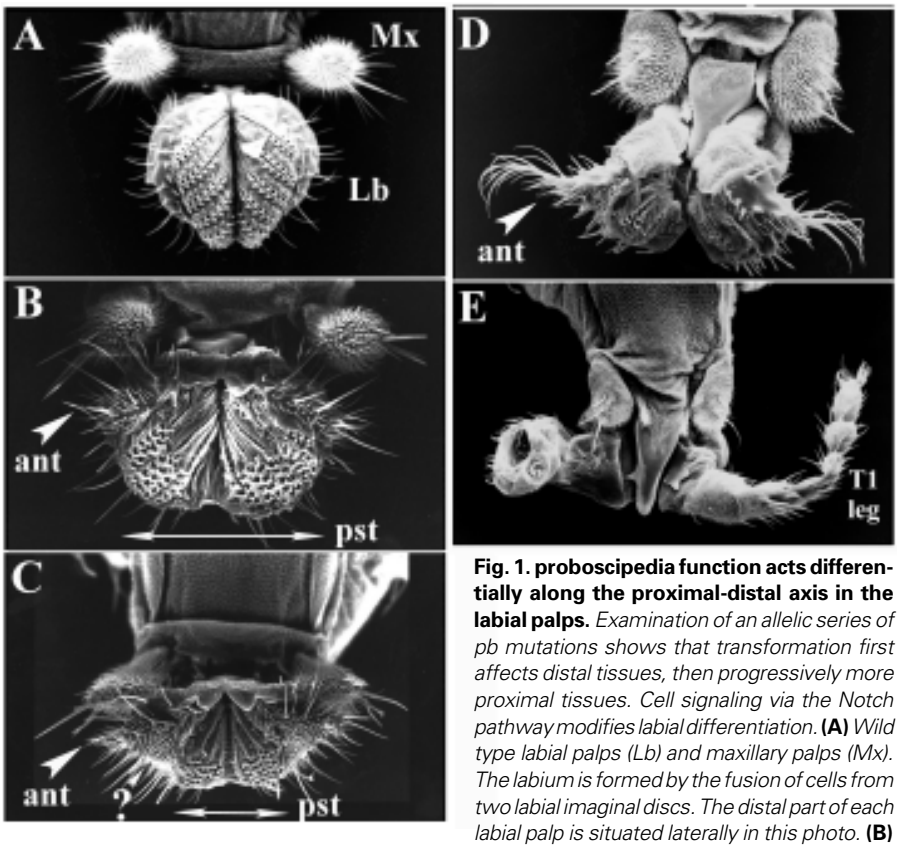

Fig. 1. proboscipedia function acts differentially along the proximal-distal axis in the labial palps. *Examination of an allelic series of pb mutations shows that transformation first affects distal tissues, then progressively more proximal tissues. Cell signaling via the Notch pathway modifies labial differentiation.* **(A)** *Wild type labial palps (Lb) and maxillary palps (Mx). The labium is formed by the fusion of cells from two labial imaginal discs. The distal part of each labial palp is situated laterally in this photo.* **(B)** *The allelic combination pb^5/pb^{13} (null/weak hypomorph) leads to the beginning of a distal transformation of labium to antennal arista (arrowhead, marked ant). The extent of the medial pseudotracheal rows (pst) is indicated by the arrow.* **(C)** *The labium of this $N^{55e11}/+$; pb^5/pb^{13} individual shows the limited labial to antennal transformation as in B (arrowhead), but with reduced pseudotracheal rows (arrow) and between the two a region of apparent epiderm (arrowhead, ?).* **(D)** *The pb^4 allele is an intermediate hypomorphic mutation that transforms distal labial palps to antennal aristae (arrowhead, ant). Some pseudotracheal tissue remains on the proximo-medial part of each palp.* **(E)** *The null condition (here, a pb^5 homozygote) results in replacement of the labial palps by distal prothoracic (T1) legs.*

protein expression in the labial discs (Randazzo *et al.,* 1991) leads to a differentiated structure including distinctive medial pseudotracheae (pst) for drinking. Loss-of-function *pb* mutations can be ordered in an allelic series yielding qualitatively distinct transformations of the labium to leg or antenna. Increasingly severe loss-of-function alleles lead to the progressive transformation of distal then more proximal tissue, as seen from the appearance of first of antennal aristae then prothoracic leg, and a diminishing number of medial pseudotracheal rows (Fig. 1A,B,D,E). Null loss-of-function mutations lead to the complete transformation of the labial palps to distal prothoracic legs (compare Fig. 1. A,E). Despite essentially uniform normal expression of Pb protein, distal labial elements are more sensitive to a reduction in *pb* activity than are more proximal elements.

Ectopic Pb expression from a transgenic mini-gene element (Cribbs *et al.,* 1995) results in several dose-sensitive gain-of-function phenotypes. Two of these can be viewed as classical homeotic segmental transformations. The distal adult antennae are transformed to maxillary palps (Fig. 2A-C). This transformation progresses from proximal to distal with increasing pb^+ function, by augmenting either transgene copy number (Fig. 2B,C) or Pb activity (Boube *et al.,* 1997). The antenna-to-maxillary transformation [Fig. 2B,C; (Cribbs *et al.,* 1995)] reflects normal pb^+ function required for development of the maxillary palps as well as the labial palps (compare Fig. 1A,E). A second partial segmental transformation induced by Pb protein can be obtained in individuals carrying a hsp70 promoter-pb cDNA transgene. Administering multiple heat shocks during larval development leads to the transformation of distal T1, T2 and T3 legs to antennal aristae. This mixed leg-antennal structure resembles the mixed transformation obtained for the labium with some $pb^{antennal}/pb^{leg}$ allelic combinations (see Pultz *et al.,* 1988, or Boube *et al.,* 1997 for examples). Distal transformation, but not proximal, was observed with this regime yielding uniform expression. Thus as for the transformation of antenna to maxillary palp, and of labial palps to antenna or leg, differing susceptibilities are observed for cells at different positions along the proximal-distal axis. However, it is not clear whether this transformation may best be interpreted as a positive selector function of *pb*, or rather an antimorphic activity opposing leg formation.

Several other dose-sensitive phenotypes result from ectopic expression of the Pb protein driven by the HSPB transgene. Under the presumption that segmental identity can be treated as an ensemble of ordered cell identity problems, we have interpreted these phenotypes as transformations of cell identity at positions especially sensitive to Pb accumulation within a generally refractory segmental context. One defect provoked by ectopic Pb expression is a dose-sensitive eye loss (Benassayag *et al.,* 1997). Another phenotype induced by ectopic Pb expression is the appearance of a second, more distal sex comb on the second tarsal segment (Boube *et al.,* 1997). These specialized bristles occupy equivalent A-P and D-V positions but differ in position along the P-D axis. Yet another phenotype is the disappearance of the dense mechanosensory (MS) bristles from the anterior wing margin (compare Fig. 2E,F). A marked preferential loss of distal MS bristles from the triple row is observed (the chemosensory bristles of the triple row are only slightly affected).

Screens for dominant Modifier mutations affecting dominant pb phenotypes

The criterion of dose-sensitive phenotypic interactions has been used as a basis to search for functional partners of proboscipedia in homeotic function. Our assumption is that employed by García-Bellido and collaborators in their "gene-dose titration" analysis (Botas *et al.,* 1982), namely that synergistic, dose-sensitive interactions are likely to reflect functional specificity. In this light we searched for partners as dominant Modifiers of any of the dominant pb phenotypes. The validity of such putative

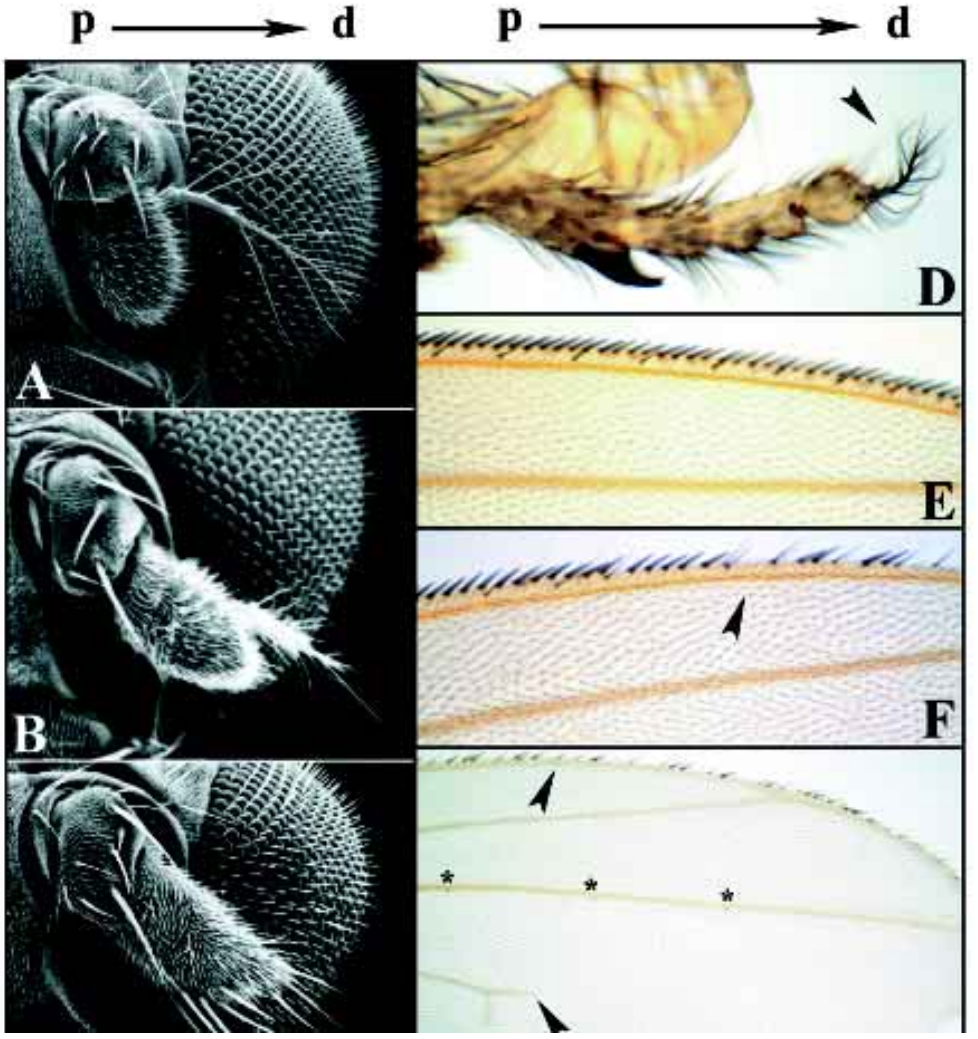

Fig. 2. Ectopic Proboscipedia expression leads to segment and cell identity changes. *Misexpression of Pb leads to diverse dose-sensitive defects including of the antennae, the legs and wings.* **(A-C)** *Progressive transformation of the antennae to maxillary palps is obtained on increasing the copy number of transgenic HSPB elements from zero (A), to one (B), to two (C) copies. This transformation does not affect the most proximal antennal tissues, and acts differentially along the proximo-distal axis from proximal (p) toward distal (d; indicated by the arrow at the top).* **(D)** *The distal prothoracic leg of a male fly carrying a Hsp70-pb cDNA construct and subjected to repeated heat shocks during larval and pupal development. The distalmost leg structures have been transformed to antennal arista (arrowhead), while more proximal tissue retains T1 leg identity (note the black row of sex comb teeth).* **(E,F)** *The anterior wing margins of flies carrying one (E) or two (F) HSPB copies show marked differences in the differentiation of the row of mechanosensory bristles. The wing margin is normal in flies carrying a single copy of the transgene, whereas the loss of many distal, but not proximal, bristles is noted in flies with two copies (F, arrowhead).* **(G)** *Mild wing phenotypes are provoked by one HSPB copy in heterozygous combination with either Ras1- or H- alleles. In this triple heterozygote (HSPB H-/Ras1-), a non-additive interaction is observed. The anterior wing margin is strongly affected (arrowhead on top, compare with E), the longitudinal wing veins L4 (arrowhead) and L5 (not visible in this photo) in the posterior compartment are partially deleted from distal toward proximal. The posterior crossvein (partially deleted here, arrowhead) is in some cases removed entirely. Also, the placing of the Campaniform sensilla (marked by asterisks) on L3 of the wing blade is irregular and displaced proximally. This result represents formal genetic evidence for functional linking of the Notch/Hairless and Ras1 signaling pathways.*

partners can then be tested in the normal lieu of pb^{+} function in the adult mouthparts, by making double mutants with pb^{lf}.

Several screens have now been employed. One was carried out with a set of characterized autosomal deficiencies and the HSPB transgene. A second, similar screen was for new DEB-induced Modifier-of-HSPB mutations. A third screen was for dominant Enhancers of the eye loss provoked by expression of a mutant Pb protein called $Pb^{sans\ yeux}$ (Benassayag *et al.,* 1997).

Each screen has yielded different Modifier loci. (1) The first screen, employing the characterized deficiencies of the autosomal Deficiency Kits (Indiana Univ. *Drosophila* Stock Center), led to the identification of the signal transduction molecules *Ras1* and *Gap1* as modifiers of *pb*. Both genes modify *pb* function in segment and cell specification, in normal as well as ectopic situations (Boube *et al.,* 1997). $Ras1^{lf}$ mutations reduce the dominant HSPB antenna-to-maxillary phenotype, from distal toward proximal, while $Gap1^{lf}$ mutations have the opposite effect. In the mouthparts, the use of $Ras1^{lf}$ alleles in combination with *pb* hypomorphic alleles suggests that distal cells are more sensitive to homeotic transformation than are more proximal cells. The attribution of specific cell identities along the P-D axis (sex comb teeth and distal claws) is sensitive to the level of $Ras1^{+}$ function. (2) In the second screen, DEB-induced mutations identified as modifying HSPB phenotypes included an allele of *Hairless* enhancing MS bristle loss on the anterior wing margin. HSPB +/+ H^{DMu4} led to a phenotype like that in Figure 2F (due to two HSPB copies) with a marked distal reduction of MS bristles, rather than to a single HSPB copy (Fig. 2E). Equivalent results were obtained with HSPB in combination with a null allele. *Hairless* is known to participate in the *Notch (N)* pathway where it opposes N^{+} function, and indeed the wing margin phenotype can be reversed in heterozygotes carrying a N^{lf} allele such as N^{55e11} (N^{-}/+; HSPB +/+ H^{-}). (3) In a third screen, we employed the dose sensitive dominant eye loss induced by the mutated element $HSPB^{sans\ yeux\ (sy)}$ (Benassayag *et al.,* 1997) to screen for dominant Enhancer mutations. Females carrying two transgene copies have essentially normal eyes, whereas with four transgenic copies the

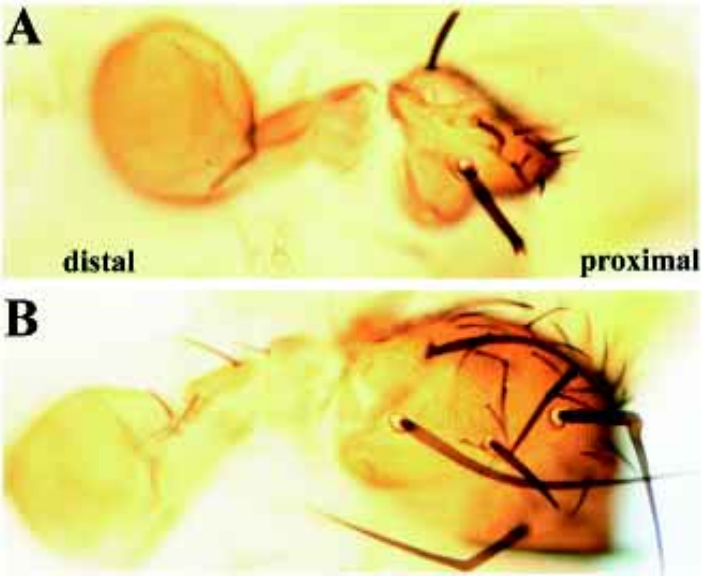

Fig. 3. *Ultrabithorax* function along the proximo-distal axis is sensitive to *Ras1* activity. *Compared are thoraces from Gap1⁻ Ubx⁻/bx and Gap1⁻ Ubx⁻/Ras1⁻ bx. adult flies. Reducing Ras1⁺ activity leads to an enhancement of the Ubx⁻/bx mutant phenotype readily seen as an extension of the notum (T2) in place of normal T3 structures. This effect, much more pronounced proximally on the body wall than on the haltere appendage, supports a role for Ras1 signaling activity in differentiation of the T3 segment under Ubx selector control.*

eyes are strongly reduced or abolished. After mutagenesis with EMS or DEB, female flies carrying two transgene copies but with strongly reduced eyes were selected. Among our collection of $HSPB^{sy}/HSPB^{sy}$; En/+ mutant stocks, three mutant *Notch* alleles were identified. The null N^{55e11} allele yielded equivalent results to the strongest *N* Enhancer allele. Marked eye reduction was likewise observed in N^{-} $HSPB^{sy}/+$ $HSPB^{sy}$ individuals, associated with occasional duplication of the maxillary palps and antennae. To test whether the interaction observed between *N* and $HSPB^{sy}$ in an ectopic and inappropriate situation corresponds to a normal interaction in the mouthparts, N^{+} activity was reduced in a *pb* hypomorphic combination. As seen in Figure 1B,C, the level of N^{+} activity can modulate differentiation of the adult labial palps. In mutants carrying the protein null pb^{5} allele together with the weak hypomorph pb^{13}, only a slight transformation of the distal labium toward antenna is detected, and several apparently normal pseudotracheae are fabricated more medially (Fig. 1B). In contrast, in $N^{-}/+$; pb^{-}/pb^{13} individuals, a proximal expansion from the distal antennal tissue is seen to occur at the expense of more proximal pseudotracheal rows (Fig. 1C). Curiously, this expansion does not yield a more fully elaborated arista, but is composed of apparent epidermal tissue. The most direct explanation for these observations appears to involve a binary choice between normal pseudotracheal tissue or its absence (here, an epidermal differentiation), modulated by the level of N^{+} function in the cells giving rise to the adult labium.

Ultrabithorax/Ras1 interactions along the proximal-distal axis in metathorax development

We previously identified *Ras1* and *Gap1* mutations as phenotypic modifiers of various gain-of-function and loss-of-function alleles of the HOM loci *Antp*, *Sex combs reduced* and *Ultrabithorax* alleles (Boube *et al.,* 1997, and unpublished results). The haploinsufficient $Ubx^{-}/+$ genotype leads to a mild haltere toward wing transformation aggravated by recessive *bithorax (bx)* mutations. On examining various combinations of Bithorax Complex mutations, we noted a new and marked effect of *Ras1* on metathoracic development proximo-medial to the haltere appendage itself (as seen in Figure 3, where the deletion allele Ubx^{109} was placed in combination with a regulatory *bx* mutation). A marked difference in metathoracic development was obtained when $Ras1^{+}$ activity was varied. In Ubx^{-}/bx flies with full $Ras1^{+}$ activity, only limited transformation of the metathorax toward mesothorax was observed immediately medio-proximal to the halteres, with weak expressivity and partial penetrance (~60%). In contrast, on using the $Ras1^{C40b}$ null allele for the genotype $Ras1^{-}$ $bx/+$ Ubx^{-} the penetrance for this proximal phenotype rose to about 90% and the transformation was markedly stronger, as seen for the representative adult cuticles in Figure 3. The more complete transformation observed in individuals with reduced *Ras1* activity correspond to outgrowths of notal tissue from mesothoracic epidermis. It is not clear at what level *Ras1* activity may act. The augmented transformation might be due to the inclusion of an inappropriately high cell number in the haltere anlagen or alternatively, it could reflect locally increased cell proliferation. Which of these possibilities is correct (if either) remains to be examined. If the observation of more complete transformation in individuals with reduced *Ras1* activity proves to correspond to increased local cell proliferation, this would raise the interesting possibility of novel $Ras1^{+}$ functions that oppose rather than facilitate proliferation.

Interactions among signaling pathways?

Dose-sensitive dominant cell identity phenotypes provoked by ectopic expression of Pb protein have been used to probe for how homeotic genes organize pattern within the confines of a segment. Screens for dominant modifier mutations of Pb activity have identified several loci encoding signaling molecules, including *Ras1*, *Gap1*, *Notch* and *Hairless*. The three former have now been shown to modify *pb* function in labial development, validating their identification in ectopic environments. We have begun to test whether these signaling pathways are fully independent, or whether they might rather be functionally connected by "cross-talk" mechanisms. Independence of action of two pathways should correspond to addition of phenotypes. In contrast, if these pathways are functionally linked, it may be possible to detect this by a synergistic, non-additive phenotypic response. We have tested several triple mutant combinations so far employing the HSPB transgene (though none in the mouthparts, where the triple mutant combinations including pb^{lf} alleles have been non-viable). The example shown in Figure 2G is a triple heterozygote comprising HSPB H^{E31}/Ras^{e1F}. Each mutation alone is without visible effect on wing development. For each pairwise combination, small deletions in the distal portions of wing veins L4 and L5 are sometimes observed. (Also, as noted above, the HSPB/H^{-} leads to loss of distal bristles on the anterior margin.) On combining mutations of the *Ras1* and *Notch* pathways with HSPB, the distal portions of veins L4 and L5 are deleted up to and including the posterior crossvein. The positions of some other specific cell identities are also altered as seen, for example, for the

Campaniform sensilla (marked by asterisks in Fig. 2G) whose spacing and position are altered along the P-D axis on vein L3. The phenotypes of several tests effected to date for eye and wing development appear non-additive. This observation is consistent with a heretofore unknown functional link between the *Ras1* and *Notch* pathways.

Discussion

Screens based on cell identity transformations to dissect normal homeotic function

The novelty of our approach resides in its premise: that cell identity changes in an inappropriate segment and with no obvious relation to "normal" homeotic function, may serve as a reliable guide to identify new relationships in the segmental differentiation pathway. In practice, we make use of distinctive position-specific cell markers such as sex comb teeth, but also of any other cell type. Screens for modifiers of the homeotic segmental antenna-to-maxillary transformation have allowed us to identify roles for *Ras1* and *Gap1* in normal *pb* homeotic function in labial development (Boube *et al.,* 1997). It is important to note that *Ras1* and *Gap1* interactions with ectopically expressed Pb lead to other non-homeotic cell transformations that are more reliable than the effects of heterozygous mutations on antennal transformation. *Notch* and *Hairless* were identified as Modifiers of ectopic HSPB activity in screens based on very different cell identity phenotypes in the eyes and wings. The analysis above provides a formal validation of this approach for *Notch*, as previously shown for *Ras1* and *Gap1* (Boube *et al.,* 1997). Several other genes have now been identified by these and related screens. Most have been found to interact with *pb* in labial or maxillary palps development; the others have yet to be examined by clonal analysis. These observations are encouraging for the study of homeotic genes expressed in small imaginal discs such as the labial discs, since they suggest that large, highly differentiated structures such as the wings and eyes can be used to effectively conduct initial screens for genes that would be difficult to identify directly via their mouthparts phenotypes. For example, the interaction of *Notch* with *pb* in the labium has been initially identified by the enhancement of an eye loss (inappropriate tissue) provoked by Pb^{sy} (a mutant protein). The role of *Notch* in differentiation of the P-D axis of the labial palps, albeit clear, would be less accessible to detection in the context of a screen.

Segmental differentiation and cell autonomy

The simple expression of a homeotic selector protein is not sufficient to reprogram the differentiation of a segment. Indeed, most segments are impervious to HOM protein expression, at least for the expression regimes employed. The observation of which segments are susceptible to homeotic transformation and which are not, has led to the formulation of the notion of "posterior prevalence" whereby more posteriorly expressed HOM proteins are functionally dominant to those more anterior (Duboule and Morata, 1994). This concept is buttressed by a variety of examples from embryogenesis of flies and mice. But this rule appears not to be universal, the role of *pb* in adult development representing an exception. Apart from the transformation of antennae to maxillary palps, ectopic Pb expression can induce the clear transformation of all three pairs of distal legs, to antennal aristae (Fig. 2).

Mutations altering the activities of *Ras1* and *Notch* signaling pathways markedly sensitize wing and eye disc differentiation to the presence of Pb protein, as seen for the wing in Figure 2. Such observations may have at least two novel implications. One is that homeotic function may not be entirely autonomous, since a role for cell signaling in homeotic function suggests previously undetected dialogue between the cells in the confines of a segment. The results of previous analyses of this question have generally supported a cell autonomous role for the nuclear HOM transcription factors - for example, (Morata *et al.,* 1983) - though in one case, a similar analysis of the *Antennapedia* locus and its role in adult development yielded evidence for non-autonomous action (Struhl, 1981). A second implication is that cell signaling pathways may be part of a "context" that helps to progressively reinforce correct developmental decisions. It has long been noted that normal development is highly reproducible, whereas developmental mutants and especially gain-of-function mutations are more erratic. Perhaps multiple rounds of cell signaling, by an as yet unknown number of signaling molecules, contribute to consolidate the normal pathway.

Subdividing the segment: the proximal-distal axis, the appendage and cell signaling

The antero-posterior and dorso-ventral axes are established during early embryogenesis from localized maternal determinants. Proximo-distal axis specification occurs somewhat later in the imaginal disc primordia giving rise to most external structures of the adult fly, and involves expression of the homeobox gene *Distal-less (Dll)* required for correct distalization of the appendages (Cohen and Jürgens, 1989; Cohen *et al.,* 1989). Distal-less is a target, probably direct, of the homeotic proteins Ultrabithorax and Deformed (Vachon *et al.,* 1992; O'Hara *et al.,* 1993). The initial establishment of the proximal-distal axis involves the localized activation of *Distal-less* reflecting an interpretation of positional cues within the segment by the HOM selector. In the labial imaginal primordium, *Dll* activation depends on *wingless* (Cohen, 1990). *Dll* activity is required for correct development of the labial palps and antennae (Cohen and Jürgens, 1989). Curiously, no modification of the dose-sensitive Pb-induced adult phenotypes employed here was observed in trans-heterozygous combinations with mutant *Dll* alleles (not shown), even though Pb acts differentially along the P-D axis both in the adult mouthparts and elsewhere (Figs. 1,2). We suggest that early HOM function may create a progressive environment by controlling instructive target genes whose functions may establish subsequent positional information utilized for ongoing homeotic function. For example, transcriptional repression of *Dll* by *Ubx* in the abdomen is temporally restricted, the *Dll* regulatory element becoming refractory to *Ubx* function before mid-embryogenesis (Castelli-Gair and Akam, 1995). Selector function may thus involve a progressive step-by-step coordinating activity, and repeated deployment in successively more detailed versions of a segment, to establish the final adult form.

Acknowledgments

We thank Stéphane Noselli and Alain Ghysen for their critical readings of the manuscript and many helpful comments. M.B. and L.S. were both supported by fellowships from the Ministère de l'Education Supérieur et de la Recherche (MESR) and from the Association pour la Recherche contre le Cancer (ARC). This work was supported by ongoing funding from the CNRS, as well as by grants from the MESR and the ARC.

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