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Homeotic *proboscipedia* function modulates *hedgehog*-mediated organizer activity to pattern adult *Drosophila* mouthparts

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Abstract

Drosophila proboscipedia (*pb; HoxA2/B2* homolog) mutants develop distal legs in place of their adult labial mouthparts. Here we examine how *pb* homeotic function distinguishes the developmental programs of labium and leg. We find that the labial-to-leg transformation in *pb* mutants occurs progressively over a 2-day period in mid-development, as viewed with identity markers such as *dachshund (dac)*. This transformation requires *hedgehog* activity, and involves a morphogenetic reorganization of the labial imaginal disc. Our results implicate *pb* function in modulating global axial organization. Pb protein acts in at least two ways. First, Pb cell autonomously regulates the expression of target genes such as *dac*. Second, Pb acts in opposition to the organizing action of *hedgehog.* This latter action is cell-autonomous, but has a nonautonomous effect on labial structure, via the negative regulation of *wingless*/dWnt and *decapentaplegic*/TGF-β. This opposition of Pb to *hedgehog* target expression appears to occur at the level of the conserved transcription factor *cubitus interruptus*/*Gli* that mediates *hedgehog* signaling activity. These results extend selector function to primary steps of tissue patterning, and lead us to suggest the notion of a homeotic organizer.

Keywords: Homeotic; *Proboscipedia*; *Hedgehog*; Organizer; Signaling; *Wingless*; *Decapentaplegic*

Introduction

Homeotic selector genes organize development in regions along the anterior–posterior (A–P) axis of highly diverse animal species, conferring identity to groups of contiguous cells. In *Drosophila*, homeotic mutants with altered selector function provoke transformations of one body segment to another. This developmental reprogramming, a defining property of homeotic genes (Bateson, 1894), is believed to reflect the altered expression of downstream target "realisator" genes (Garcia-Bellido, 1975). In accord with this prediction, Hox genes code for homeodomain transcription factors thought to control segment morphology and cell identity through the regulation of batteries of target genes (Mann and Morata, 2000; McGinnis and Krumlauf, 1992). A growing number of homeotic target genes has now been identified (Graba et al., 1997; Weatherbee et al., 1998). Several recent studies have provided compelling evidence that *Drosophila* Hox selectors discriminate among alternative cell fates, presumably through gene regulation, as a function of the cellular context (Brodu et al., 2002; Estrada and Sanchez-Herrero, 2001; Rozowski and Akam, 2002). Though extensive duplication complicates functional analysis in vertebrates, the Hox genes individually and the gene complexes they compose are remarkably conserved in a wide variety of metazoans (for review, see McGinnis and Krumlauf, 1992). Numerous studies of vertebrate Hox genes have described complex and dynamic developmental roles in regional and cellular specification within the central nervous system, in limb and muscle development, or in organizing and confining plasticity in the neural crest (Maconochie et al., 1996;

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Trainor and Krumlauf, 2001). Further, some vertebrate Hox mutants show clear developmental transformations (Maconochie et al., 1996) that are viewed as analogous to *Drosophila* homeotic mutants.

Adult legs and wings are often-used models for patterning by homeotic genes in *Drosophila* development, and legs are exemplary in this regard. They originate from the ventral leg imaginal discs through a shared patterning mechanism defined by a complex genetic cascade. A–P imaginal disc subdivision reflects the posterior expression of the segment polarity gene *engrailed* (*en*). Localized expression of the En homeodomain protein that defines the posterior compartment activates transcription of *hedgehog* (*hh*) in posterior cells, providing a source of secreted Hh protein that serves as a short-range morphogen across the A–P compartment boundary. A nexus of the *hh* pathway is at the level of the Cubitus interruptus (Ci) zinc-finger protein expressed in anterior cells. Differential cleavage of Ci results in alternative protein forms that are dynamically cycled between the cytoplasm and nucleus: a noncleaved activator form near the source, or a processed repressor form in more distant anterior cells (Aza-Blanc and Kornberg, 1999; Aza-Blanc et al., 1997). Active Ci near the frontier contributes in turn to D–V patterning by activating the mutually antagonistic *decapentaplegic* (*dpp*) and *wingless* (*wg*) genes that express secreted TGF-β and Wnt family proteins in the anterior compartment (Basler and Struhl, 1994; Brook and Cohen, 1996; Jiang and Struhl, 1996; Penton and Hoffmann, 1996). The expression of the Dpp and Wg morphogens along the A–P boundary of the leg disc, in the dorsal and ventral territories, respectively, permits their joint action in patterning the proximo-distal (P–D) axis through the activation [*Distal-less* (*Dll*) and *dachshund* (*dac*)] or the repression [*homothorax* (*hth*)] of target genes (Abu-Shaar and Mann, 1998; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). Each resulting leg pair is distinct, with the segment-specific differences in morphology and specific bristle pattern attributed to a unique *Hox* selector gene: *Sex combs reduced (Scr)* in the T1, or prothoracic segment, *Antennapedia (Antp)* in T2 (mesothorax), and *Ultrabithorax (Ubx)* in T3 (metathorax), respectively (Struhl, 1982).

A related process takes place in the dorsal imaginal discs giving rise to wings and halteres. There, activator Ci contributes to A–P patterning through the localized activation of *dpp* in anterior cells adjacent to the A–P boundary; the resulting gradient of secreted TGF-β morphogen diffusing from this source contributes to establishing wing size and pattern. Contrary to leg discs, however, wing imaginal discs contain a second, Hh-independent dorsal–ventral (D–V) organizer. There, the activity of the *apterous*-encoded transcription factor (Diaz-Benjumea and Cohen, 1993) contributes to the localized expression of the dWnt growth factor encoded by *wingless* (*wg*), whose diffusion leads to a second morphogen gradient complementary to the *dpp* signal.

Surprisingly little is known of the nature of a Hox network involved in specifying segment form and identity, and this problem has remained stubbornly refractory to our understanding. Until rather recently, homeotic functions have most often been viewed as essentially cellular. However, homeotic genes can have a nonautonomous effect on segment development implying intercellular communication, as described for the role of *Antennapedia* in distinguishing leg and antennal developmental programs (Struhl, 1981). Important new insights from studies of the role of the *Drosophila* homeotic *Ubx* locus in differentiating haltere from wing have renewed the question of a homeotic network. The Ubx homeodomain protein is now seen as playing a role in the global elaboration of the haltere imaginal disc, where it regulates diverse genes involved at multiple levels in signaling pathway output (Weatherbee et al., 1998). One potentially important level of *Ubx* action is nonautonomous and global, by restraining *wingless*/dWnt signaling from a localized dorso-ventral (D–V) organizer influencing wing growth, patterning, and cell differentiation (Shashidhara et al., 1999).

The labial palps, the drinking and taste apparatus of the adult fly head, are highly refined ventral appendages homologous to legs and antennae. As for most adult structures, these mouthparts are derived from larval imaginal discs, the labial discs. Wild-type *proboscipedia* (*pb; HoxA2/B2* homolog) selector function acts together with a second *Hox* locus, *Scr*, to direct the development of the labial discs giving rise to the adult proboscis. In the absence of *pb* activity, the adult labium is transformed to distal prothoracic (T1) legs, reflecting the ongoing expression and function of *Scr* in the same disc (Abzhanov et al., 2001; Percival-Smith et al., 1997). Though the *pb* locus shows prominent segmental embryonic expression, as for the other *Drosophila* homeotic genes of the Bithorax and Antennapedia complexes, it is unique in that it has no detected embryonic function (Pultz et al., 1988) and null *pb* mutants eclose as adults that are unable to feed. Thus, normal *pb* selector function is required relatively late, in the labial imaginal discs that proliferate and differentiate during larval/pupal development to yield the adult labial palps. Though the genetic pathway guiding development of the ventral labial imaginal discs to adult mouthparts remains relatively unexplored both in flies and elsewhere, one recent study of P–D patterning identified several genes subject to *pb* regulation in the labial discs (notably *Dll*, *dac*, and *hth*) and indicated a distinct organization of normal labial discs compared to other imaginal discs (Abzhanov et al., 2001).

Here we have pursued an investigation of how *pb* homeotic function distinguishes between labial and leg developmental programs. Our results implicate *pb* function at the level of global axial organization. Employing identity markers such as *dachshund (dac)*, we identify a 2-day

period late in larval development when normal *pb* function is required for labial development. The labial-to-leg transformation occurs during the third larval instar stage, involves a progressive morphogenetic reorganization of the labial imaginal disc, and is *hedgehog*-dependent. Our analysis of the transformation indicates that normal *pb* action is required at least at two distinct levels. One is in the cell-autonomous regulation of target genes such as *dac* likely to be implicated in cell identity. A second level involves an autonomous action with a nonautonomous effect on labial structure, through the negative regulation of *wingless*/dWnt and *decapentaplegic*/TGF-β downstream of *hh* signaling. This opposition to *hh* targets is likely to occur at the level of the transcription factor *cubitus interruptus*/*Gli*, a crucial and conserved mediator of *hh* signaling activity. These results lead us to propose that homeotic function may exist in intimate functional contact with the *hedgehog* organizer signaling system: the "homeotic organizer".

Materials and methods

Drosophila strains and culture

Drosophila stocks were maintained at 22°C on standard cornmeal/agar medium unless otherwise noted. The mutant chromosomes employed, generated by standard genetic recombination, were maintained as heterozygotes with the TM6B, *Hu Tb* balancer chromosome.

The transgenic *pb*GAL4 P element was constructed as described in Benassayag et al. (2003), and insertion lines were obtained by standard P-element-mediated transformation procedures (Rubin and Spradling, 1982). Among several independent lines tested for their ability to direct localized expression from a UAS:*lacZ* reporter element, the *pb*GAL4(10) insertion (on chromosome 2) was retained for our analyses. This line directs labial expression resembling wild type (though it is stronger distally than proximally). A recombinant chromosome 2 carrying both the *pb*GAL4(10) and UAS:Flp insertions served as a labial-directed source of Flp recombinase for clonal analysis. GAL4 transcriptional activity varies with temperature. In experiments where components of the Hh pathway were misexpressed under pbGAL4 control from UAS transgenes, labial phenotypes were examined for adults raised at several temperatures ranging from 18°C to 28°C.

Histology, immunohistochemistry, and antibodies

Antibody stainings were carried out essentially as described previously (Pattatucci and Kaufman, 1991). Imaginal discs were dissected and mounted on microscope slides in 0.5× PBS–50% glycerol, then viewed using a confocal microscope (Zeiss LSM 410 or Leica TCS SPII). Antibodies were employed at the following dilutions: mouse anti-Dac, 1:100; rabbit anti-Hth, 1:500; mouse anti-Dll, 1:500; mouse anti-βgal, Promega, 1:5000; mouse anti-Wg, 1:100; rabbit anti-En, 1:100. The secondary antibodies, directed against mouse or rabbit IgGs, were coupled to rhodamine, Cye-5 or FITC fluorochromes.

Clonal analysis

Mitotic recombination events giving rise to clones were induced by the Flp/FRT system (Xu and Rubin, 1993). Non-tissue-directed clones of pb^- cells were obtained in larvae of genotype *y w* hs-Flp; FRT^{82B} *Ki* pb^5/FRT^{82B} P[Ub-GFP], where clones were induced by heat-shock during the first or second larval instars (2 h at 37.5°C). Localized expression of Flp recombinase under control of a *pb*GAL4 driver (above) allowed us to obtain mitotic clones in the small labial imaginal discs at high frequency (≥1 clone/disc). To favor large clones, this tissue-specificity was coupled with the Minute technique (Morata and Ripoll, 1975) conferring a specific growth advantage to pb^- labial cells. The composite CyO-TM6B balancer employed to ensure co-segregation of *pb*GAL4 UAS:Flp (chromosome 2) with the FRT-harboring chromosome 3 of interest is unstable for parts of the third chromosome, and such stocks must be regenerated periodically. Clones were induced in larvae from crosses of *pb*GAL4(10) UAS:Flp; FRT^{82B} *M(3)rps3* P[Ub-GFP]/T(2;3)CyO-TM6B, *Hu Tb* flies with the following stocks: FRT^{82B} *Ki* pb^5/TM6B, *Hu Tb;* FRT^{82B} hh^{AC}/TM6B, *Hu Tb;* FRT^{82B} *Ki* pb^5 hh^{AC}/TM6B, *Hu Tb*.

Directed misexpression in the labial discs was driven by the *pb*GAL4(10) element. UAS constructs employed, singly or in combination, expressed lacZ, GFP, Pb, Ptc, Dpp, and/or Wg (Bloomington stock collection), Hh and full-length Ci (provided by Bruno Glise). Gain-of-function clones were generated by the Flip-out technique (Struhl and Basler, 1993), in one of two ways. (1) After crossing UAS:pb or UAS:HhCD2 transgenic lines with act > CD2 > GAL4; hs-Flp *Sb*/TM6B, *Tb* flies, Flp recombinase expression was induced by heat shock (37°C, 45 min) in L2 or early L3 larvae. Resulting act > GAL4 clones overexpress normal Pb protein, or membrane-tethered Hh (HhCD2). Overexpression of HhCD2 in a pb^5 context was carried out in the same conditions on flies of genotype hs-Flp/+; act > CD2 > GAL4/+; UAS:HhCD2 FRT^{82B} *Ki* pb^5/FRT^{82B} *Ki* pb^5. (2) Alternatively, the *pb*GAL4 UAS:Flp was used to overexpress Wg from corresponding Flp-out constructions (Struhl and Basler, 1993).

Adult cuticle analysis

Phenotypes were initially examined under a stereomicroscope. Flies of interest were stored in ethanol until dissection. Detailed examinations of dissected heads and legs were performed by light microscopy (Zeiss Axiophot) after mounting in Hoyer's medium.

Results

Larval pb activity regulates cell identity, target gene expression, and global positional information

While imaginal disc development leading to adult form (notably the large discs yielding wings and legs) in the fruit fly has been subjected to intense scrutiny, the labial discs yielding the adult feeding apparatus have attracted comparatively limited attention. Nevertheless, they offer a useful model for studying homeotic function since mouthpart form in *Drosophila* is highly plastic and depends on the state of *pb* selector function: diminishing *pb* activity in labial cells results in their reprogramming, to antennae in hypomorphs, or to T1 legs in null mutants.

To better understand how *pb* selector function acts in directing labial development, we induced mitotic clones of pb^- cells then examined the consequences on adult structure and larval gene expression. Clones induced during the first and second larval stages, using an inducible *hs-Flp* transgene as the source of recombinase (Xu and Rubin, 1993), led to discrete changes in cell identity from normal labium

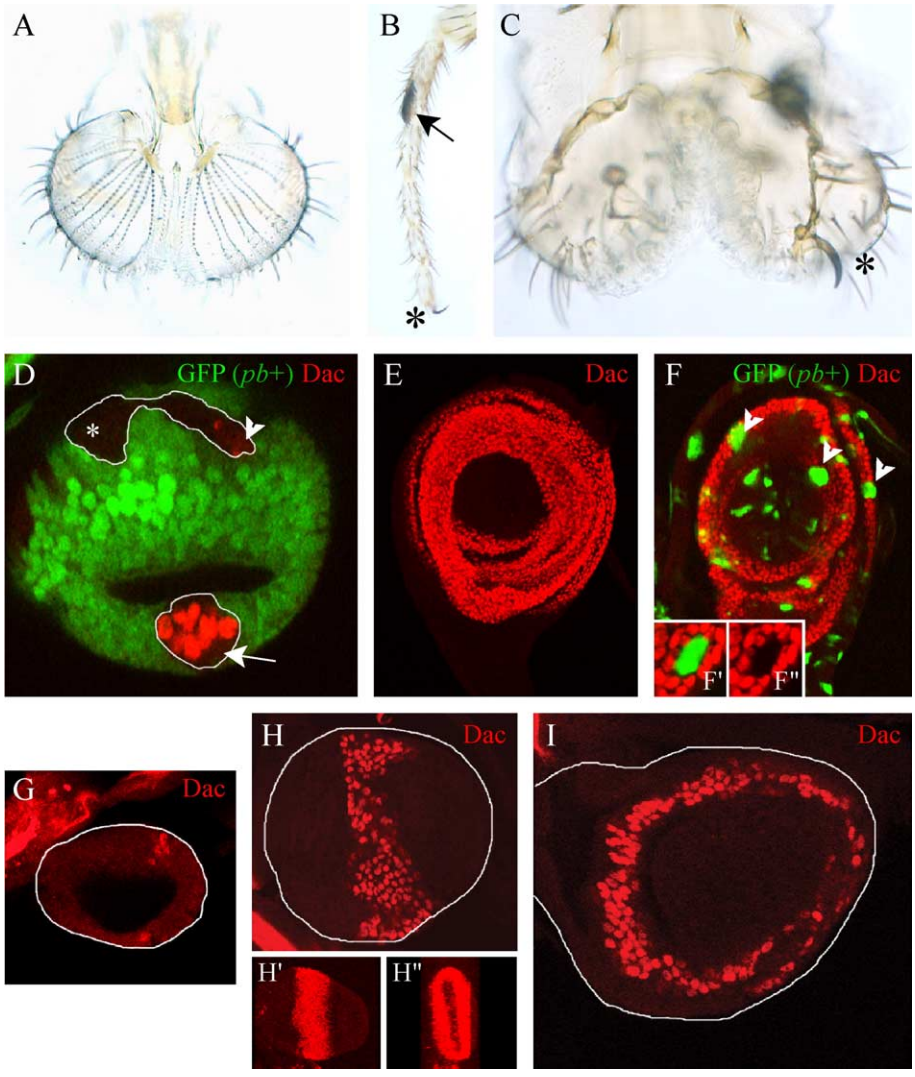


Fig. 1. *pb* regulates *dac* in a context-dependent manner. (A) Wild-type mouthparts, with every wild-type hemi-labium showing five to six pseudotracheal rows. (B) Wild-type distal T1 male leg, with sex comb teeth (arrow) and claw (asterisk). (C) Clones of pb^- cells lead some cells to adopt leg fate, as for this distal claw (asterisk). (D) *dac* is activated nonuniformly in pb^- clones located in a labial disc. Mutant cells visualized by the absence of GFP differentially express Dac (red): strongly (bottom); at low levels suggesting recent onset (arrowhead); or no detectable expression (asterisk). The arrow, bottom, indicates two cells in a pb^- clone that lack detectable Dac, in contrast to expressing neighbors. (E, F) *pb* function blocks *dac* activation in otherwise competent leg disc cells. (E) Wild-type Dac (red) accumulation in a leg disc. (F) Dac (red) accumulation is autonomously repressed in clones of Pb-expressing leg disc cells (green; Flip-out GAL4 > UAS:*pb*/UAS:GFP). (F′, F″) Higher magnification of a clone of Pb-expressing cells (green) located in the *dac*-expressing domain (red), showing the disappearance of Dac from all cells expressing Pb. A progressive homeotic/morphogenetic transformation from labium to leg. Dac is absent from wild-type labial discs. In a pb^- labial disc, (G) Dac is first detected in early L3 larvae in two patches of cells (arrowheads); the expression (H) expands to comprise a band of cells, which (I) resolves to a leg-like ring of Dac expressing cells (cf. E, F). (H′, H″) Three-dimensional confocal reconstructions of Dac expression in a mid-L3 mutant disc: in H′, Dac expression sections are compiled in the *z* axis (comparable to B); in H″, the same 3D reconstruction is rotated 90° to show that the apparent band of Dac-expressing cells is in fact a ring prefiguring the final flattened disc form (I).

(Fig. 1A) toward T1 leg (Fig. 1B), as shown by the appearance of a distal leg claw (asterisk, Fig. 1C) or of sex comb teeth (not shown). Such results confirm a role for *pb* in attributing cell identity.

To explore the molecular basis of these cell identity transformations, we employed recently identified markers including *dac* (Abzhanov et al., 2001). The nuclear Dac protein is required in multiple developmental processes, and the ring of Dac expression in the leg discs (Fig. 1E) reflects its role in medial leg differentiation (Mardon et al., 1994). Contrary to leg discs, *dac* is not expressed in the wild-type labial disc (Abzhanov et al., 2001). However, Dac protein can be detected within clones of pb^- cells in labial discs. As seen in Fig. 1D (arrow), most but not all pb^- cells of this clone expressed Dac at high levels. However, in a second clone in the same disc, Dac was expressed only weakly (arrowhead) or not at all (asterisk). Taken together, these data confirm the negative regulation of *dac* expression by Pb in the labial disc, but the observed disparities in *dac* expression (strong, weak or undetectable in pb^- cells) suggest that activation requires the absence of Pb protein over an extended period of time, in the presence of position-specific information.

Cells that normally express *dac* in other discs possess all required environmental signals. We therefore asked whether the addition of Pb protein suffices to extinguish *dac* expression. Cell autonomous repression of *dac* was observed in clones of Pb-expressing cells generated by the "flip-out" method (Struhl and Basler, 1993) in leg (see Figs. 1F, F′, F″), wing, antennal and eye discs (not shown). These results support a cell-autonomous role for Pb in repressing *dac* expression, downstream of environmental activating signals.

However, a careful examination of *dac* gene expression in *pb* mutants led us to reconsider this position. Dac protein is normally absent from pb^+ labial cells, but accumulates in a subset of labial cells in mature pb^- L3 larvae (Abzhanov et al., 2001). *pb* is expressed in the larval/pupal labial disc but also in the embryo, where it has no known function. To identify the temporal requirement for pb^+ in repressing *dac*, we examined Dac protein accumulation in *pb* mutants. Dac protein expression is first detected in a small number of labial disc cells in early L3 larvae (Fig. 1G). This group is enlarged in mid-L3 to an apparent band of expressing cells (Fig. 1H); this is in fact a ring of cells around the pouch-like labial disc as is readily seen in three-dimensional reconstructions (compare Fig. 1H with H′, H″). Finally, the ring perpendicular to the image resolves to a ring in the plane of the photo late in larval development (Fig. 1I). Thus, *dac* expression initiates then evolves dynamically during the L3 time frame. These observations provide two sorts of new information concerning pb^+ homeotic function. First, they identify a limited time frame, the larval third instar period, for a homeotic transformation. Second, this homeotic function appears to act at the level of global tissue organization. The dynamic changes in Dac indicate its environment is changing alongside. These data thus support an ongoing requirement for *pb* in maintaining the global organization of the labial disc.

A progressive homeotic/morphogenetic transformation

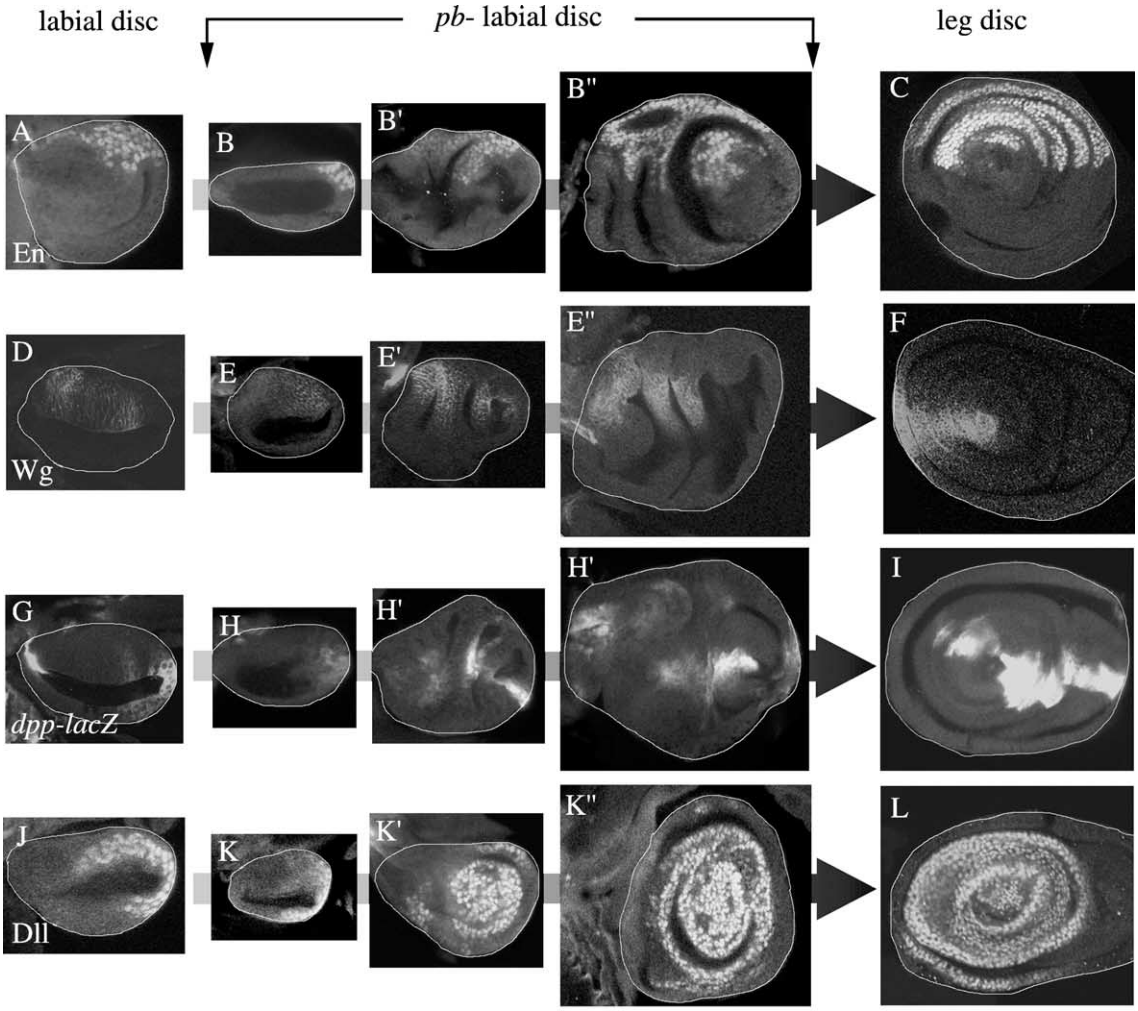
The preceding observations led us to envisage the possibility that global signals change over time in *pb* mutant L3 discs. We therefore compared the axial organization of the wild-type labial disc with that of the leg disc. Normal labial discs differ morphologically from leg discs in several respects, as the labial disc is considerably smaller, the single layer of epithelial labial disc cells is organized as a small pouch with a lateral furrow rather than the flattened structure of the leg disc, and labial discs lack the peripodial membrane of large, flattened epithelial cells seen in other imaginal discs.

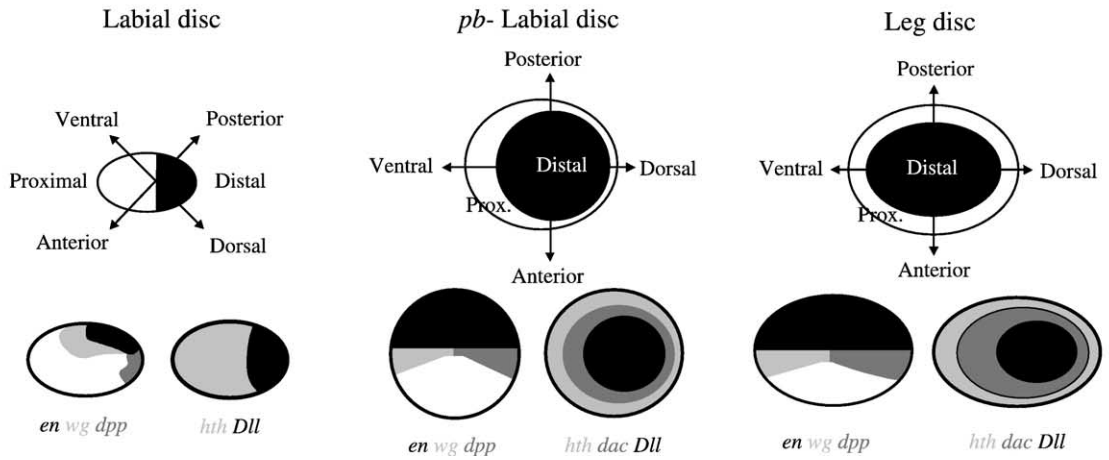
Toward a molecular description of labial disc organization, we first examined expression of the A–P marker gene *engrailed* (*en*). The posterior, En-expressing region (adjacent to the furrow: Fig. 2A) is small, comprising only about 20% of the cell population rather than half in the leg (Fig. 2C). All cells accumulating the nuclear En homeodomain protein co-express *hh* as seen with a *hh-lacZ* marker. Next, we examined the expression of the D–V markers *wg* and *dpp*. Accumulation of Wg protein (Fig.

Fig. 2. A progressive homeotic/morphogenetic labial-to-leg transformation. (A, D, G, J) En, Wg, *dpp* (visualized with a *dpp-lacZ* reporter) and Dll distributions in a wild-type labial disc. (B–B″, E–E″, H–H″, K–K″) Progression of En, Wg, *dpp* (visualized with a *dpp-lacZ* reporter), and Dll expression, respectively, during the third larval instar (L3) in pb^- labial discs. B–E–H–K: early L3; B′ –E′ –H′ –K′ : mid-L3; and B″–E″ –H″–K″ : late L3 discs. (C, F, I, L) En, Wg, *dpp-lacZ* reporter and Dll expression in a wild-type leg disc. (A) En distribution in a wild-type labial disc, where posterior cells represent only about 20% of the cell population. (B–B″) Progressive expansion of the En-expressing population in a *pb* background. At the end of the L3 stage, En-expressing cells represent nearly half of the cell population, as for the leg disc. (C) Unlike the labial disc, En is detected in half of the leg disc cell population. (D) Wg is expressed at a very low level in cells located near the A–P frontier in a wild-type labial disc. (E–E″) Evolution of Wg expression expands and intensifies during L3 in a pb^- mutant to adopt a "leg-like" pattern. (F) Strong localized Wg expression in a leg disc defines the ventral part of the future leg. (G) *dpp* is expressed in small groups of cells located near the antero-posterior frontier. These *dpp*-expressing cells do not express Wg morphogen. (H–H″) *dpp* expression is increased and expanded during the L3 larval instar to adopt a "leg-like" pattern of expression. (I) *dpp* expression pattern in a wild-type leg disc, defining the dorsal part of the tissue. (J) *Dll* expression defines the distal part of a wild-type labial disc. (K–K″) Evolution of *Dll* expression in a pb^- mutant disc. This pattern evolves from a wild-type pattern at the beginning of L3 to a leg-like pattern where *Dll* accumulates in the center of the tissue. (L) *Dll* expression in the center of a normal leg disc. At the bottom is a summary of the axial structures of normal and mutant labial discs, and of normal leg discs. In each case, different axes are represented in the bottom part, distal and proximal part appear respectively in black and white. In the bottom line, paired imaginal discs show the expression patterns for *en*, *wg* and *dpp* (left), or *Dll*, *dac* and *hth* (right) for a wild-type labial disc (left column), a pb^- labial disc (central column) or a leg disc (right column).

2D), of β-galactosidase from a *wg-lacZ* P insertion (not shown) or from a *dpp-lacZ* transgene (Fig. 2G), are detected in the anterior part of the disc adjacent to the En/Hh-expressing cell population, but at markedly lower levels compared to other imaginal discs of the same animals. The P–D marker genes *Dll* and *hth* are expressed in largely exclusive groups of cells, as in the leg (not shown). These observations support the existence of a *hh*-inspired signaling cascade in the labium related to that in the leg.

The differences between normal and *pb* mutant discs result from multiple changes that appear during L3 larval development. Mutant labial discs appear normal in early L3 larvae (~72 h), but are two-fold or more enlarged compared





to wild-type and show leg-like form by the end of L3 (~120 h). This can be attributed at least in part to augmented proliferation in mutant cells visualized with an antibody recognizing the phosphorylated histone 3 form (not shown). During the same interval the posterior (En-expressing) portion of the disc expands progressively, from ~20% initially to encompass nearly half the enlarged mutant disc in late L3 (Figs. 2B, B′ and B″). Expression of both *wg* and *dpp* adjacent to the A–P border is initially low, as in the wild-type labial disc, but expands anteriorly and intensifies during L3 to reach cellular levels similar to leg discs (Figs. 2E, E′ and E″ and Figs. 2H, H′ and H″). Dpp and Wg are known to act in P–D patterning of leg discs through the modulated expression of specific downstream target genes: *Dll, dac* and *hth* (Abu-Shaar and Mann, 1998; Diaz-Benjumea et al., 1994; Lecuit and Cohen, 1997). Apart from *dac* (described above), *Dll* expression is likewise modified progressively during L3 larval development, the normal pattern of early discs (Fig. 2J) resolving to a leg-like pattern (Fig. 2L) by the end of L3 (Figs. 2K, K′ and K″). Comparable results were obtained for *hth* (not shown). The axial structures of normal and mutant labial discs are summarized and compared with normal leg discs at the bottom of Fig. 2. As visualized with these marker genes for all three axes, the temporally restricted homeotic transformation of a labial disc toward leg identity in *pb* mutants involves a global morphogenetic reorganization of the mutant tissue.

The morphogenetic labial-to-leg transformation is Hh dependent

The diffusible Hedgehog molecule encoded by *hh* is considered as a primary patterning effector in the leg disc, and we therefore examined the role of *hh* in the adult labial-to-leg transformation. Since *hh* mutations are recessive embryonic lethals, we used mitotic recombination. Clones in the labial discs were obtained at high frequency by targeting the expression of the FLP recombinase (*pb*-GAL4 + UAS:FLP; "*pb* > FLP"), in the presence of a *Minute* mutation to enhance mutant cell growth (Materials and methods). A wild-type adult labium and its component pseudotracheal rows is shown in Fig. 3A, with the approximate A–P compartment boundary indicated (Hama et al., 1990; Struhl, 1977). Induction of growth-enhanced clones for the null allele pb^5 by *pb* > FLP led to a reliable transformation comparable to pb^- homozygotes (Fig. 3C). Inducing hh^- clones in the same manner resulted in discrete deletions within pseudotracheae near the A–P compartment boundary (Fig. 3B). This result confirms that *hh* function is required for normal labial development, and shows that the tissue most sensitive to the clones is near the A–P boundary. In labial palps with pb^- hh^- double mutant clones induced by *pb* > FLP, distinct leg tarsi were no longer formed (Fig. 3D). The residual tissue clearly retains prothoracic leg identity (sex comb teeth), presumably due to the continued presence of Scr protein, but the abnormally small tissue size coupled with leg cell identity led us to infer an abortive tissue transformation. We conclude that the successful transformation of labium to leg requires *hh* function.

The effect of *hh* activity on the homeotic/morphogenetic transformation of labium to leg was confirmed using the temperature-sensitive allele hh^{ts2} (Ma et al., 1993) to eliminate *hh* function in pb^- larvae. When *hh* activity was removed throughout the normal two day duration of the L3 stage, the pb^- hh^{ts2} disc appeared labial rather than leg-like, both by morphological and molecular criteria. Disc size was not augmented, morphology remained pouch-like, cellular En accumulation was unchanged and the expansion of En+ cells observed in pb^- labial discs was no longer seen (compare Figs. 3E and 2B″). When *hh* function was abolished for the second half of the L3 stage only, the number and proportion of *en*-expressing cells increased (Fig. 3F). Similarly, while *dac* expression was not detected in mutant larvae that were maintained at the restrictive temperature throughout L3 (Fig. 3G), when mutant larvae were shifted to the restrictive temperature at mid L3 stage the *dac* expression pattern at the end of L3 was the lateral stripe typical of mid-L3 *pb* mutant discs (compare Fig. 3H with Fig. 1H). Results obtained with anti-Dll as a marker supported the same conclusion (Figs. 2J–L). We conclude that *hh* activity is required, initially to activate *dac* expression and subsequently for progression (spots → band → ring). Further, the morphogenetic movements and underlying gene expression patterns indicate that reshaping the pb^- mutant labial disc to a leg-like form requires ongoing input from *hh*.

pb acts to limit hh pathway signaling activity

The preceding analysis implicating *hh* in labial development supports two conclusions. First, labial development employs a Hh-mediated organization similar to legs. Second, the successful homeotic/morphogenetic transformation of labial to leg tissue requires ongoing *hh* activity. The dichotomy between the mild effect of reducing *hh* function in pb^+ labial tissue *versus* the dramatic effect on leg formation observed in *pb* mutants might be explained if *hh* expression, or its relevant signaling activity, is required at significantly lower levels in wild-type than in mutant tissue: the effect of removing function might be less if little activity is normally expected.

To position their respective roles, we first compared *hh* expression in pb^- mutants with wild type, in two ways: (i) by the β-galactosidase accumulation from a *hh*-lacZ allele that recapitulates the *hh* pattern; and (ii) by following Hh expression directly with a monospecific antibody. As shown in Fig. 4, the cellular level of β-gal under *hh* control is not detectably altered in pb^- labial discs (compare Figs. 4A, B). The same result was obtained using anti-Hh sera. We conclude that *hh* gene expression itself is not regulated by *pb*.

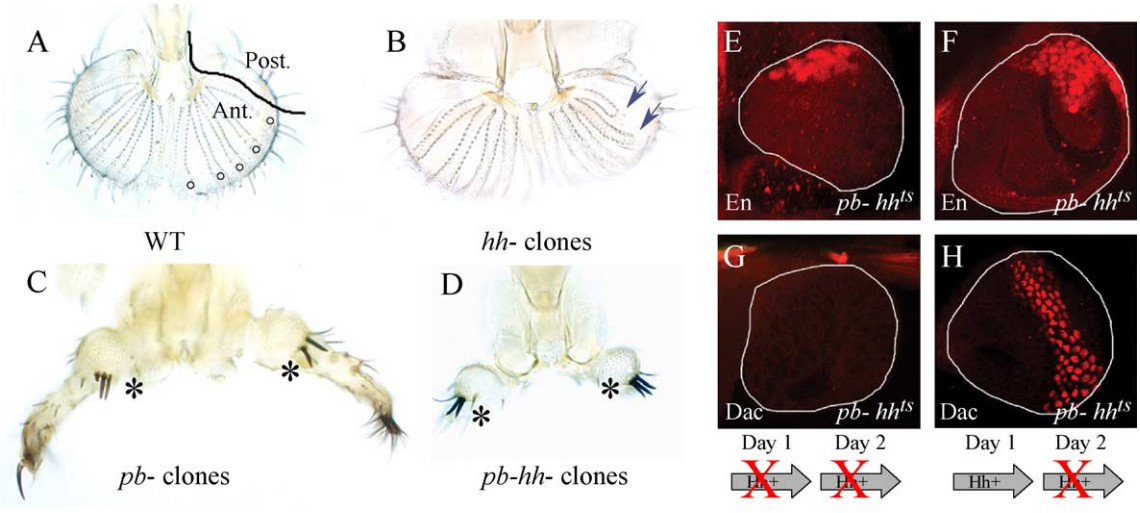


Fig. 3. *hh* pathway activity is required for homeotic/morphogenetic labial-to-leg transformation. All clones shown in this figure were induced using the *pb*GAL4 > UAS:Flp targeting system (Materials and methods). (A) Wild-type adult labium. The A–P boundary is indicated. (B) In hh^- clones, some pseudotracheal cells localized near the A–P boundary are absent (arrow). (C) Adult labial-to-T1 leg transformation with targeted clones of pb^- cells appears complete (asterisks indicate T1-specific male sex comb teeth). (D) In pb^-hh^- double mutants, the labial-to-T1 leg transformation is modified. Sex comb teeth (asterisks) typical of T1 identity are present, but overall tissue size is strongly reduced compared to C. (E–H) All discs are from pb^-hh^{ts2} climbing larvae ~5 days of age (end of L3). (E) En expression remains limited to ~20% of the labial cells on removing *hh* function throughout L3 stage, whereas (F) a partial expansion is observed when *hh* function is removed during the second day of L3. (G) Dac expression is not detected in a pb^-hh^{ts2} labial disc on removing *hh* function throughout the L3 stage, while (H) Dac expression typical of mid-L3 (band of expression, as in Fig. 2H′) is observed on removing *hh* function during the second day of L3.

We then asked how Pb might modulate *hh* activity from new organizing centers: clones of anterior cells expressing a membrane-tethered form of Hh protein that acts at a single-cell range (HhCD2 (Strigini and Cohen, 1997)). Hh-expressing clones generated by the flip-out technique were examined in three situations: anterior leg disc (no Pb), anterior normal labial disc (Pb) and anterior mutant labial disc (no Pb). Two measures of the activity of Hh signaling were employed: activation of the *wg* target gene (de Celis and Ruiz-Gomez, 1995; Guillen et al., 1995; Struhl et al., 1997), and Hh-dependent feedback activation of *engrailed* in anterior cells. For clones in anterior cells of a normal leg disc, *wg* was activated and vesicles of Wg protein were detected within and around Hh-expressing cells (Fig. 4C). En protein accumulation was likewise detected in the subset of Hh-expressing anterior cells with highest GFP accumulation (Fig. 4F). When equivalent clones expressing HhCD2 protein were induced in the anterior compartment of wild-type labial discs (Pb^+), *wg* activation was barely detectable and *en* was not induced (Figs. 4D, G). In stark contrast, when clones of cells expressing HhCD2 were induced in pb^- anterior labial cells, Wg accumulated strongly in and around the clone while En was activated within the clone as for the leg disc (Figs. 4E, H). These data show that *pb* regulates Hh-mediated activation of *wg* and the feedback activation of *en* in labial disc cells.

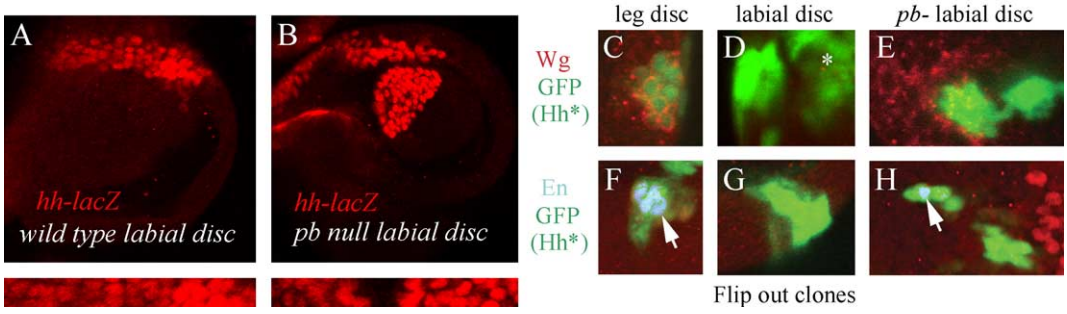


Fig. 4. *pb* controls *hh* activity but not expression. (A–B) A *hh-lacZ* reporter is expressed at indistinguishable levels in cells of (A) a wild-type labial disc, or (B) a pb^- labial disc. (C–H) Cellular effects of anterior Flip-out clones expressing tethered HhCD2 in the presence or absence of Pb. In anterior leg cells (no Pb): (C) the expression of HhCD2 (green, GFP^+) induces Wg (red) accumulation, and (D) leads to En activation in a subset of Hh-expressing cells (blue). In anterior labial disc cells (Pb+): (E) HhCD2-expressing cells (green) induce ectopic Wg (red) at barely detectable levels (asterisk), and (F) fail to activate En (blue). In anterior cells of a pb^- "labial" disc, HhCD2 (green) induces (G) ectopic Wg accumulation (red) in surrounding cells, and (H) En expression (blue) within the clone.

Normal pb function acts to limit hh function in anterior cells at the A–P boundary

Since Pb protein regulates *hh* activity rather than expression, we sought to position *pb* relative to the *hh* pathway in labial development. Pb protein is accumulated uniformly in all cells of the labial discs (Kapoun and Kaufman, 1995; Randazzo et al., 1991). By misexpressing molecules with known roles in the *hh* signaling cascade in normal leg development we asked which, if any, are able to bypass the effects of Pb protein. To this end, a *pb*-GAL4 driver coupled with UAS constructs was used to direct labial expression of Hh, Ptc, Ci, Dpp and Wg. Hh expression was without effect at all temperatures tested (compare Figs. 5A, B). Expressing the Hh receptor encoded by *patched* (*ptc*) was without visible effect at 22°, but at 28° led to a discrete pseudotracheal shortening near the A–P boundary that closely resembles that of hh^{-} mutant clones (Fig. 5C). We consider that this phenotype likely reflects titration of free Hh by excess Ptc receptor. Ci misexpression under *pb*GAL4 control provokes much more extensive defects of the labial palps, including a shortening and disorganization of the pseudotracheal rows (Fig. 5D). Thus deregulating the *hh* pathway can perturb global labial organization. Directed expression of Dpp (Fig. 5E) led to related labial defects and the near-complete absence of pseudotracheae. *pb*GAL4-driven expression of *wg* was fully lethal in all conditions tested. We therefore employed *pb* > FLP as a recombinase source to generate "flip-out" clones of Wg-expressing cells (Struhl and Basler, 1993).

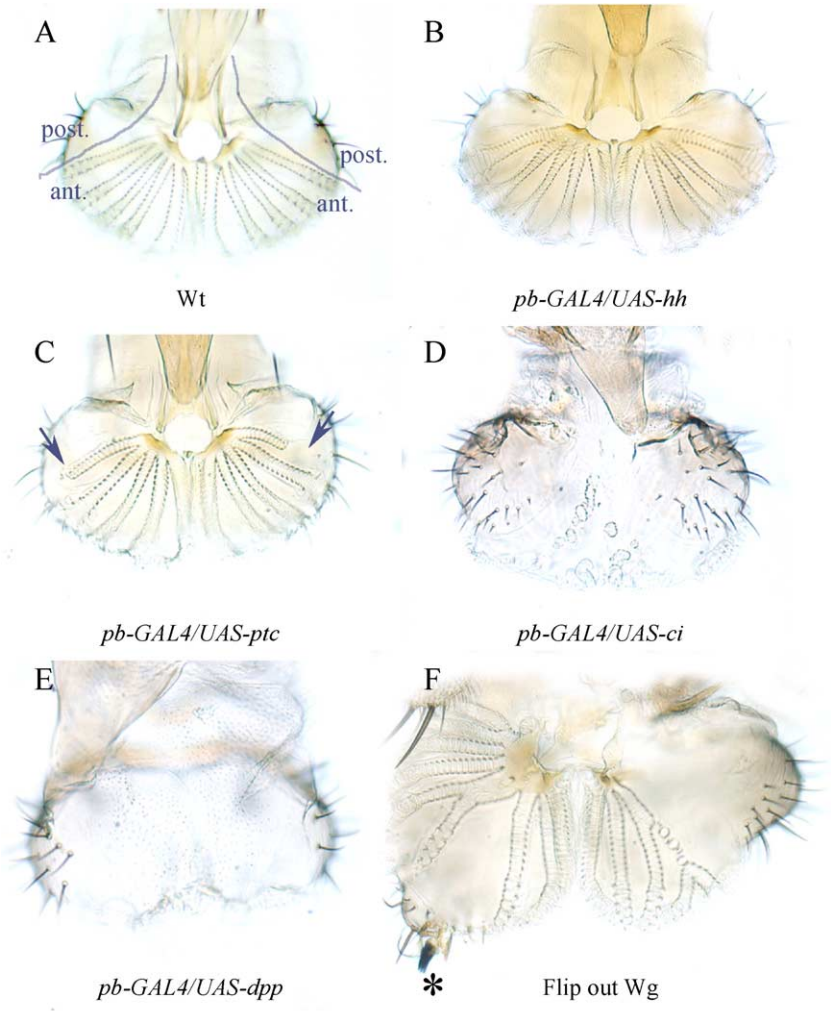


Fig. 5. Dissecting the functional relationship of *pb* and *hh*. Hh, Ptc, Ci, Dpp, and Wg were misexpressed in wild-type labial discs from UAS transgenes directed by a *pb*GAL4 driver (Materials and methods). The adult flies shown in this figure were raised at 28°C. (A) Wild-type adult labium. The position of the A–P compartment boundary in the adult labium (Struhl, 1977) is shown as a dotted line, and pseudotracheal rows are indicated (o). (B) Hh overexpression in these conditions does not affect labial palp development. (C) Ptc overexpression results in the truncation of some pseudotracheal rows near the A–P boundary. (D) Ci overexpression leads to a dose-dependent loss of pseudotracheal rows in the anterior compartment. (E) Dpp misexpression also results in the loss of pseudotracheal rows. (F) Wg overexpression under the same conditions as the other constructs was 100% lethal. Misexpressing Wg via the Flip-out technique (*pb*GAL4 > UAS:Flp; act > CD2 > Wg; Zecca et al., 1995), led to pharate adults with overgrown and disorganized labial palps. In a minority of cases, discrete groups of cells adopted a T1 leg fate as seen for the distinctive sex comb tooth here (asterisk).

No viable adults eclosed, but some pharate adults were obtained. These were found to possess oversized, misorganized labial palps with up to 11 pseudotracheal rows (compared with the 5 or 6 normally). Further, these palps sometimes harbored leg cells (e.g., the sex comb tooth in Fig. 5F) typical of the pb^- condition. Thus Wg misexpression can influence labial palp size and identity (Maves and Schubiger, 2003). Taken together these results suggest that Pb counters *hh*-signaling in anterior cells, acting downstream of Hh and Ptc but upstream of or parallel to the transcription factor Ci and its potential targets *wg* and *dpp*.

If *pb* acts in anterior cells to regulate *hh*-induced expression of the *dpp*- and *wg*-coded morphogens, then adding Pb homeodomain protein to anterior leg cells where *dpp* and *wg* are activated by *hh* signaling should diminish their expression. We tested this possibility by inducing mitotic clones of Pb-expressing cells (GFP+) in leg imaginal discs, then examining the effect of Pb on *dpp* or *wg* expression in anterior compartment cells (*dpp-lacZ* transgene or anti-Wg sera, respectively). For *dpp-lacZ*, the cell-autonomous reduction in β-gal accumulation is detectable even in very small clones such as the two-cell clone in Figs. 6A, A′. Wg protein accumulation was also diminished in Pb-expressing cells (Figs. 6B, B′). This result likewise supports a role for Pb in reducing Wg (though diffusion of Wg protein may obscure its expression pattern). Thus, Pb expressed within a leg organizer region appears capable of countering Hh-mediated activation of both *dpp* and *wg* expression.

To test whether Pb is involved in this regulation of *dpp* and *wg* in labial imaginal discs, we generated clones of pb^- cells and examined their effects using *dpp-lacZ* and Wg

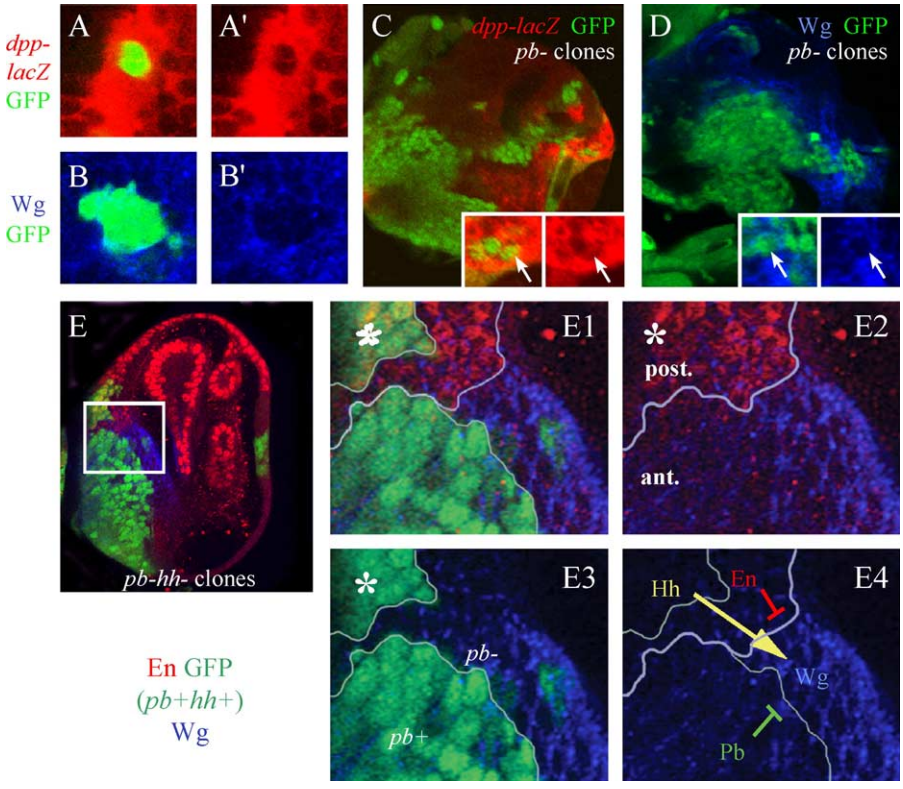


Fig. 6. Pb acts in anterior cells to limit *hh* organizer activity. (A–B) Overexpression of Pb in anterior leg cells led to a cell-autonomous decrease (A–A′) in *dpp-lacZ* reporter expression and (B–B′) in Wg accumulation. (C–D) Large pb^- clones were induced in larvae of genotype *pb*GAL4 UAS:Flp/+; FRT^{82B} *Ki* pb^5 p^p/FRT^{82B} *M(3)rps3* P[Ub-GFP]. pb^- (GFP^-) cells detected in labial discs harboring such clones showed (C) increased *dpp-lacZ* reporter expression and (D) prominent Wg accumulation. The insets in C enlarge the boxed part of the figure. A cell autonomous action of *pb* on the *dpp* promoter is indicated by β-gal expression from the *dpp-lacZ* marker (red), that is substantially lower in pb^+ cells (green, GFP+; marked with arrows) than in surrounding pb^- cells. The same conclusion is supported by Wg protein accumulation (blue) in pb^+ (green) versus pb^- cells (see arrow). (E) *pb* acts between *hh* and *wg*. In this triple labeling, posterior cells are marked by nuclear En expression (red), Wg protein appears in blue, and the pb^+ hh^+ chromosome arm expresses GFP (green). A localized Hh source was generated by pb^-hh^- clones that removed the capacity to make functional Hh from most posterior cells, leaving only a small number of pb^+ hh^+ cells (red and green) capable of expressing Hh. Cells not expressing GFP are part of the pb^- hh^- clones. Wg protein accumulation (blue) is limited essentially to the anterior (En^-) compartment, where it is observed more strongly in pb^- cells. Detailed views of the boxed inset in E are presented in E1–E4. (E1) The small number of remaining posterior pb^+ hh^+ cells (asterisk) are the sole source of Hh. Wg accumulation (blue) is detected in both anterior and posterior pb^- cells, but is markedly enhanced in anterior cells near the *hh* source (~10 cell diameters) lacking both En and Pb proteins, compared with less distant pb^- cells containing En. (E2) Wg accumulates as a function of *pb* activity. pb^+ cells are marked with GFP (green), Wg with blue. Wg accumulates in pb^- cells, but is barely detectable in pb^+ cells in agreement with negative regulation by Pb. (E3) Wg accumulation (blue) is observed in all pb^- (GFP^-) cells near the Hh source (asterisk), but is enhanced anteriorly (no En) compared to posterior, En-expressing cells. (E4) Summary of regulatory elements acting in labial development. Hh-mediated activation of *wg* is impeded by the En and Pb homeoproteins.

markers. In discs harboring large clones, *dpp-lacZ* expression was strongly enhanced in a zone deduced to be the A–P boundary region (Fig. 6C). This enhanced expression of β-gal marker from the *dpp* promoter appears cell-autonomous, since much lower levels of marker expression were seen in interspersed pb^+ cells (Fig. 6C insert). The same analysis with anti-Wg led to the same conclusion: Wg accumulation was strongly enhanced in a localized zone of the labial disc (Fig. 6D), but accumulated substantially less in pb^+ than in neighboring mutant cells (Fig. 6D insert). Similarly, Patched (Ptc) protein accumulation is also markedly enhanced near the A–P boundary in *pb* mutant discs (not shown). Thus *pb* appears to regulate multiple downstream targets of *hh*, including *dpp* and *wg*, in cells near the A–P boundary.

The above data suggested that Pb may intervene to reduce the response of *dpp* and *wg* to a Hh signaling gradient. Since one confounding factor is the distances to which morphogens can diffuse, we sought to limit the size of the endogenous *hh* source then re-examined the effect of Pb on *wg* with the same markers. To this end we generated and identified mosaic labial discs harboring large $pb^- hh^-$ clones. Some of these encompass most but not all of the posterior compartment. This leads to a localized, identifiable source of Hh: the En-expressing, *hh*-endowed ($pb^+ hh^+$) posterior cells. For one $pb^- hh^-$ clone situated near the antero-posterior frontier, robust accumulation of Wg protein is seen only in mutant anterior cells ($Pb^- En^- Hh^-$) located near Hh-secreting $pb^+ hh^+$ cells (Figs. 6E; 6E1–E3, asterisk). Wg accumulation is modulated as a function of genotype and position, since pb^+ anterior cells show little accumulation of Wg compared to mutant anterior cells (Figs. 6E1, E3, E4). Accumulation is similarly low in posterior (En^+) pb^- cells (6E1,E2,E4). In contrast, it is markedly increased in anterior (En^-) pb^- cells further distant from the Hh source (Figs. 6E1, E3, E4). These observations, summarized in Fig. 6E4, firmly support that *pb* acts to limit the response of *wg* to incoming Hh signal.

Finding the right balance: Pb and Ci in labial development

The preceding results show a role for pb^+ in regulating output of a *hh*-dependent labial organizer, and suggest that *pb* normally acts to confer labial rather than leg disc form *via* regulation of endogenous *hh* organizer activity. This regulation occurs in anterior cells at the A–P boundary, i.e., the same cells where the Ci transcription factor activates *hh* targets including *dpp*, *wg* and *ptc* (Methot and Basler, 2001; Muller and Basler, 2000). Overexpressing Ci in pb^+ labial discs induced variable defects in adult labial palps as a function of the growth temperature, and thus perhaps of the relative levels of Ci and Pb. Further, the preceding clonal analysis ascribes an activity to *pb* at the A–P boundary in opposition to Hh signal from the posterior compartment, while overexpressing Ci in pb^+ labial discs induced adult defects near the A–P boundary.

A direct hypothesis to account for these observations is to invoke simultaneous but antagonistic roles for the homeodomain and zinc finger transcription factors encoded by *pb* and *ci* in regulating targets such as *dpp* and *wg,* with Ci activating and Pb repressing the same targets. If correct, altering the relative doses of the two proteins should modulate the effective output. Ci expression directed by *pb*GAL4 at 22° leads to limited loss of pseudotracheal rows near the A–P boundary of the adult labium (Fig. 7A). This

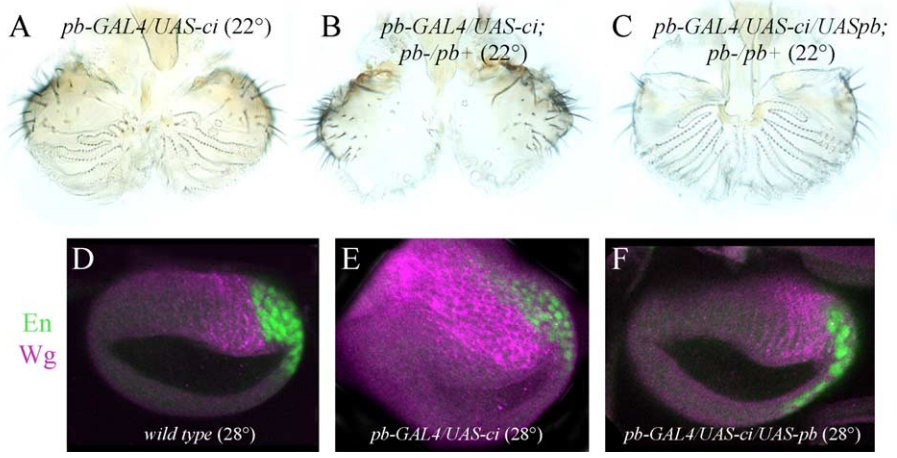


Fig. 7. Dose-sensitive antagonism between Ci and Pb in form and *wg* transcription. (A–C) Adult labial palp form is modified by the relative levels of Ci and Pb proteins. (A) Misexpression of Ci in normal labial discs (*pb*GAL4 > UAS:*ci*) at 22° induced shortening of the pseudotracheae near the A–P boundary. Note that the effect is less severe than at 28° (Fig. 5D). (B) Expressing Ci at 22° in larvae with reduced endogenous Pb (pb^-/pb^+ heterozygotes; normally fully recessive) markedly enhanced the effect of Ci misexpression. (C) Adding additional Pb through a UAS:pb construct in the same pb^-/pb^+ background ameliorated the phenotype to yield a nearly wild-type outcome. (D–F) Modulating the Ci/Pb ratio changes the expression of *wg*. (D) Normal Wg protein accumulation (violet) is seen in the anterior compartment near the posterior Hh source (En-expressing cells, green). Larvae raised at 28°. (E) Misexpressing Ci under *pb*GAL4 control at 28° led to a markedly enhanced expression of Wg in the anterior compartment. We estimate this overexpression at roughly 5–10× based on the settings used for confocal microscopy. Note that the imaginal disc is overgrown and somewhat misshapen. (F) Misexpressing both Ci and Pb at 28° renders Wg expression nearly normal. These data indicate that the ratio of Ci/Pb is central to regulating Wg expression.

effect on labial palp development was markedly enhanced on expressing Ci in pb^{-}/pb^{+} heterozygotes (increased Ci/Pb ratio; Fig. 7B). Reducing the dose of Pb thus potentiates the dominant effect of transgenically supplied Ci. To reduce the preceding Ci/Pb ratio, *pb*GAL4 driver was used to jointly direct UAS:*ci* and UAS:*pb* transgenes in a *pb* heterozygous context. Here, the transgenic supplement of Pb largely counters the effect of Ci and the morphological defects due to Ci were annulled (Fig. 7C). These observations thus support the importance of relative Pb and Ci activities in directing tissue organization.

If the defects associated with different Ci/Pb ratios reflect *hh* organizer output, they should result in altered expression of strategic Hh target genes. We therefore examined Wg protein accumulation for differing Ci/Pb ratios. Over-expressing Ci in pb^{+} labial cells is indeed associated with enhanced Wg accumulation (roughly 5–10× increased cf. wild type, based on the confocal microscopic conditions used; compare Figs. 7D, E). This effect is reversed on co-expressing UAS-supplied Ci plus Pb, and Wg accumulation is much closer to wild-type levels (Fig. 7F). These data thus support an essential and antagonistic role whereby Pb opposes Ci in the *hedgehog* segment organizer, and thereby modulates *wg* expression and its resulting morphogen gradient.

Discussion

Sculpting an appendage involves multi-tiered selector action

Segmental organization in the imaginal discs involves the reiterated deployment of segment polarity genes that organize the fundamental segmental form. This involves a cascade proceeding from posteriorly expressed Engrailed protein through a short-range Hh morphogen gradient in anterior cells favoring the activator form of Ci transcription factor, which in turn activates *wg* and *dpp* to establish two concurrent, instructive concentration gradients that structure gene expression along the proximo-distal axis.

In contrast with this elaborate choreography of the segment polarity genes, the homeodomain proteins encoded by Hox genes are expressed in a segmental register, which obscures how they can direct the differentiation of distinct cell types within the segment. The present investigation of homeotic *proboscipedia* function during labial palp formation indicates a multipronged action for *pb* in the labial disc. Pb acts cell-autonomously in the negative regulation of target genes including *dac*, which is normally extinguished in Pb-expressing cells of labial or leg imaginal discs but is activated in labial discs in the absence of *pb* activity. This activation of *dac* in mutant labial cells is *hh*-dependent and is likely a response to *wg* and *dpp* morphogen signals as for leg discs (Abu-Shaar and Mann, 1998). Our data further indicate that *pb* acts cell autonomously to regulate the level of both *wg* and *dpp* expression in response to *hh*. Thus, *pb* appears to negatively regulate *dac* expression directly, but also by withholding positive instructions from Wg and Dpp morphogens. The interweaving of homeotic selector proteins with strategic target genes including morphogens (*wg*, *dpp*) and targets of signaling activity (*dac*, *Dll*) may influence segment patterning from global size and shape to specific local pattern and cell identity. This positioning offers a powerful yet economical mode of selector function that helps to better understand how a single selector gene can integrate global patterning with cellular identity (see Fig. 8).

This view invoking multiple and overlapping modes of regulation by a homeotic selector protein supports and extends the vision from analyses by Weatherbee et al. (1998) seeking to explain how *Ultrabithorax* (*Ubx*) selector function differentiates between the serially homologous wing and haltere appendages. This analysis supports a role for *Ubx* in fruit flies transforming a dorsal default state (wing) to haltere, by repressing the accumulation of Wg in the posterior part of the haltere, and by regulating a subset of Dpp or Wg activated targets such as *vestigial* and *spalt related.* Additionally, Shashidhara et al. (1999) presented clear evidence for a nonautonomous action of Ubx via its activity in cells of the D–V organizer where *wg* is expressed. Ubx thus acts to down-regulate *wg* in the haltere, but also intervenes to modulate the expression of targets of both *dpp* and *wingless* signaling pathways (Prasad et al., 2003; Shashidhara et al., 1999; Weatherbee et al., 1998). A recent analysis of mutants for *maxillopedia* (*mxp*), the Tribolium *pb* homolog, revealed augmented transcription of flour beetle *wg* within the transformed labial segment (DeCamillis and Ffrench-Constant, 2003). This observation, in full accord with the above mentioned results for *Ubx*, and for

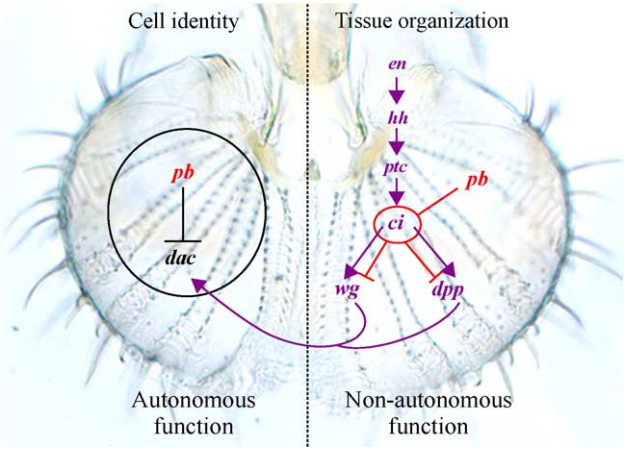


Fig. 8. Homeotic *proboscipedia* functions at multiple levels in labial palp specification. *pb* controls cell identity via the regulated expression of target genes involved in this process, including *dac*. A second role, with nonautonomous consequences for overall tissue structure, involves the ongoing control of *hh*-dependent signaling pathways required for tissue organization. Pb acts as a "homeotic organizer" at the level of Ci, where its functional antagonism serves to limit the levels of *wg* and *dpp* expression.

our results for *Drosophila pb*, supports a conserved role for homeotic regulation of nonautonomous signaling input in appendage development. At the same time, *mxp* mutants show a precocious maxilla-to-leg transformation in larvae, demonstrating a prior, embryonic requirement for *mxp*. This result is of particular interest since it highlights a temporal aspect of *pb* action in the fly labial disc: the absence of *pb* function early has no apparent effect on the labial discs in early L3 larvae, which appear normal. It is only subsequently that these diverge toward leg structure. Thus, the globally conserved activity of *mxp*/*pb* in equivalent beetle or fly organs is nonetheless employed in temporally different ways among species. Though it is not clear whether this reflects the existence of species-specific cofactors or rather of the effects of expression dosage and timing, such modifications might offer important possibilities for changing form. Variations on all these themes can probably contribute to the diversification of organism form, within and among species.

Developmental control and the modulation of signaling pathway levels

The roles of diffusible Wg and Dpp morphogens induced by Hh at the A–P boundary, and the transcriptional programs they induce according to their concentrations within a gradient, are considered central to organizing the group of cells constituting a segment. The present work indicates that *pb* normally acts downstream of Hh within the organizer, where it maintains Wg and Dpp at low levels in labial imaginal tissue. Overexpressing Wg or Dpp in the labial discs results in malformed, overgrown or transformed "labial" tissue (Fig. 5). These observations support the viewpoint that limiting morphogen accumulation is essential to ensuring that the labial program is correctly applied. Our study underlines the potential importance of the absolute levels of *wg* and *dpp*-encoded signaling molecules deployed for tissue organization. While a gradient may in principle be formed from any source, part of the spectrum of threshold levels necessary for stimulating specific gene responses is likely removed from the repertoire in the labial environment. The absolute level of activation or inhibition of diverse signaling pathways thus may be in itself a tissue-specific property, allowing gradients of related form but with different instructive capacities that can be a distinctive element in guiding tissue formation and specifying ultimate identity. This integration of diverse sorts of information—the *hh* organizer linked to the Hox selector—may confer order to tissue organization and identity.

The fine-tuning of morphogen signals by Hox selectors coupled with the concomitant regulation of downstream targets thus appears to offer a strategic control point for achieving reliable developmental control coupled with evolutionary flexibility. The modulation of different cell signaling pathways by *pb* activity implies it can regulate both the tissue "context" generated by the signaling pathways activated in a tissue, and the cellular response to this context. This capacity to meld large-scale patterning with cellular identities merits emphasis.

An evolutionarily conserved Hox organizer?

While the logic described above appears to be conserved, its application leads to widely different results according to the species and the tissue. Quite recently, an analysis of vertebrate Hox function has led to the identification of an intimate developmental link between Hox selector function and *hedgehog* signaling. This analysis revealed a direct physical interaction between the mouse Ci homolog Gli and Hox homeodomain transcription factors (Chen et al., 2004). It thus provides a compelling complement to the present work, since the molecular framework of a direct link between Gli and Hox proteins goes far to rationalise the dose-sensitive interplay between Ci and Pb that we observed in *Drosophila*. If Hox proteins indeed compete for available nuclear Gli/Ci, as suggested by the results of Chen et al. as well as our own, this molecular mechanism may also help to understand other phenomena including phenotypic suppression in flies (Morata, 1993) or posterior prevalence in mice (Lufkin et al., 1992). Correspondingly, our own data place Pb in antagonism to Ci within the *hedgehog* organizer, where it modulates output from the *wg* and *dpp* genes and the instructive morphogens they encode. These complementary observations from insect and vertebrate models suggest the existence of an evolutionarily conserved machinery with enormous potential for generating morphological diversity. It will be exciting to know more about how the homeotic selector function is integrated in known cascades that make use of conserved molecules both to ensure the fidelity of normal form, as well as to generate new form.

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