Research Article

The Noncell Autonomous Requirement of Proboscipedia for Growth and Differentiation of the Distal Maxillary Palp during Metamorphosis of Drosophila melanogaster

Anthony Percival-Smith, Gabriel Ponce, and Jacob J. H. Pelling

Department of Biology, University of Western Ontario, London, ON, Canada N6A 5B7

Correspondence should be addressed to Anthony Percival-Smith; aperciva@uwo.ca

Received 11 November 2016; Accepted 18 January 2017; Published 5 March 2017

Academic Editor: Norman A. Doggett

Copyright © 2017 Anthony Percival-Smith et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The Drosophila maxillary palp that develops during metamorphosis is composed of two elements: the proximal maxillary socket and distal maxillary palp. The HOX protein, Proboscipedia (PB), was required for development of the proximal maxillary socket and distal maxillary palp. For growth and differentiation of the distal maxillary palp, PB was required in the cells of, or close to, the maxillary socket, as well as the cells of the distal maxillary palp. Therefore, PB is required in cells outside the distal maxillary palp for the expression, by some mechanism, of a growth factor or factors that promote the growth of the distal maxillary palp. Both wingless (wg) and hedgehog (hh) genes were expressed in cells outside the distal maxillary palp in the lancinia and maxillary socket, respectively. Both wg and hh were required for distal maxillary palp growth, and hh was required noncell autonomously for distal maxillary palp growth. However, expression of wg-GAL4 and hh-GAL4 during maxillary palp differentiation did not require PB, ruling out a direct role for PB in the regulation of transcription of these growth factors.

1. Introduction

The life cycle of Drosophila has two distinct free-living forms: the larva and adult. During embryogenesis a larva is formed, and during the larval stages and metamorphosis the imaginal cells proliferate and differentiate to form an adult. The head of the larva and adult fly are highly derived relative to the archetypical insect head [1]. The important function of the mouthparts in adapting to distinct ecological niches [2] explains the large diversity of morphology of mouthparts in insects. The morphogenesis of the adult Drosophila mouthparts, the maxillary palp and proboscis, requires four Hox genes: labial (lab), Deformed (Dfd), pb, and Sex combs reduced (Scr) [3–7]. The diversity of the structure and function of insect mouthparts observed during evolution of the lineages leading to Drosophila, Tribolium, and Oncopeltus is reflected in distinct requirements of HOX proteins for mouthpart development. The requirements of LAB, PB, DFD, and SCR in maxillary palpus development and the maxillary palpus phenotype due to the loss of these HOX proteins are distinct in Drosophila, Tribolium, and Oncopeltus [5, 6, 8, 9].

Even within the Drosophila life cycle, the requirements of HOX proteins for mouthpart development are distinct [10]. During embryogenesis PB is expressed in, but not required for, mouthpart development; SCR patterns the labial segment and DFD patterns the maxillary segment [11]. In adults, PB is required for patterning the maxillary palpus and PB with SCR is required for patterning the proboscis [12, 13].

The Drosophila maxillary palp is a highly derived sensory appendage. The establishment of the adult maxillary palpus developmental field requires temporal regulation of wingless (WG) expression during the larval stages [14]. Although DFD expression during second and third stadium larvae defines a maxillary field, it is the delayed expression of WG that specifies maxillary palpus versus antennal identity. Precocious expression of WG in the maxillary primordia results in a maxillary palpus to antenna homeotic transformation. The maxillary palpus has a proximal-distal axis. Proximal-distal axis formation of the legs is well described in Drosophila [15, 16]. In the first step, the anterior and posterior compartments are established by the expression of Engrailed (EN) and Hedgehog (HH) in the posterior compartment.
HH activates the expression of Decapentaplegic (DPP) in a sector of dorsal cells and the expression of wingless (WG) in a sector of ventral cells. The expression of the DPP and WG morphogens patterns the proximal-distal axis by regulating the expression of genes such as Distalless (Dll) and homothorax (hth) [16].

Determination and differentiation is easy to observe during embryogenesis and larval imaginal disc development but not during metamorphosis, because the pupae are opaque, the larval tissue is undergoing histolysis and the developing imaginal tissue is fragile. Although easy to identify body parts that have undergone overt differentiation in fixed pupal material, undifferentiated cells are hard to assign an origin and future. Finally dynamic temporal changes in gene expression are hard to identify by comparing one static, fixed and dissected pupal stage against another. The development of live imaging of metamorphosis allows access to the events of metamorphosis [17, 18]. In this paper, we show that PB is required noncell autonomously for growth of the distal maxillary palp but not by regulation of the transcription of two growth factor genes wingless (wg) and hedgehog (hh).

2. Materials and Methods

2.1. Drosophila Stocks and Crosses. The fly strains were maintained on standard medium. All genotypes were generated by standard Drosophila crosses. The stocks used in this study are listed in Table 1.

| Name   | Genotype                                                                 | Origin |
|--------|---------------------------------------------------------------------------|--------|
| APS303 | y w; P(hspFLP); pb03/TM6B, P[wallY]                                       | [12]   |
| APS304 | y w, P[w+ , pb03>y >Tubabl>D; pb03/TM6B, P[wallY]                       | [12]   |
| APS202 | y w; P[r+y , nec0, FRT] 82B pb27/TM6B, P[wallY]                          | [12]   |
| APS205 | y w; P[r+y , nec0, FRT]; 82B Sh6Mb M(3)95A2 P[r+y , 96E/TM6B, P[wallY]   | This work |
| APS201 | y w; P[r+y , nec0, FRT]; 82B pb27 Sc2 P[w+, 90E/TM6B, P[wallY]           | [12]   |
| APS21  | y w; P[r+y , nec0, FRT]; 82B pb27 Sh6Mb M(3)95A2 P[y+, 96E/TM6B, P[wallY] | [12]   |
| DJ103  | y w; P[r+y , nec0, FRT]; 82B M(3)95A2 P[y+, 96E/TM6B, P[wallY]           | [19]   |
| GS902  | y w, P(hspFLP)122, P[UASermC292A T451A, w+]                               | G. Struhl |
| APS402 | y w; P[UASermC292A T451A, w+], P[r+y , nec0, FRT] 82B pb27 hh-GAL4/TM6B, P[wallY] | This work |
| APS403 | y w; P[UASermC292A T451A, w+], P[r+y , nec0, FRT] 82B pb27 hh-GAL4/TM6B, P[wallY] | This work |
| APS404 | y w; P[UASEGFP, w+], pb03/TM6B, P[wallY]                                 | This work |
| APS405 | y w; P[r+y , nec0 , FRT] 82B pb27 Sc2 e hh+/TM6B, P[wallY]               | This work |
| CB10   | w1118; P[pb-GAL4, w+], P[UASlacZ, w+/CyO]                                | [20]   |
| GS30   | w1118, P[r+y , neo0, FRT] 82B e hh+/TM2                                    | G. Struhl |
| GFP    | w1118, P[UASEGFP, w+].                                                   | Bloomington stock center |
| VDRC60010 | w1118, P[UASdicer2, w+]; Pin/CyO                                        | [21]   |
| GPI    | y w; P[UASYFP, w+]; P[Usb GFP]                                           | This work |
| APS454 | y w; P[UAS YFP, w+]; P[r+y , neo0 , FRT] 82B pb27/TM6B, P[w+wallY]       | This work |
| APS455 | y w; pb03, P[Ubi GFP, w+]                                                | This work |
| KBI    | y w, P(hspFLP); wgGAL4270                                                | [22]   |
| S491   | w1118, P[dpp-GAL4, w+]                                                   | [23]   |

2.2. Immunolocalization of PB/SCR and Detection of GFP/RFP. Staged prepupae and pupae were dissected from the pupal case in Drosophila Ringer’s solution, and the pupal membrane was torn along the dorsal side of the thorax to allow penetration of the fixative. The prepupae and pupae were fixed for 20 min in PBS and 4% formaldehyde. Pupae were dissected further to remove more of the pupal membrane and histolysed larval tissue, and refixed for 20 min. For immunolocalization, rabbit anti-PB E9 polyclonal antibody and mouse anti-SCR monoclonal antibody were used to detect PB and SCR expression [24, 25]. The primary antibodies were visualized with donkey FITC conjugated anti-rabbit and Texas-red conjugated anti-mouse antibodies (Jackson laboratories). In the case of detection of GFP and RFP, fixed material was counterstained with DAPI. Images were collected on a Zeiss confocal microscope in the Biotron integrated microscopy facility. Detection of β-galactosidase activity was performed with the X-gal substrate using conditions as described in [26].

2.3. Live Imaging of Metamorphosis. Similar methods were used as described in [17] with one important modification: white prepupae were suspended in a small drop of halocarbon
oil on a coverslip of a humidity chamber to improve greatly the live imaging of metamorphosis. Images of the time lapse were collected at approximately 2.53 minute intervals with a Hamamatsu digital camera mounted on a Leica DMRBE microscope and 2.5 minute intervals with a Zeiss confocal microscope. All images were exported as.tif files, and to preserve relative intensities, all images of the time lapse were simultaneously adjusted for brightness, contrast and size with Adobe Photoshop. For the experiments with hh-GAL4 and wg-GAL4 in wild type and pb27/pb20 pupae, the pupae were mounted side by side during image capture, and after adjustment for intensity, the image of each pupa of the set was separated and used to make a movie in Adobe After Effects. These movies were synchronized in Adobe After Effects such that head eversion of the pupa occurred in the same frame. A movie of the appropriate time stamp was imported into Adobe After Effects and also synchronized to head eversion. The final movie was rendered and exported as a QuickTime (MPEG4) file.

2.4. Phenotypic Analysis of Drosophila Heads. Heads were dissected from eclosed or pharate adults. For bright field microscopy the heads were incubated with 80% acetic acid 20% glycerol overnight at 60°C. The heads were mounted on slides in 1:1 Hoyer’s mountant: lactic acid [27]. For scanning electron microscopy, the heads were critical point dried and sputter gold coated. Images were collected on a Hitachi 3400-N variable pressure scanning electron microscope in the Biotron integrated microscopy facility.

2.5. Mosaic Analyses. Flip-mediated mitotic recombination was used to generate all clones of mutant tissue [28]. Larvae were heat shocked for 1 h at 36.5°C. For marking the clones on the adult cuticle, either the FRT82B Sb M y+ or FRT82B M y+exd+ chromosome were used screening for Sb+ M+ y+ cells (Table 4) [12, 19]. The pb27 clones in pupae expressing RFP driven by the hh-GAL4 driver were generated in the genotype y w; P[UASRFP] / P[hsFLP]; P[UASIGsc292A T453A], FRT82B pb27 hh-GAL4/ FRT82B [UbiGFP] and homozygous pb27 cells identified by lack of expression of GFP.

2.6. Marking Adult Cuticle for Expression of hh-GAL4. The reporter P[UASIGsc292A T453A] was used [29]. Cells that express TRC: S292A T453A had multiple stunted tricorns that were detected with a scanning electron microscope.

2.7. RNAi Reduction of Expression. RNAi lines were obtained from Vienna Drosophila RNAi Center and virgin females crossed with w1118, P[UASdicer2, w+]; P[pb-GAL4, w+], P[UASlacZ, w+]/CyO males (VDRC60010 X CB10) [20, 21]. The crosses were reared at 29°C. The heads were mounted in Hoyer’s mountant [27], and the length of the maxillary palps measured in Openlab 3.1. Five independent biological replicates were set up, and the mean lengths of male and female distal maxillary palps from the replicates were analyzed with an ANOVA for statistical significance in SSPS v. 16.0.

3. Results

3.1. The Requirement of PB for Maxillary Palpus Development. The maxillary palpus and the proboscis constitute the mouthparts of Drosophila. The maxillary palpus is composed of two pieces: the distal maxillary palp, the mobile sensory appendage, and the proximal maxillary socket into which the distal maxillary palp is inserted (Figure 1A). The formation of the adult mouthparts required the Hox gene pb (Figure 1), and PB protein was specifically expressed in the developing
maxillary palps and proboscis (Figures 2(C) and 2(D)). The HOX protein SCR, required for proboscis development but not required for maxillary palpus development, was expressed in the developing proboscis (Figure 2(D)). PB was expressed in both the cells of the distal maxillary palp and the cells surrounding the distal maxillary palp. This latter expression of PB outside the distal maxillary palp initiated a close examination of the pb null phenotype.

The cells surrounding and outside the distal maxillary palp primordium give rise to the proximal maxillary socket and lacinia. In null pb^{27}/pb^{20} mutant adults both the distal maxillary palp and the proximal maxillary socket were reduced, but the lacinia was unaffected (Figure 1(B)). The reduction of the proximal maxillary socket was also associated with the loss of the proximal palpus bristles and the 7.5 fold reduction of tricomb length from 9.64 ± 0.77 μm to 1.28 ± 0.09 μm (± SEM, n = 12) on maxillary socket cells [30] (Figures 1(A) and 1(B)). Therefore, cells surrounding the distal maxillary palp were also affected by loss of PB expression.

3.2. Live Imaging of Maxillary Palpus Development. Live imaging of metamorphosis was employed to observe mouthpart development (Figure 2; Supplemental Data Movies 1 and 2 in Supplementary Material available online at https://doi.org/10.1155/2017/2624170) [17]. The prepupal and planerocephalic stages of metamorphosis were recorded, and the major event observed in the maxillary palp primordium is the fusion of the larval maxillary socket primordia (Figure 2(I)). During the prepupal stage important for mouthpart development, a pb-GAL4 driver was used to drive expression of GFP or YFP [20]. pb-GAL4 has many sites of ectopic expression in addition to expression in the mouthparts [20]. In wandering third stadium larvae, pb-GAL4 is expressed in a small ring of cells in the aristal primordia, which was observed in the early prepupa (Figure 2(F)). In addition, pb-GAL4 is expressed in the wings, legs, larval salivary glands, brain, and peripheral nervous system (Figures 2(F)–2(M); Supplemental Data Movie 1).

Live imaging revealed that the levels and pattern of pb-GAL4 expression were dynamic during metamorphosis. During the prepupal stage, pb-GAL4 was expressed strongly in the salivary glands, brain, PNS (Kellin’s organs and an anterior sensory complex potentially the labial sensory organ), and labial imaginal discs (Figure 2(F)). The major event observed with pb-GAL4 during the prepupal stage important for mouthpart development was the fusion of the labial imaginal discs, which was associated with strong expression of pb-GAL4 (Figures 2(A), 2(G), and 2(H)). Before head eversion, the imaginal tissue of the eye antennal, clypeolabral, and labial discs had fused, and pb-GAL4 was expressed strongly in the fused labial discs, in the antenna, and in the primordia of the maxillary palpus (Figure 2(A)). However, we were unable to detect pb-GAL4 expression in the maxillary palpus during the prepupal stage in live imaging, and therefore, do not know exactly when pb-GAL4 is first expressed in the maxillary palpus.

After head eversion, differentiation of the mouthparts continues forming readily identifiable mouthparts (Figure 2(M)). Expression of pb-GAL4 in the mouthpart primordia was obscured for a few hours after head eversion by the histolysis of the salivary glands in the movie using fluorescence optics (Figure 2(J)). Using confocal microscopy, pb-GAL4 expression was detected in the maxillary palp and proboscis primordia after head eversion (Figures 2(N)–2(Q) Supplemental Movie 2). As the GFP/YFP signal expressed from the larval salivary glands degraded, two bright spots of pb-GAL4 expression appeared in the mouthpart primordia (Figure 2(K), Supplemental Movies 1 and 2). The expression of pb-GAL4 intensified in the two spots and the cells of the developing proboscis became more visible (Figure 2(I)). A bright spot of pb-GAL4 expression appeared to be pushed dorsally during the differentiation of distal maxillary palps. Distal maxillary palp growth occurred between 7:37 and 27:40 h AHE. The bright spot of GFP expression was in cells of the maxillary socket (Figure 2(B)). In the mouthparts, the pb-GAL4 driver reproduces the expression pattern of PB well (Figures 2(B), 2(C), and 2(M)). Importantly both pb-GAL4 and PB are expressed in cells surrounding the distal maxillary palp as well as the cells of the developing distal maxillary palp.

Antennal differentiation was illuminated by ectopic expression of pb-GAL4. Just before head eversion the expression of pb-GAL4 in the antenna went from a small circle in the arista primordia to throughout the antenna becoming more intense (Figure 2(H), Supplemental Movie 1). After head eversion the expression of pb-GAL4 was very strong (Figure 2(I)). Between 3:45 and 17:55 h AHE, the antenna continued differentiation and migrated toward the centerline with the ongoing differentiation of the head.

3.3. Noncell Autonomous Requirement of PB for Growth of the Distal Maxillary Palp. Using FLP-mediated mitotic recombination to generate genetically mosaic flies with clones of pb^{27} mutant cells showed that PB is required noncell autonomously in cells of, or close to, the proximal maxillary socket for distal maxillary palp growth, as well as being required in the cells of the distal maxillary palp for growth [12, 28]. All pb^{27} clones (Sb^{+} M^{+}) in the distal maxillary palps were reduced (Figure 3(A)) (Table 2), suggesting that PB is required in the distal maxillary palp for growth. Interestingly enough, one-quarter of the Sb M (pb^{+}) distal maxillary palps were also reduced, suggesting that PB is also required noncell autonomously in cells outside the distal maxillary palp for growth (Figure 3(B)) (Table 2). To determine which cells outside the distal maxillary palp PB was required in, a second mosaic analysis using FRT pb^{27} Scr^{2} and FRT M y^{exd} chromosomes was performed scoring the y^{+} phenotype of the tricombs and maxillary socket bristles [19]. In vestigial maxillary palps the tricombs of the socket cells were reduced 7.5 fold in length and were too small to assess the y^{+} phenotype (Figures 1(A) and 1(B)). Of the 189 maxillary palps examined in genetically mosaic flies, 22 had y^{+} wild type distal maxillary palps (Figure 3(C)). In all 22 of these examples, the maxillary socket cells were(pb^{+}), as both the tricombs and maxillary socket bristles had the y^{+} phenotype, suggesting that PB expressed in the ectoderm cells of, or very
Figure 2: The expression of PB and pb-GAL4 during metamorphosis. Panel (A) is the expression of pb-GAL4 recorded using the UASlacZ reporter gene fixed just before head eversion. The arrows indicate the fused labial discs, asterisks indicate the maxillary palp primordia, and the arrowheads show the antennal primordia. Panel (B) is the expression of pb-GAL4 in developing mouthparts (approximately 18 h AHE) using UASEGFP as the reporter gene (green). The arrowheads indicate the distal maxillary pals. The tissue is stained with DAPI (blue). Panels (C) and (D) are the expression of PB (C) and the expression of PB (green) and SCR (red) (D) at approximately 36 h AHE. The arrowheads indicate the distal maxillary palp. Panel (E) is the time line of metamorphosis indicating the stages and major events observed. The start and stop point for labial fusion, antenna migration, and maxillary palp morphogenesis are estimates based on first evidence of movement. The letters indicate the relative time of the images shown in panels (F)–(M). Panels (F)–(M) are individual frames from live imaging shown in Supplemental Data Movie 1. The time the image was recorded is indicated (h: min: sec BHE or AHE) and the arrows indicate the developing labial segment and the arrowheads indicate the aristal primordia and antennal primordia expression. Panels (N)–(Q) are the first 7.5 minutes AHE of YFP expression driven by pb-GAL4 shown in Supplemental Data Movie 2. The arrowheads indicate the proboscis primordia and the arrows the maxillary palpus primordia. In panels (A)–(D) and (I)–(Q) the dorsal side of the head is at the top and the ventral is at the bottom.
Figure 3: The maxillary palpus phenotypes of three independent mosaic analyses. Panels (A) and (B) are scanning electron micrographs of the effects of clonal loss of PB function generated in flies with the genotype y w; P(hspFLP)/+; P(ry', neo', FRT)82B pb57+/P(ry', neo', FRT)82B Sb63b M(3)95A2 P(y', ry')96E. In panel (A), a Sb+ pb27 clone in the distal maxillary palp is shown and the maxillary palp is reduced. In panel (B), Sb pb+ distal maxillary palps are shown; the right is wild type, and the left is reduced indicated by the arrow. In panels (A) and (B), the arrows indicate Sb bristles and the arrowheads Sb+ bristles. Panels (C) and (D) are bright field micrographs of clonal loss of PB function generated in the genotype y w; P(hspFLP)/+; P(ry', neo', FRT)82B pb57 Scrm P(w', ry')90E/P(ry', neo', FRT)82B M(3)95A2 P(y', ry')96E P(exd+, w'). Panel (C) is one of the 22 pb57/+ wild type distal maxillary palps with the y+ proximal palpus bristle indicated by the arrow and y+ maxillary socket tricoms indicated with the arrowhead. Panel (D) shows a y+ pb57 Scrm maxillary palp. Panels (E)–(G) are bright field micrographs of distal maxillary palps from the clonal ectopic expression of PB in a pb57/pb57 mutant background generated in the genotype y w, P(w', pb57>y' tubal) B; P(hspFLP)/+; pb57/pb57. Panel (E) is a rescued y− and PB expressing (pb57>y' tubal) maxillary palp, panel (F) is a reduced y− and PB expressing (pb57>y' tubal) maxillary palp, and panel (G) is a reduced y+ pb− (pb57>y' tubal) maxillary palp.
Table 2: Distribution of phenotypes in the pb loss-of-function mosaic analysis.

|                      | Sb M y⁺(pb⁺) | Reduced maxillary palps | Sb⁺ M⁺ y⁺ (pb⁺) | Reduced maxillary palps |
|----------------------|--------------|--------------------------|------------------|--------------------------|
| Wild type maxillary palps | 40⁺          | 13                        | 0                | 96                        |

The numbers only include distal maxillary palps that were completely Sb⁺ or Sb; distal maxillary palps that were a mix of genotypes were not included.

Table 3: Distribution of phenotypes in PB ectopic expression rescue mosaic analysis.

|                      | y⁺ (pb⁺) | Reduced maxillary palps | y⁺ (pb⁻) | Reduced maxillary palps |
|----------------------|----------|--------------------------|----------|--------------------------|
| Rescued maxillary palps | 63⁺      | 10                        | 0        | 31                        |

The numbers only include distal maxillary palps that were completely y⁺ or y⁻; distal maxillary palps that were a mix of genotypes were not included.

close to, the maxillary socket is required for growth of the distal maxillary palp.

A potential hypothesis for the noncell autonomous role of PB is that PB expressed in the proximal maxillary socket cells is required for transcription of the pb gene in the distal maxillary palp. A simple model for this hypothesis is that PB expression in the proximal maxillary socket cells is required for the expression of a secreted factor that binds and acts on the distal maxillary palp cells to induce transcription of the pb gene, and this expression of PB in the distal maxillary palp cells directs growth and differentiation. A mosaic analysis with PB expressed from a Tubulin α1 pb fusion gene was used to test this hypothesis [12]. Flip recombinase was used to excise the y⁺ gene from a Tubα1>y⁺>pb⁺ construct (>FRT site) to create a Tubα1>pb fusion gene expressing PB in a pb²⁷/pb²⁰ mutant background. In all y⁺ distal maxillary palps, which do not express PB from the Tubα1 pb fusion gene, the distal maxillary palp was reduced indicating that PB expression in the distal maxillary palp cells is required for rescue (Figure 3(G)). 87% of the y⁺ and PB expressing distal maxillary palps were rescued (Figure 3(E)). But 13% of the y⁺ and PB expressing distal maxillary palps were not rescued, suggesting that expression of PB in the distal maxillary palp is not sufficient to rescue growth. (Figure 3(F)) (Table 3). This was the same phenomena observed with the generation of pb²⁷ clones, and more importantly if PB is required noncell autonomously for pb transcription in the distal maxillary palp, then all distal maxillary palps with PB being expressed from the Tubulin α1 promoter would have been rescued.

3.4. Expression of Wingless, Decapentaplegic, and Hedgehog during Maxillary Palpus Differentiation. The three secreted proteins WG, DPP, and HH are required for establishing the proximal-distal axis of the leg, wing, and antenna. These three proteins are potential candidates for a PB-regulated factor secreted from cells within or close to the maxillary socket that promotes growth of the distal maxillary palp along the proximal-distal axis. The expression patterns of these secreted factors were assessed using GAL4 driver lines during metamorphosis (Figure 4) [22, 23, 31]. Both hh-GAL4 and wg-GAL4 were expressed strongly in cells outside the developing distal maxillary palp and less so in some of the distal maxillary palp cells (Figures 4(B) and 4(C)). However, dpp-GAL4 was strongly expressed in the distal maxillary palp cells ruling out DPP as a candidate for the PB-regulated growth factor (Figure 4(D)).

Although the site of expression of wg-GAL4 outside the distal maxillary palp is the developing lancinia, the site of hh-GAL4 expression outside the distal maxillary palp is unclear. To mark the adult cells that had strongly expressed hh-GAL4, the UAStricorneredS²⁹²A T⁴⁵³A fusion gene was used [29]. The TRCS²⁹²A T⁴⁵³A protein inhibits TRC⁺ protein activity resulting in multiple short tricombs on each cell that express TRCS²⁹²A T⁴⁵³A. This was most easily observed when expression of TRC⁵⁹²A T⁴⁵³A was driven by hh-GAL4 in the posterior compartment of the wing (Figure 4(E)). The cells of the anterior compartment had long single tricombs on each cell, but the cells of the posterior compartment expressing GAL4 had multiple short tricombs (Figure 4(E)). The tricombs of the distal maxillary palp were unaffected when TRC⁵⁹²A T⁴⁵³A was expressed using the hh-GAL4 driver. However, ventral cells of the maxillary socket had multiple short tricombs indicating high levels of TRC⁵⁹²A T⁴⁵³A expression had occurred in these cells (Figures 4(F) and 4(G)).

3.5. Requirement of wg and hh for the Growth of the Distal Maxillary Palp. Both WG and HH are required for many processes at many stages of development. Particularly relevant to this study is the importance of WG expression for the establishment of the maxillary palp field during larval development [14]. Therefore, to target reduction of expression of WG and HH to the developing maxillary palpus, the pb-GAL4 driver and UASRNAi lines were used. The use of the pb-GAL4 driver restricted expression of RNAi molecules to the maxillary palpus during pupal development, and in Drosophila RNAi mediated reduction of expression is cell autonomous [21]. To increase the activity of GAL4 expressed from pbGAL4, the flies were grown at 29°C [32]. Three HH RNAi lines were obtained: two of which (ID# 1402 and 1403) were predicted to have one off target (CG4637); and one line (ID# 43255) was predicted to have five off targets.
Table 4: The effect of mouthpart-specific, RNAi mediated inhibition of components of the WG and HH pathways on distal maxillary palp length.

| RNAi line | Construct | Targeted mRNA | Length of the distal maxillary palp (μm) ± SEM* |
|-----------|-----------|---------------|-----------------------------------------------|
|           |           |               | Female (UASdicer2) | Male (UASdicer2) |
| y w       | —         | —             | 160 ± 3* (5) | 139 ± 1* (5) |
| 1402      | 193       | HH            | 144 ± 3 (5) | 141 ± 2* (5) |
| 1403      | 193       | HH            | 126 ± 1* (5) | 127 ± 3 (5) |
| 43255     | 6242      | HH            | 138 ± 2h (5) | 136 ± 3 (5) |
| y w       | —         | —             | 156 ± 3* (5) | 134 ± 1* (5) |
| 13351     | 5007      | WG            | 134 ± 3 (5) | 135 ± 4* (5) |
| 39676     | 5007      | WG            | 130 ± 6b (2) | 127 ± 2* (3) |
| 7767      | 1372      | ARM           | 138 ± 1b (5) | 141 ± 1* (4) |
| 107344    | 102545    | ARM           | — (0)        | 108 ± 8b (4) |
| 25940     | 10429     | PAN           | 143 ± 3 (5) | 138 ± 2* (5) |
| 9542      | 577       | SMO           | 136 ± 2h (4) | 134 ± 2* (5) |

* Data in the same column with the same letter are not significantly different (P > 0.05).

(CGI7450, CG32819, CG32820, CG86655, CG9934) [21, 33, 34]. The predicted off targets were not shared between the two constructs. As a result of the crossing scheme, females expressed Dicer from UASdicer2, but males did not. In Dicer expressing females, all HH RNAi lines exhibited a significant reduction in the length of the distal maxillary palp (Table 4). The only significant reduction observed in males, which do not express Dicer, was with RNAi line 1403, which showed the strongest effect in females. Two WG RNAi line were obtained (1D# 13351 and 39676). Both carried the same construct with no predicted off targets and showed a significant reduction in the length of the distal maxillary palp. Using RNAi lines to target the reduction of expression of components of the WG and HH signal transduction pathways, Armirdillo (ARM) and Pangolin (PAN) of the WG pathway and Smoothened (SMO) of the HH pathway were shown to be required for distal maxillary palp growth (Table 4).

3.6. The Requirement of HH in the Growth of the Distal Maxillary Palp. The hh gene encodes a secreted ligand, and therefore, hh mutant alleles behave noncell autonomously in a mosaic analysis [35]. To determine whether HH is required noncell autonomously for maxillary palpus development as expected from hh-GAL4 expression (Figure 4), we induced hh* mutant clones using FLP-mediated mitotic recombination. The right palp in Figure 5(A) had hh* mutant clone of cells in the distal maxillary palp marked by the Sb+ bristles and exhibited a wild type phenotype indicating that hh was not required in the cells of the distal maxillary palp for growth. All other distal maxillary palps shown in Figures 5(A) and 5(B) were shortened or absent confirming the RNAi results that HH was required for distal maxillary palp growth. These two observations also show that HH was required noncell autonomously for distal maxillary palp growth.

Although both pb and hh were required for the growth of the distal maxillary palp, the pb and hh phenotypes were distinct: loss of PB expression resulted in a vestigial maxillary palpus; whereas, loss of HH expression resulted sometimes in a complete deletion of the maxillary palpus (Figures 5(A) and 5(B)). Using FRT82 pb27/Dr Sl2 hh* and FRT82 pb27/Dr Sl2 Sb M y* chromosomes, hh* clones were induced in a pb mutant background (Figure 5(C)). As observed with hh* clones in a wild type background, hh* clones in a pb mutant background also resulted in loss of the vestigial palpus indicating that growth of the vestigial palpus is HH-dependent (Figure 5(C)).

3.7. Expressions of wg-GAL4 and hh-GAL4 Were Not PB-Dependent. The expression of wg-GAL4 and hh-GAL4 were assessed in parallel live imaging experiments where both wild type and pb27/pb20 prepupae were mounted side by side and allowed to undergo metamorphosis. The expression of GAL4 was detected with a UAS-GFP reporter gene and all cells of the pupae were marked with GFP expressed from UbiGFP. In parallel live imaging of wg-GAL4 expression in wild type and pb27/pb20 prepupae and pupae, wg-GAL4 was strongly expressed in lacinia of both the wild type and pb mutant (Figure 6; Supplemental Data Movie 3) indicating that wg is not regulated by PB. In parallel live imaging of hh-GAL4 expression in wild type and pb27/pb20 prepupae, hh-GAL4 was strongly expressed in the salivary glands of wild type but not pb27/pb20 prepupae indicating that hh expression in the salivary gland is PB-dependent (Supplemental Data Movie 4). However, in wild type pupae hh-GAL4 was expressed strongly in the cells of the maxillary socket and hh-GAL4 expression was only expressed a little less in the maxillary palp socket cells of the vestigial maxillary palpus of pb mutants (Figure 6 Supplemental Data Movie 4). Although in other repeat experiments, a greater difference between expression of hh-GAL4 in wild type and pb mutants was observed, hh-GAL4 is still expressed in pb mutants. The variation observed between experiments could be due to the pb mutant mouthpart cells not being very healthy resulting...
in a nonreproducible level of hh-GAL4 expression. Or as clearly observed in the movies, once the maxillary palps of wild type and pb mutants start to differentiate they are very different from one another early in differentiation and the lower level of hh-GAL4 expression may reflect divergence of the structure of the wild type and mutant palps. To investigate further whether PB was required for hh-GAL4 expression, a FLP-mediated mosaic analysis was performed. The hh-GAL4 allele is an insertion of a GAL4 enhancer detector into the hh locus, and both the pb and hh loci are on the right arm of chromosome 3. Therefore, the FRT82 pb27 hh-GAL4 chromosome created to perform the mosaic analysis resulted
in 3 distinct cellular genotypes that were most easily observed in the posterior compartment of the wing (Figures 7(A)–7(D)): parental RFP and GFP expressing cells, loss of GFP expression (pb\(^{+/−}\)hh-GALA) but not RFP expression, and gain of GFP expression but loss of RFP expression due to the loss of hh-GALA (FRT UbiGFP). In pb\(^{+/−}\) clones in the maxillary socket cells, RFP, and therefore hh-GALA, was still expressed at a high level (Figures 7(E)–7(H)). PB is not required cell autonomously for hh-GALA expression.

4. Discussion

4.1. Noncell Autonomous Requirement of PB in Maxillary Palpus Development. PB is required for the development of both the adult proboscis and the maxillary palp [3]. At the wandering third instar larval stage, PB is expressed in the labial imaginal disc but not in the eye antenna imaginal disc that harbors the primordia for the maxillary palp [20]. During the prepupal stage pb-GAL4 expression intensifies in the differentiating labial discs, and is expressed in the maxillary palp primordia. During the first thirty hours of pupal development the mouthparts undergo major events of morphogenesis forming a structure that is easily recognized as adult mouthparts. During this stage of pupal development pb-GAL4 expression is dynamic and intense. The expression of PB and pb-GAL4 are not restricted to the proboscis and distal maxillary palp, but are also expressed in the cells of the maxillary socket and surrounding tissue. Expression of PB in the cells of the maxillary socket, or cells close to it, is required for the growth of the distal maxillary palp. This noncell autonomous requirement of PB in cells outside the distal maxillary palp is not due to the transcriptional regulation of genes that encode the growth factors HH and WG, even though WG and HH are required for growth of the distal maxillary palp.

Although the HOX protein PB is a transcription factor, and is expected to have a cell autonomous role in regulation of PB-regulated genes, these regulated genes can function on pathways involved in cell-cell communication. This phenomenon is well described in a number of HOX systems in Drosophila. In morphogenesis of the embryonic gut, Ultrabithorax (UBX) is required for the expression of the growth factor DPP [36]. Also SCR and other HOX proteins are required noncell autonomously for induction of ectopic tarsi, and Antennapedia is required noncell autonomously for leg determination [12, 37, 38]. PB is required in a complex combination of cell autonomy and noncell autonomy in the regulation of WG and HH pathways during proboscis determination [39, 40]. For regulation of the growth of the haltere UBX is required noncell autonomously and UBX regulated genes involved in mediating this noncell autonomy are identified [41, 42].

PB is also required in the distal maxillary palp cells for growth. In our mosaic analysis we were unable to assess the growth phenotype of pb\(^{+/−}\) and pb\(^{−/−}\) cells in palps of mixed pb\(^{+/−}\)pb\(^{−/−}\) genotypes, and therefore, we were unable to assess whether PB is required cell autonomously in the developing distal maxillary palp cells. If PB is required cell autonomously in the distal maxillary palp cells, then it is possible that PB also regulates the expression of the components (receptor, signal transduction, etc.) that receive the noncell autonomous PB-regulated signal coming from cells outside the distal maxillary palp.

4.2. PB and Proximal-Distal Axis Formation. The major maxillary palpus phenotype caused by loss of PB expression is the loss of growth of the distal maxillary palp along the proximal-distal axis resulting in a vestigial stump. This phenotype suggested the possibility that PB regulates the expression of
4.3. The Derived Drosophila Maxillary Palpus. The maxillary palpus of Drosophila is a highly derived structure relative to that proposed for the archetypical insect head [1, 44, 45]. This high level of derivation may be reflected in two other observations. First, analysis of mitotic clones did not detect anterior-posterior compartment formation in the maxillary palpus even though HH is expressed in a spatially restricted domain during maxillary palpus differentiation [30, 46]. Second, Dil-GAL4 expression in the distal maxillary palp and maxillary socket [17] suggests that the derived maxillary palpus may be of telopodite origin. These two observations may suggest that the compartmental boundaries are established during metamorphosis and the maxillary palpus is homologous to the distal arista and tarsus of the antenna and leg, respectively. Therefore, PB may be regulating the Epidermal Growth Factor Receptor (EGFR) pathway, which is important for determining the proximal-distal axis of the distal segments of the legs and antennae [47, 48]. This is supported by the identification of components of the EGFR signal transduction pathway as being important for the antenna to maxillary palp transformation caused by ectopic expression of PB [49]. In addition, analysis of the pathways involved in proximal-distal axis formation of Tribolium castaneum mouthparts has shown an involvement of the EGFR pathway [45]. Although an interesting possibility, when considering the ligands of
Figure 7: Clonal analysis of the requirement of PB in hh-GAL4 expression. All clones were generated in the genotype y w; P[UASRFP]/P(hspFLP); P[UAStrcS292AT453A], FRT82B pb27 hh-GAL4/FRT82B P(UbiGFP). Panels (A)–(D) are a pupal wing and panels (E)–(H) are a pupal maxillary palp. Panels (A) and (E) are GFP expression; panels (B) and (F) are GFP expression (green) and nuclei visualized with DAPI (red); panels (C) and (G) are RFP expression; and panels (D) and (H) are GFP (green) and RFP (blue) expression with the nuclei visualized with DAPI (red). The two arrowheads in panels (A)–(D) indicate clones of cells that are homozygous for UbiGFP and have lost RFP expression due to loss of hh-GAL4. In panels (E)–(H), the arrow indicates the developing distal maxillary palp, and the arrowhead indicates a pb27 mutant clone that shows strong expression of RFP indicating strong expression of hh-GAL4.

5. Conclusions

The HOX transcription factor PB is required both in the cells of the distal maxillary palp and in cells of, or close to, the adjacent maxillary socket for growth of the distal maxillary palp. Therefore, an important role of PB in the growth of the distal maxillary palp is the regulation a cell-cell communication pathway(s). The genes wg and hh are expressed in cells outside the distal maxillary palp and are required for growth of the maxillary palp. Although WG and HH are good candidates for mediating the noncell autonomous requirement of PB, transcription of the wg and hh genes is not directly regulated by PB. But the option remains that PB may be required for activation of either WG or HH protein activity, or that PB may regulate the expression of another signaling pathway altogether.

Competing Interests

The authors have no competing interests.

Authors’ Contributions

Anthony Percival-Smith designed and performed the experiments presented in Figures 1, 2, 3, 4, 5, and 7 and wrote the first drafts of the manuscript. Gabriel Ponce developed the flies for dual live imaging, assisted with collection of the live imaging data, and revised the manuscript. Jacob J. H. Pelling processed the live imaging data to make the movies and revised the manuscript.

Acknowledgments

The authors thank Gary Struhl, the Bloomington Stock Center, and the Vienna Drosophila RNAi Center for fly stocks, and Anthony Percival-Smith thanks Markus Affolter for lab space during a sabbatical leave where some of this work was performed. The authors thank Gary Struhl for suggesting using UASrc S292A T453A to mark GAL4 expressing cells, Sheila Macfie for assistance with statistical analysis,
References

[1] R. E. Snodgrass, Principles of Insect Morphogenesis, McGraw-Hill Book Co., New York, NY, USA, 1935.
[2] P. R. Grant and B. R. Grant, "Unpredictable evolution in a 30-year study of Darwin's finches," Science, vol. 296, no. 5568, pp. 707–711, 2002.
[3] T. C. Kaufman, "Cytogenetic analysis of chromosome 3 in Drosophila melanogaster: isolation and characterization of four new alleles of the proboscipedia (pb) locus," Genetics, vol. 90, no. 3, pp. 579–596, 1978.
[4] G. Struhl, "Genes controlling segmental specification in the Drosophila thorax," Proceedings of the National Academy of Sciences of the United States of America, vol. 79, no. 23, pp. 7380–7384, 1982.
[5] V. K. L. Merrill, F. R. Turner, and T. C. Kaufman, "A genetic and developmental analysis of mutations in the Deformed locus in Drosophila melanogaster," Developmental Biology, vol. 122, no. 2, pp. 379–395, 1987.
[6] V. K. L. Merrill, R. J. Diederich, F. R. Turner, and T. C. Kaufman, "A genetic and developmental analysis of mutations in labial, a gene necessary for proper head formation in Drosophila melanogaster," Developmental Biology, vol. 135, no. 2, pp. 376–391, 1989.
[7] D. L. Cribs, C. Benassayag, F. M. Randazzo, and T. C. Kaufman, "Levels of homeotic protein function can determine developmental identity: evidence from low-level expression of the Drosophila homeotic gene proboscipedia under Hsp70 control," EMBO Journal, vol. 14, no. 4, pp. 767–778, 1995.
[8] R. W. Beeman, J. J. Stuart, M. S. Haas, and R. E. Denell, "Genetic analysis of the homotic gene complex (HOM-C) in the beetle Tribolium castaneum;" Developmental Biology, vol. 133, no. 1, pp. 196–209, 1989.
[9] C. L. Hughes and T. C. Kaufman, "RNAi analysis of deformed, proboscipedia and sex combs reduced in the milkweed bug Oncopeltus fasciatus: novel roles for Hox genes in the Hemipteran head," Development, vol. 127, no. 17, pp. 3683–3694, 2000.
[10] T. C. Kaufman, M. A. Seeger, and G. Olsen, "Molecular and genetic organization of the antennapedia gene complex of Drosophila melanogaster," Advances in Genetics, vol. 27, pp. 309–362, 1990.
[11] M. A. Pultz, R. J. Diederich, D. L. Cribs, and T. C. Kaufman, "The proboscipedia locus of the Antennapedia complex: a molecular and genetic analysis," Genes & Development, vol. 2, no. 7, pp. 901–920, 1988.
[12] A. Percival-Smith, J. Weber, E. Gilfoyle, and P. Wilson, "Genetic characterization of the role of the two HOX proteins, Proboscipedia and Sex Combs Reduced, in determination of adult antennal, tarsal, maxillary palp and proboscis identities in Drosophila melanogaster," Development, vol. 124, no. 24, pp. 5049–5062, 1997.
[13] L. Sivathanarajah and A. Percival-Smith, "Analysis of the sequence and phenotype of Drosophila Sex combs reduced alleles reveals potential functions of conserved protein motifs of the Sex combs reduced protein," Genetics, vol. 182, no. 1, pp. 191–203, 2009.
[14] G. Lefebvre, C. Faucher, D. L. Cribs, and C. Benassayag, "Timing of Wingless signalling distinguishes maxillary and antennal identities in Drosophila melanogaster," Development, vol. 135, no. 13, pp. 2301–2309, 2008.
[15] W. J. Brook, F. J. Diaz-Benjumea, and S. M. Cohen, "Organizing spatial pattern in limb development," Annual Review of Cell and Developmental Biology, vol. 12, pp. 161–180, 1996.
[16] T. Lecuit and S. M. Cohen, "Proximal-distal axis formation in the drosophila leg," Nature, vol. 388, no. 6638, pp. 139–145, 1997.
[17] R. E. Ward, P. Reid, A. Bashirullah, P. P. D’Avino, and C. S. Thummel, "GFP in living animals reveals dynamic developmental responses to ecdysone during Drosophila metamorphosis," Developmental Biology, vol. 256, no. 2, pp. 389–402, 2003.
[18] C. Vinegoni, C. Pitsouli, D. Razansky, N. Perrimon, and V. Ntziachristos, "In vivo imaging of Drosophila melanogaster pupae with mesoscopic fluorescence tomography," Nature Methods, vol. 5, no. 1, pp. 45–47, 2008.
[19] A. Percival-Smith and D. J. Hayden, "Analysis in Drosophila melanogaster of the interaction between sex combs reduced and extradenticle activity in the determination of tarsus and arista identity," Genetics, vol. 150, pp. 189–198, 1998.
[20] C. Benassayag, S. Plaza, P. Callaerts et al., "Evidence for a direct functional antagonism of the selector genes proboscipedia and eyeless in Drosophila head development," Development, vol. 130, no. 3, pp. 575–586, 2003.
[21] G. Dietzl, D. Chen, F. Schnorrer et al., "A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila," Nature, vol. 448, no. 7150, pp. 151–156, 2007.
[22] O. Gerlitz, D. Nellen, M. Ottiger, and K. Basler, "A screen for genes expressed in Drosophila imaginal discs," International Journal of Developmental Biology, vol. 46, no. 1, pp. 173–176, 2002.
[23] K. Staeling-Hampton, P. D. Jackson, M. J. Clark, A. H. Brand, and F. M. Hoffmann, "Specificity of bone morphogenetic protein-related factors: cell fate and gene expression changes in Drosophila embryos induced by decapentaplegic but not 60A," Cell Growth and Differentiation, vol. 5, no. 6, pp. 585–593, 1994.
[24] D. L. Cribs, M. A. Pultz, D. Johnson, M. Mazzulla, and T. C. Kaufman, "Structural complexity and evolutionary conservation of the Drosophila homeotic gene proboscipedia," EMBO Journal, vol. 11, no. 4, pp. 1437–1449, 1992.
[25] M. A. Glicksman and D. L. Brower, "Expression of the Sex combs reduced protein in Drosophila larvae," Developmental Biology, vol. 127, no. 1, pp. 113–118, 1988.
[26] H. J. Bellen, C. J. O’Kane, C. Wilson, U. Grossniklaus, R. K. Pearson, and W. J. Gehring, "P-element-mediated enhancer detection: a versatile method to study development in Drosophila," Genes & Development, vol. 3, no. 9, pp. 1288–1300, 1989.
[27] E. Wieschaus and C. Nusslein-Volhard, "Looking at embryos," in Drosophila: A Practical Approach, D. B. Roberts, Ed., pp. 199–228, IRL Press, Oxford, UK, 1986.
[28] T. Xu and G. M. Rubin, "Analysis of genetic mosaics in developing and adult Drosophila tissues," Development, vol. 117, no. 4, pp. 1223–1237, 1993.
[29] Y. He, X. Fang, K. Emoto, Y.-N. Jan, and P. N. Adler, "The tricornered Ser/Thr protein kinase is regulated by phosphorylation and interacts with furry during Drosophila wing hair development," Molecular Biology of the Cell, vol. 16, no. 2, pp. 689–700, 2005.
[30] J. L. Haynie and P. J. Bryant, “Development of the eye-antenna imaginal disc and morphogenesis of the adult head in Drosophila melanogaster,” Journal of Experimental Zoology, vol. 237, no. 3, pp. 293–308, 1986.

[31] H. Tanimoto, S. Itoh, P. Ten Dijke, and T. Tabata, “Hedgehog creates a gradient of DPP activity in Drosophila wing imaginal discs,” Molecular Cell, vol. 5, no. 1, pp. 59–71, 2000.

[32] T. E. Haerry, O. Khalsa, M. B. O’Connor, and K. A. Wharton, “Synergistic signaling by two BMP ligands through the SAX and TKV receptors controls wing growth and patterning in Drosophila,” Development, vol. 125, no. 20, pp. 3977–3987, 1998.

[33] M. M. Kulkarni, M. Booker, S. J. Silver et al., “Evidence of off-target effects associated with long dsRNAs in Drosophila melanogaster cell-based assays,” Nature Methods, vol. 3, no. 10, pp. 833–838, 2006.

[34] Y. Ma, A. Creanga, L. Lum, and P. A. Beachy, “Prevalence of off-target effects in Drosophila RNA interference screens,” Nature, vol. 443, no. 7109, pp. 359–363, 2006.

[35] J. Mohler and K. Vani, “Molecular organization and embryonic expression of the hedgehog gene involved in cell-cell communication in segmental patterning of Drosophila,” Development, vol. 115, no. 4, pp. 957–971, 1992.

[36] K. Immerglück, P. A. Lawrence, and M. Bienz, “Induction across germ layers in Drosophila mediated by a genetic cascade,” Cell, vol. 62, no. 2, pp. 261–268, 1990.

[37] A. Percival-Smith, W. A. Teft, and J. L. Barta, “Tarsus determination in Drosophila melanogaster,” Genome, vol. 48, no. 4, pp. 712–721, 2005.

[38] M. A. Crickmore and R. S. Mann, “Hox control of organ size by regulation of morphogen production and mobility,” Science, vol. 313, no. 5783, pp. 63–68, 2006.

[39] M. A. Crickmore and R. S. Mann, “Hox control of morphogen mobility and organ development through regulation of glypican expression,” Development, vol. 134, no. 2, pp. 327–334, 2007.

[40] R. K. Mann and P. A. Beachy, “Novel lipid modifications of secreted protein signals,” Annual Review of Biochemistry, vol. 73, pp. 891–923, 2004.

[41] D. R. Angelini and T. C. Kaufman, “Insect appendages and comparative ontogenetics,” Developmental Biology, vol. 286, no. 1, pp. 57–77, 2005.

[42] D. R. Angelini, F. W. Smith, A. C. Aspiras, M. Kikuchi, and E. L. Jockusch, “Patterning of the adult mandibulate mouthparts in the red flour beetle, Tribolium castaneum,” Genetics, vol. 190, no. 2, pp. 639–654, 2012.

[43] G. Morata and P. A. Lawrence, “Development of the eye-antenna imaginal disc of Drosophila,” Developmental Biology, vol. 70, no. 2, pp. 355–371, 1979.

[44] G. Campbell, “Distalization of the Drosophila leg by graded EGF-receptor activity,” Nature, vol. 418, no. 6899, pp. 781–785, 2002.