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## Pox neuro control of cell lineages that give rise to larval poly-innervated external sensory organs in Drosophila

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### ABSTRACT

The Pox neuro (Poxn) gene of Drosophila plays a crucial role in the development of poly-innervated external sensory (p-es) organs. However, how Poxn exerts this role has remained elusive. In this study, we have analyzed the cell lineages of all larval p-es organs, namely of the kölbchen, papilla 6, and hair 3. Surprisingly, these lineages are distinct from any previously reported cell lineages of sensory organs. Unlike the well-established lineage of mono-innervated external sensory (m-es) organs and a previously proposed model of the p-es lineage, we demonstrate that all wild-type p-es lineages exhibit the following features: the secondary precursor, plla, gives rise to all three support cells-socket, shaft, and sheath, whereas the other secondary precursor, pIIb, is neuronal and gives rise to all neurons. We further show that in one of the p-es lineages, that of papilla 6, one cell undergoes apoptosis. By contrast in Poxn null mutants, all p-es lineages have a reduced number of cells and their pattern of cell divisions is changed to that of an m-es organ, with the exception of a lineage in a minority of mutant kölbchen that retains a second bipolar neuron. Indeed, the role of Poxn in p-es lineages is consistent with the specification of the developmental potential of secondary precursors and the regulation of cell division but not apoptosis.

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#### Introduction

The peripheral nervous system (PNS) of Drosophila with its sensory organs provides a simple paradigm for the study of a variety of complex processes, such as pattern formation, cell determination and differentiation, asymmetric cell division, axonal pathfinding, or the formation and evolutionary diversification of organs in different segments and species (Jan and Jan, 1993; Bellaïche and Schweisguth, 2001; Betschinger and Knoblich, 2004; Lai and Orgogozo, 2004). Like all holometabolous insects, Drosophila has a larval and an adult PNS. The former develops during embryogenesis and consists of only a few types of sensory organs that are arranged in a segmentally repeated, stereotyped pattern (Hertweck, 1931; Dambly-Chaudière and Ghysen, 1986; Ghysen et al., 1986; Bodmer and Jan, 1987; Hartenstein, 1988; Campos-Ortega and Hartenstein, 1997). They consist of few cells, which in most cases only contain a single neuron. They are either internal, such as chordotonal organs or single multidendritic (md) neurons, or external sensory (es) organs, which are further subdivided into mono- (m-es) or poly-innervated (p-es) es organs, depending on whether they are innervated by a single bipolar neuron or by two or three bipolar neurons. While most es organs are mono-innervated

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and form either a dome-like campaniform sensillum (papilla) or a trichoid-type sensillum (hair), two p-es organs are present in each thoracic and abdominal hemisegment. In the thorax, these are peglike basiconical sensilla, named kölbchen (Hertweck, 1931), and are located in a ventral and dorsal position in T1, or in a ventral and lateral position in T2 and T3. In the abdomen (A1–A7), the p-es organs consist of a small dorsal hair, h3, and a lateral papilla, p6 (Dambly-Chaudière and Ghysen, 1986) that are homologous to those of the thorax, as evident from their transformation into kölbchen in bithoraxoid embryos (Dambly-Chaudière and Ghysen, 1987).

Each sensory organ is derived from a sensory organ precursor (SOP) cell. During early neurogenesis, groups of cells in the neuroectoderm acquire the potential to become neuronal precursor cells by the expression of proneural genes, such as the genes of the achaete-scute complex. From each group, a single cell is selected to become a neuronal precursor through a process called "lateral inhibition", which is mediated by the neurogenic genes, like Notch and Delta, which silence the proneural genes in all cells of the proneural group except the SOP. The identity of an SOP cell is further determined by the action of neuronal precursor genes and neuronal precursor-type selector genes that are required to initiate the precise developmental program of a particular sensory organ (for a review, see Jan and Jan, 1993). Once an SOP cell is determined, it will follow a series of asymmetric cell divisions to give rise to a number of cells, which include the external sensory organ cells (three support cells and one or several bipolar neurons)









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and an md neuron (Brewster and Bodmer, 1995; Orgogozo et al., 2001; Lai and Orgogozo, 2004). Of these lineages the one that gives rise to the larval m-es organ and md neuron is best characterized (Orgogozo et al., 2001). Specifically, the SOP cell divides to give rise to two secondary precursor cells, plla and pllb. The plla cell is the precursor of the socket and shaft cell, while the pllb cell divides to generate an md neuron and the pllb cell, which further divides to generate the sheath cell and a bipolar neuron. For p-es organs, it has been proposed that the pllb cell goes through additional divisions to produce multiple neurons (Bodmer et al., 1989; Brewster and Bodmer, 1995).

The Pox neuro (Poxn) gene, a member of the Drosophila Pax gene family, encodes a transcription factor with a DNA-binding paired domain (Bopp et al., 1989; Noll, 1993). Poxn plays essential roles in many aspects during the development of the PNS in both larvae and adults (Dambly-Chaudière et al., 1992; Awasaki and Kimura, 1997, 2001; Boll and Noll, 2002). It is expressed in all larval p-es organs as well as in a subgroup of m-es organs in a temporally and spatially controlled fashion (Dambly-Chaudière et al., 1992; Awasaki and Kimura, 2001; Boll and Noll, 2002). In Poxn null mutants, larval p-es organs are transformed into m-es organs (Dambly-Chaudière et al., 1992; Awasaki and Kimura, 2001). These transformed mutant p-es organs form papillae or hairs that are frequently shifted in position, whereby the shafts of the hairs are lost after the first larval instar. Thus, all transformed p-es organs appear as papilla-like m-es organs during the second and third larval instars (Awasaki and Kimura, 2001; W.B., unpublished data). In addition, m-es hairs are transformed in Poxn null mutants to papilla-like organs that have no shafts, a phenotype that again appears only in second and third, but not in first, instar larvae (Awasaki and Kimura, 2001; W.B., unpublished data). In adult Poxn null mutants, chemosensory sensilla are transformed into mechanosensory-like sensilla (Awasaki and Kimura, 1997; Boll and Noll, 2002). Null mutants of *Poxn*,  $Poxn^{\Delta M22}$ , generated in our lab, are viable, but males are sterile due to the absence of several Poxn functions each of which is regulated by a separate enhancer and is necessary for male fertility (Boll and Noll, 2002; Krstic et al., 2009).

Although the importance of Poxn during the development of the p-es organs had been observed long ago, the function(s) of Poxn during this process remained elusive. In this study, we describe novel cell lineages for all larval p-es organs, i.e., the thoracic kölbchen and their abdominal homologs, the lateral p6 and dorsal h3. In all wildtype larval p-es lineages, the SOP cells divide to generate two secondary precursors, the pIIa and pIIb cells. Notably, in these lineages the pIIa cell is the precursor of all support cells, whereas the pIIb cell gives rise to all neurons. This is in striking contrast to the lineage of m-es organs where the pIIb precursor generates both epidermal - the sheath support cell - and neuronal cells. In addition, we find that one cell undergoes apoptosis in the wild-type p6 lineage. On the other hand, Poxn null mutants show altered lineages, where the p-es division pattern is transformed into that of an m-es lineage. Moreover, apoptosis is no longer observed in any transformed p-es lineage of *Poxn* mutants. Together our results suggest that Poxn plays a decisive role in the specification of the cell lineages during the development of p-es organs in Drosophila larvae.

#### Materials and methods

#### Fly stocks

The following fly strains were used:

*y w*, *y w*; *E*7-2-36 (Bier et al., 1989), *w*; *UAS-p*35 (kindly provided by Bruce Hay), *y w*;  $Poxn^{\Delta M22}/CyO$ ,  $y^+$ ,

w; 
$$Poxn^{\Delta M22}/CyO$$
;  $Poxn-CD8::GFP^{3-3}$ ,  
w;  $Poxn-Gal4^{ups1f}/TM3$ ,  
y w; UAS-CD8::GFP/CyO,  
y w; UAS-CD8::GFP;  $Poxn-Gal4^{ups1f}/TM3$ ,  
y w hs-flp; UAS > CD2 y<sup>+</sup> > CD8::GFP/CyO;  $TM2/TM6B$ ,  
y w hs-flp; UAS > CD2 y<sup>+</sup> > CD8::GFP/CyO;  $Poxn-Gal4^{ups1f}/TM6B$ ,  
y w hs-flp;  $Poxn^{\Delta M22}$  UAS > CD2 y<sup>+</sup> > CD8::GFP/CyO;  $Poxn-Gal4^{ups1f}/TM6B$ ,  
y w hs-flp;  $E7-2-36$  UAS > CD2 y<sup>+</sup> > CD8::GFP/CyO;  $Poxn-Gal4^{ups1f}/TM6B$ ,  
y w hs-flp;  $Poxn^{\Delta M22}$  E7-2-36/CyO;  $Poxn-Gal4^{ups1f}/TM6B$ ,  
y w hs-flp;  $Poxn^{\Delta M22}$  E7-2-36/CyO;  $Poxn-Gal4^{ups1f}/TM6B$ ,  
y w hs-flp;  $Poxn^{\Delta M22}$  UAS - p35/CyO;  $Poxn-Gal4^{ups1f}/TM6B$ ,

Generation of flip-out clones in the embryonic PNS

Flies were crossed to generate a stock that combined three transgenes (Fig. 2A): (i) *hsp70-flp*, the yeast recombinase gene *flp* under control of the heat-inducible *hsp70* promoter (Struhl and Basler, 1993); (ii) *Poxn-Gal4<sup>ups1f</sup>* (*Poxn* transgene *EvK* (Boll and Noll, 2002), the coding region of which has been replaced by that of *Gal4*) that expresses Gal4 in all SOPs of larval p-es organs and their daughter cells; and (iii) the reporter transgene *UAS* > *CD2*  $y^+$  > *CD8::GFP* (derived from *Actin5C* > *CD2*  $y^+$  > *PKA*\* (Jiang and Struhl, 1995) and kindly provided by Gary Struhl). The stock was kept at 18°C to minimize *flp*-induced recombination in the germline.

To collect embryos, flies were allowed to lay eggs on apple juicecontaining plates, supplemented with a small amount of yeast, for 2 h at 18°C. Plates were kept at 18°C for 4, 6, 8, 10, and 12 h, and embryos were collected in a fine custom-built nylon sieve. The age of the collected embryos thus corresponded to 2–3, 3–4, 4–5, 5–6, and 6-7 h of development at 25°C. Embryos were rinsed several times with cold tap water before they were heat-shocked by placing the nylon sieve into a pre-heated waterbath and keeping the embryos submerged for 30 min. After heat shock under five different conditions (30 min or 15 min at 34°C; 25 min or 30 min at 32°C; 30 min at 30°C), the average numbers of GFP-labeled clones per embryo were determined (data not shown). A 30-min heat shock at 32°C produced on average about one thoracic clone per embryo, thus labeling only one of the twelve kölbchen lineages, which was taken as an indication that only a single recombination event had taken place in this lineage. Therefore, these heat-shock conditions were used throughout our studies. After heat shock, embryos were rinsed briefly with cold tap water and placed, still on the nylon sieve, into a humidified chamber. Embryos were allowed to develop at  $18\,^\circ\text{C}$ until they reached what corresponds to 14-15 h of development at 25°C (mid stage 16) before they were fixed and immunostained.

#### Antibodies and immunohistochemistry

The following primary antibodies were used: rabbit anti-Poxn (1:50; Bopp et al., 1989); rabbit anti-D-Pax2 (1:100; Fu and Noll, 1997); chicken anti-GFP (1:500; Abcam); mouse anti-CD2 (1:100; Serotec); rabbit anti-Su(H) (1:100; Santa Cruz Biotechnology); mouse anti-Prospero (1:10; batch MR1A, Developmental Studies Hybridoma Bank, University of Iowa (DSHB)); mouse anti-22C10 (1:100; DSHB); rabbit anti- $\beta$ -galactosidase (1:1000; Cappel); rabbit anti-BarH1 (1:100; Higashijima et al., 1992); rabbit anti-cleaved Casp-3 (Asp175) (1:100; Cell Signaling Technology); and rat anti-Elav (1:200; DSHB). The secondary antibody used in Fig. 1 was biotiny-lated goat anti-rabbit IgG (VECTASTAIN ABC Kit, Vector Laboratories PK-4001); other secondary antibodies used in this study were: Alexa 488-, Alexa 594-, and Alexa 647-conjugated anti-chicken, -rabbit, -mouse, or -rat IgG (1:500; all from Molecular Probes).

Embryos were fixed and stained as described previously (Gutjahr et al., 1993; Boll and Noll, 2002).



**Fig. 1.** Expression of *Poxn* during embryogenesis. Poxn protein is visualized by staining with anti-Poxn antiserum in lateral views of whole-mount embryos at late stage 10 (A), stage 11 (B), early (C) and late stage 12 (D), stage 13 (E), stage 14 (F), stage 15 (G), and stage 16 (H). Arrowheads in G and H indicate the *Poxn*-expressing bipolar neurons innervating the kölbchen. Embryos are oriented with their anterior to the left and dorsal side up. Abbreviations: T, thoracic segments; A, abdominal segments; dk, dorsal kölbchen; lk, lateral kölbchen; vk, ventral kölbchen; h3, hair 3; p6, papilla 6; br, brain; CNS, central nervous system; epid, epidermis.

### Quantification of cell number in kölbchen of Poxn null mutants

In *Poxn*<sup> $\Delta$ M22</sup> embryos, two types of kölbchen lineages were observed, producing five (Fig. 5A) or six cells (Fig. 5B). To test whether this variation in cell number is an artifact of Gal4 that induces an additional mitosis in some of the lineages and to quantify both lineages, we analyzed the number of GFP-positive cells in *Poxn*<sup> $\Delta$ M22</sup>; *Poxn-CD8::GFP*<sup>3-3</sup> embryos in which expression of CD8:: GFP (Lee and Luo, 1999) is controlled by the same *Poxn* promoter and enhancer as *Poxn-Gal4*<sup>ups1f</sup> (see above). In these embryos, again two types of kölbchen lineages were found of which 93/144 consisted of five and 51/144 of six cells, thus excluding the possibility that the variation in cell number was the result of a Gal4 artifact.

#### Microscopy and image processing

Poxn protein was visualized in wild-type embryos by immunohistochemistry and recorded with a Zeiss Axiophot microscope equipped with a Hamamatsu CCD camera. Pictures of immunofluorescent proteins in clonal analyses were taken with a Leica TCS SP1 confocal microscope and a Zeiss LSM 710 confocal microscope. All immunofluorescent images are maximal projections of confocal Z-stacks that were processed with LCS Lite (Leica), Zen software (Zeiss), ImageJ, and Photoshop (Adobe Systems).

#### Results

# Embryonic Poxn expression versus the Poxn mutant phenotype of larval p-es organs

The expression of *Poxn* during embryonic development is highly dynamic (Dambly-Chaudière et al., 1992), as illustrated in Fig. 1 in greater detail. Initially, a single dorsal cell of each thoracic and abdominal hemisegment expresses Poxn protein at late stage 10 (Fig. 1A), followed by a second ventral cell during stage 11 (Fig. 1B).



**Fig. 2.** Strategy of marking clones and its application to the kölbchen lineage. (A) Labeling of flip-out clones in p-es organs in a time-dependent manner. Expression of CD2 in developing larval p-es organs is regulated by Gal4 under control of the *Poxn* enhancer and promoter in the *Poxn-Gal4<sup>ups1f</sup>* transgene (in the text abbreviated as *Poxn-Gal4*). A moderate heat shock (hs) activates the *hsp70* promoter that controls the yeast *flp* gene encoding FLP recombinase, which excises CD2 by recombination of flanking FRT sites during cell division and thereby brings CD8::GFP (green) under the control of Gal4. Note that cells of the p-es lineages in which no recombination was induced express CD2 (red). ((B)–(P)) Labeled clones in dorsal ((I), (M) and (N)) or lateral (all other panels) kölbchen of stage 16 embryos are shown, which were induced at *2-3* ((B)–(F); except (E), where no clones were induced but all cells of a kölbchen were marked by CD2), 3–4 ((G) and (H)), 3–5 (I), 4–5 ((J) and (K)), and 5–6 h AEL ((L)–(P)) and immunostained with antibodies directed against the proteins indicated in the corresponding colors below the panels. Overlaps of D-Pax2 or Elav (blue) with Pros or β-gal (red) are purple ((D) and (I)), while those of GFP (green) with 22C10 (red) are yellow (K). Genotypes of embryos are *y* w *hs-flp* (homozygous or over *Y*); *UAS* > *CD2 y* + > *CD8::GFP* (homozygous or over *CyO*); *Poxn-Gal4<sup>ups1f</sup>* (homozygous or over *TM6B*) and include (in (F), (1) and (P)) the enhancer trap marker *E7-2-36* on the second chromosome. For a detailed description of clones, see text. Here and in subsequent figures as well as in supplementary figures, panels include explanatory sketches that illustrate individual cells of the clones (derived also labeled by the antibodies belong to either adjacent m-es organs or the same p-es organ, as e.g., in Fig. S2G.

These cells are the SOPs of the larval p-es organs (Dambly-Chaudière et al., 1992), which divide (Fig. 1B and C) and give rise to two clusters of *Poxn*-expressing cells per hemisegment by the end of germband retraction (Fig. 1D). During germband retraction (stage 12), the dorsal clusters of *Poxn*-expressing cells in T2 and T3 are located ventrally to those of the remaining segments (arrows in Fig. 1C and D). At the onset of stage 13, expression of *Poxn* begins to fade in the developing p-es organs (Fig. 1E). In the thoracic segments, however, one cell of each cluster continues to express *Poxn* very strongly at later stages until the end of embryogenesis (Fig. 1F–H; arrowheads in panels G and H). These single *Poxn*-expressing cells are bipolar neurons innervating the kölbchen. As evident from Fig. 1, *Poxn* is also expressed in several cells of the gnathal and terminal abdominal segments, in the ventral nerve cord and developing larval brain, in a

few cells that may belong to the olfactory and gustatory sensory organs of the larval head (not visible in focal planes of Fig. 1), in many epidermal cells, and, after stage 14, in single cells associated with larval mono-innervated hairs.

In larvae that are homozygous for the *Poxn* null allele,  $Poxn^{\Delta M22}$  (Boll and Noll, 2002), all p-es organs as well as a subset of m-es organs exhibit a mutant phenotype. Of the p-es organs, the kölbchen and hair 3 are transformed into papilla-like sensory organs, and the transformed ventral kölbchen, papilla 6, and hair 3 are mislocalized (data not shown). These defects are in agreement with previous analyses of larvae that are homozygous for a large deficiency, *Df(2R)WMG*, uncovering *Poxn* (Dambly-Chaudière et al., 1992), or a strong hypomorphic *Poxn* allele, *Poxn*<sup>70</sup> (Awasaki and Kimura, 2001).

# Experimental strategy and cell lineage of kölbchen in wild-type embryos

To address the role of *Poxn* during the development of larval p-es organs, we first analyzed the cell lineage of kölbchen in wild-type embryos. To this end, we designed an assay in embryos that combines temporally controlled FLP/FRT recombination (Golic and Lindquist, 1989) with spatially controlled Gal4/UAS reporter expression (Brand and Perrimon, 1993) to label flip-out clones (Struhl and Basler, 1993) in the lineage of larval p-es organs. These embryos express CD2 under the indirect control of a *Poxn* enhancer in all cells of the larval p-es lineages until activation of the FLP recombinase by a moderate heat shock deletes CD2 in flip-out clones (Fig. 2A: Jiang and Struhl, 1995). Consequently, these clones express the fusion protein CD8::GFP under the control of the Poxn enhancer (Fig. 2A). Two features of this system were essential for proper analysis of larval p-es organ lineages: (i) all cells of the lineages express Poxn, and hence the Poxn-Gal4 transgene, until stage 13 when cell division has ceased (Fig. 1); and (ii) the stability of the Gal4, CD2, and CD8::GFP proteins results in their perdurance, which permits clonal analysis at stage 16, when these cells have adopted their final fates and express cell-type specific proteins. The combinatorial analysis of such celltype specific markers and the patterns of CD8::GFP- and CD2expressing cells in a large number of individual clones, induced at different times after egg laying (AEL), enabled us to distinguish all cells produced after the last division of the lineage, to quantify each type of clone, and to derive the pattern of cell divisions.

When embryos were subjected to a moderate heat shock (30 min at  $32^{\circ}$ C) during early embryogenesis (2–3 h AEL; although embryos developed at 18°C, except during heat shock, the indicated time intervals throughout the paper correspond to development at 25°C), flip-out clones were mostly produced before SOP selection (Table S1 in Supporting information). Therefore, all cells that expressed CD8:: GFP were derived from an SOP in which a flip-out event had occurred. Since such clones consisted of seven cells in dorsal or lateral kölbchen (dk or lk) (Fig. 2B) and in the ventral kölbchen (vk) (Fig. S1A in Supporting information), it follows that the kölbchen lineage gives rise to seven cells. Further detailed analysis was restricted to clones of the dk/lk lineages because their cells were better separated from each other and hence much easier to distinguish by position (Fig. 2B) than clones of the vk (Fig. S1 in Supporting information; see below).

The seven cells could be identified by the use of markers that are expressed in a specific subset of larval es organ cells. The socket cell was marked by the transcription factor Suppressor of Hairless [Su (H)] (Gho et al., 1996) and is always located most dorsally (Fig. 2C). The shaft cell and sheath cell expressed D-Pax2 (Fig. 2D), a paireddomain transcription factor homologous to Poxn (Fu and Noll, 1997; Hill et al., 2010). At the same time, the more ventrally located sheath cell also expressed the homeodomain transcription factor Prospero (Pros) (Vaessin et al., 1991) (Fig. 2D). Finally, expression of the pan-neuronal nuclear protein Elav (Robinow and White, 1991) identified the remaining four cells as neurons (Fig. 2E), one of which, consistently located at the most ventral position, was identified as the md neuron (Fig. 2F) by its  $\beta$ -gal expression of the enhancer trap marker E7-2-36 (Bier et al., 1989; Brewster and Bodmer, 1995). This result showed that the kölbchen lineage generates three bipolar neurons and one md neuron.

When embryos were heat-shocked at a slightly later stage during embryogenesis (3–4 h AEL), the flip-out clones either labeled all seven cells or only a subset of these cells, the latter of which resulted from a FLP-induced recombination event after division of the SOP cell (Table S1 in Supporting information). When heat shock was applied even later (4–5 h AEL), nearly all flip-out clones labeled only a subset of cells of the kölbchen lineage, among which mainly two types of clones were observed (Table S1 in Supporting information). The first consisted of three cells, one of which was always located most dorsally, while the remaining two expressed D-Pax2, which suggested that these were the three support cells (Fig. 2G). The second type consisted of four cells that did not express D-Pax2 and were localized ventrally of the CD2-expressing cells in the lineage, which indicated that they were all neurons (Fig. 2H). This was confirmed by the expression of Elav in these clones, one cell of which was an md neuron that was consistently located most ventrally (Fig. 2I). In addition, observations with both types of clones were confirmed by immunostaining for the axonal and dendritic marker 22C10 (Hummel et al., 2000), which does not stain the first type (Fig. 2J) but does stain all cells of the second type (Fig. 2K). These results suggest that the socket, shaft, and sheath cells are derived from one secondary precursor, plla, whereas all neurons are derived from the other, pllb (Fig. 7A).

To determine the cell division patterns of the secondary precursors, plla and pllb, clones were induced at 5-6 h AEL. Four additional types of flip-out clones were observed (Table S1 in Supporting information), consisting of (i) a single CD8::GFPpositive cell, which is the socket cell, evident from its most dorsal position (Fig. 2L); (ii) the shaft and sheath cells, based on their expression of D-Pax2 (Fig. 2M); (iii) two bipolar neurons, according to their positions (neither most dorsal nor most ventral in the lineage) and the lack of D-Pax2 expression (Fig. 2N); and (iv) a bipolar neuron and the md neuron, since the latter was always located most ventrally (Fig. 20). As shown in Fig. 1, Poxn continues to be expressed in a single cell of the kölbchen lineage throughout embryogenesis (Fig. 1F-H). This Poxn-expressing cell is a bipolar neuron, as it formed a clone with the md neuron (Fig. 2P). The remaining two bipolar neurons could not be distinguished from each other by the expression of BarH1, unlike in the lineage of papilla p6 (see below), because although BarH1 is strongly expressed in the outer support cells, the socket and shaft cells, and a bit less in the inner support cell, the sheath cell, it is not expressed in any of the kölbchen neurons (Y.J., unpublished data).

Because individual cells could not be resolved in most cases, it was impossible to analyze the vk lineage in the same detail as that of dk/lk. However, in cases where the positions of individual cells could be distinguished (Fig. S1 in Supporting information), the vk and dk/lk lineages are identical. Thus, vk also consisted of seven cells, four of which were labeled with Elav, and hence are neurons, while the remaining three were support cells (Fig. S1A in Supporting information). Like in the dk/lk lineage, the vk support cells were derived from one secondary precursor, plla, and two of them expressed D-Pax2 (Fig. S1B in Supporting information), whereas the four vk neurons were derived from the other secondary precursor (Fig. S1C in Supporting information), pllb. In addition, one of the bipolar neurons of the vk continues to express Poxn (Fig. S1D in Supporting information and Fig. 1F–H), while one is an md neuron (Fig. S1D in Supporting information).

Surprisingly, the lineage of kölbchen derived from this analysis (Fig. 7A) differs drastically from that reported for one of its two abdominal homologs, papilla 6 (p6) (Brewster and Bodmer, 1995; Fig. 7D), which was thought to be similar to the lineage of m-es organs (Orgogozo et al., 2001; Fig. 7E). Therefore, we also analyzed the p6 lineage by the same method we used to determine the kölbchen lineage.

#### Cell lineage of papilla 6 in wild-type embryos

The p6 lineage had been proposed to generate six cells, three support cells, two bipolar neurons, and one md neuron (Brewster and Bodmer, 1995; Fig. 7D). Yet, most flip-out clones induced at 2–3 or 3–4 h AEL consisted of nine cells (Fig. 3A and Table S2 in Supporting information), three of which were located at a ventral and posterior position (marked by brackets in Fig. 3A). These nine cells formed a single clone that comprises the entire lineage



**Fig. 3.** Clones of the papilla 6 lineage. ((A)-(C) and (E)-(L)) Marked clones of stage 16 embryos are shown that were generated at 3–4 ((A)-(C)), 5–6 ((E)-(H)), and 6–7 h AEL ((1)-(L)) and immunostained with antibodies directed against the proteins indicated in the corresponding colors below the panels. Overlaps of GFP (green) with 22C10 (red) are yellow ((E), (G), (K) and (L)). Genotypes of embryos are as described in the legend to Fig. 2. For a detailed description of clones, see text. (D) Lineage of progenitor cell p0 that, upon division, generates the SOP, pI, of the p6 lineage and the precursor, pl', of the presumptive epidermal gland (Orgogozo and Schweisguth, 2004).

because (i) the vast majority of marked clones consisted of nine cells when they were labeled by heat shock long before the selection of SOPs (2-3 h AEL in Table S2 in Supporting information), (ii) heat-shock conditions had been optimized to generate only one recombination event per thorax, and (iii) no CD2-positive cells were observed in these clones (data not shown). The three ventral cells of the clone have been mentioned previously and were suggested to form an epidermal gland (Orgogozo and Schweisguth, 2004). The expression of GFP in these cells is not the result of ectopic expression of Poxn-Gal4, as Poxn is also expressed in all three cells, which are distinct from the p6 cells at stage 13 (data not shown). While none of these three presumptive epidermal gland cells expressed the neuronal markers 22C10 or Elav, two did express D-Pax2 at late embryogenesis (data not shown). When embryos were heat-shocked later (3-4 h AEL in Table S2 in Supporting information), about half of the flip-out clones consisted in equal parts of all p6 (Fig. 3B) or all presumptive epidermal gland cells (Fig. 3C). These results suggest that the SOP of p6, pI, and the precursor of the presumptive epidermal gland, pl', are derived from the same progenitor cell p0 that expresses Poxn (Fig. 3D). In the following, we restricted our analysis to the p6 lineage.

In agreement with Brewster and Bodmer (1995), we observed that the p6 lineage gives rise to six cells. Similar to kölbchen, the p6 socket cell was always located most dorsally, the shaft and sheath cells of p6 expressed D-Pax2, while the three p6 neurons were located more ventrally and expressed the neuronal marker 22C10 (Fig. 3E). However, when heat shock was applied at 5–6 h AEL to mark clones that include only part of the p6 lineage (Table S2 in Supporting information), most of the clones consisted of either all support cells (Fig. 3F) or all neurons (Fig. 3G and H), again as we have observed for kölbchen. One p6 neuron expressed E7-2-36 and hence was an md neuron (Fig. 3G and H), while only one of the two bipolar neurons expressed BarH1 (Fig. 3H) and thus can be distinguished from the other.

To examine the division patterns of the pIIa and pIIb cells of p6, clones were induced at 6–7 h AEL, which generated mainly four additional types of clones (Table S2 in Supporting information). The first included only the socket cell, as it was located most dorsally and expressed neither D-Pax2 nor 22C10 (Fig. 3I); the second consisted of the D-Pax2-expressing shaft and sheath cells (Fig. 3J); the third of the two bipolar neurons that express 22C10 but not the md neuronal marker (Fig. 3K); while the fourth included only the md neuron (Fig. 3L). Thus, the p6 lineage derived from these results is identical to the kölbchen lineage with the exception that the latter includes an additional bipolar neuron (Fig. 7A).

#### Apoptosis in the p6 lineage

This difference between the kölbchen and p6 lineages could arise either from an additional division in the kölbchen lineage or



**Fig. 4.** One cell of the p6 lineage undergoes apoptosis. (A) An apoptotic cell marked by activated Casp-3 (asterisk) is shown among ten GFP-labeled p6 and epidermal gland (bracket) cells in a *y w; UAS-CD8::GFP; Poxn-Gal4<sup>ups1f</sup>* (homozygous or over *TM3*) embryo at stage 13. The interpretation in the sketch of the staining pattern was derived from an analysis of suitable substacks of confocal laser scanning microscope sections. The sketch does not include the three epidermal gland cells. (B) Inhibition of apoptosis by P35 generates a supernumerary cell that is associated with p6 and epidermal gland (bracket) cells of a *y w hs-flp/(w* or *Y*); *UAS > CD2 y<sup>+</sup> > CD8::GFP/UAS-p35; Poxn-Gal4<sup>ups1f</sup>/+* embryo at stage 16. The sketch does not include the three epidermal gland cells. (C)–(E) Clones of stage 16 *y w hs-flp/(w* or *Y*); *E7-2-36 UAS > CD2 y<sup>+</sup> > CD8::GFP/UAS-p35; Poxn-Gal4<sup>ups1f</sup>/+* embryos, expressing P35 in p-es organs, were marked at 5–7 h AEL and immunostained with antibodies directed against the proteins indicated in the corresponding colors below the panels. Overlaps of Elav (red) with β-galactosidase of E7-2-36 (blue) are purple ((C)–(E)). Asterisks indicate the supernumerary cell. For a detailed description of clones, see text.

from apoptosis of one of the bipolar neurons in the p6 lineage. If there is apoptosis in the p6 lineage, it is expected to occur during cell differentiation between the ends of stages 12 and 15. Therefore, stage 12–15 embryos whose p-es organs were labeled by *UAS-CD8::GFP* under the control of *Poxn-Gal4* were tested for apoptosis by antibodies that recognized activated Caspase-3 (Casp-3; Yu et al., 2002). Indeed, in about 70% of the cases (43/62), a GFPlabeled cell adjacent to the p6 cells also stained for activated Casp-3 in stage 13 embryos (asterisk in Fig. 4A). This cell was supernumerary, since all other cells of the p6 and the epidermal gland lineages (bracket in Fig. 4A) were present. Moreover, when apoptosis was inhibited by the apoptosis inhibitor P35 of baculovirus (Hay et al., 1994), the combined p6 and epidermal gland lineages produced ten rather than nine cells (Fig. 4B).

To determine whether the apoptotic cell belongs to the p6 or epidermal gland lineage (Fig. 3D), clones were induced to mark the entire p6 lineage or a sub-lineage of it in embryos that express P35 in the lineage. In clones that labeled only the p6 lineage, a supernumerary cell was present, which in many cases (34/55) also expressed the neuronal marker Elav (Fig. 4E), but not the md neuronal marker E7-2-36 (Fig. 4C). By contrast, no extra cell was observed in clones that consist only of the epidermal gland cells (data not shown). Consistent with these observations, the supernumerary cell was found in clones that label only the neurons (asterisk in Fig. 4D). Finally, the smallest marked clones that included the supernumerary cell also labeled the md neuron (Fig. 4E), which demonstrates that these cells are derived from the same precursor. It follows that the p6 and kölbchen lineages are identical, except that the bipolar neuron, which continues to express Poxn in the kölbchen lineage (Fig. 7A), undergoes apoptosis in the p6 lineage (Fig. 7B).

#### Cell lineage of hair 3 in wild-type embryos

Finally, we examined the lineage of the dorsal abdominal larval p-es organ, h3. All flip-out clones induced in its primary precursor, pI or SOP, consisted of six cells, three of which were neurons expressing Elav, while two support cells expressed D-Pax2 (Fig. S2A in Supporting information), as was observed for p6 (Fig. 3E). However, in contrast to p6 whose three support cells are all located dorsally to the three neurons (Fig. 3E and F), the h3 shaft and sheath cells that express D-Pax2 are located ventrally to the two bipolar neurons (Fig. S2A in Supporting information). Clones larger than six cells, like those of the p6 and epidermal gland lineage (Fig. 3A and Table S2 in Supporting information), were never observed for h3, when induced as early as 3-4 h AEL. When flip-out was induced at a later stage in the h3 secondary precursors, pIIa or pIIb, two types of three-cell clones were observed, which were either all support cells (Fig. S2B in Supporting information) or all neurons (Fig. S2C and D in Supporting information). In the first case, only the most dorsal cell did not express D-Pax2 and was likely the socket cell, while the other cells were shaft and sheath cells. Thus, like in the p6 lineage, all three support cells of h3 are derived from the secondary precursor, pIIa, while all neurons are descendants of the secondary precursor, pIIb. Likewise, the most ventral neuron in the h3 lineage was always the md neuron (Fig. S2D in Supporting information). Flip-out clones generated even later, in tertiary precursors, included two types of two-cell clones: the D-Pax2-expressing shaft and



**Fig. 5.** Altered kölbchen lineage in *Poxn* null mutants. Clones of stage 16 *Poxn* mutant embryos were marked at 3–4 ((A)–(F)), 4–5 ((G)–(I)), and 5–6 h AEL ((J)–(L)) and immunostained with antibodies directed against the proteins indicated in the corresponding colors below the panels. Overlaps of GFP (green) with 22C10 (red) are yellow ((E) and (F)), those of D-Pax2 (blue) with Pros (red) are purple ((D) and (I)). Genotypes of embryos are *y* w *hs*-*flp* (homozygous or over *Y*); *Poxn*<sup>ΔM22</sup> *UAS* > *CD2*  $y^+$  > *CD8::GFP*/*Poxn*<sup>ΔM22</sup> *UAS* > *CD2*  $y^+$  > *CD8::GFP*/*Poxn*<sup>ΔM22</sup> *E*7-2-36; *Poxn*-*Gal4*<sup>ups1f</sup> (homozygous or over *TM6B*) ((E) and (F)). For a detailed description of clones, see text.

sheath cells (Fig. S2E in Supporting information) or the two bipolar neurons (Fig. S2F in Supporting information) that are located far dorsally to the md neuron (Fig. S2D in Supporting information). It follows that the shaft and sheath cells are generated from the tertiary precursor pIIIa, while the two bipolar neurons, the more ventral of which weakly expresses BarH1 (Fig. S2G in Supporting information), are derived from the tertiary precursor pIIIb. Thus, the lineage of h3 (Fig. 7C) is similar to the lineage of p6 (Fig. 7B), but different in two important aspects. Although division of pIIb also produces the tertiary precursor pIIIb that, like pIIIb1 in the p6 lineage, generates two bipolar neurons, pIIb does not give rise to a second tertiary precursor, since the second daughter cell of pIIb differentiates into an md neuron. In addition, apoptosis was never observed in the h3 lineage when it was analyzed for activated Casp-3 at stages 12–15 (Fig. S3 in Supporting information).

#### No apoptosis in the kölbchen lineage

To test whether apoptosis occurs in the kölbchen lineage, we analyzed GFP-labeled kölbchen cells for the presence of apoptosis in stage 12–15 embryos. None of these cells stained for activated Casp-3 (Fig. S4A and B in Supporting information, and data not shown). In agreement with this result, we never observed more than seven kölbchen cells when P35 was used to inhibit cell death

in their lineage (Fig. S4C in Supporting information). Thus, unlike in the p6 lineage, apoptosis plays no role in the kölbchen lineage of wild-type embryos.

#### Alteration of the kölbchen lineage in Poxn null mutants

Since larval p-es organs are transformed into mechanosensorylike sensilla in the absence of Poxn (Dambly-Chaudière et al., 1992), we asked how this affected their lineages. In Poxn null mutants, the ventral kölbchen localized more dorsally and its cells were much better resolved from each other. Therefore, all kölbchen were included in this lineage analysis. We initially induced clones that labeled the entire lineage. Surprisingly, we observed in dorsal/lateral as well as ventral kölbchen two types of lineages, both of which generated a reduced number of differentiating cells: Two thirds (93/144) of the mutant kölbchen produced only five cells (Fig. 5A), whereas the rest (51/144), only six cells (Fig. 5B). The five cells consisted of a most dorsally located Su(H)-expressing socket cell (Fig. 5C), a D-Pax2-positive shaft cell (Fig. 5D), a more ventrally located sheath cell that expressed both D-Pax2 and Pros (Fig. 5D), and two neurons marked by 22C10 (Fig. 5E). When the lineage produced six cells, these included the same support cells (data not shown) and an additional neuron (Fig. 5F). Like in wild-type embryos, the most ventrally located neuron of both lineages was an



**Fig. 6.** Altered p6 lineage in *Poxn* null mutants. Clones of stage 16 *Poxn* mutant embryos were marked at 3–4 (A), 4–5 ((B)–(F)), and 5–6 or 6–7 h AEL ((G) and (H)) and immunostained with antibodies directed against the proteins indicated in the corresponding colors below the panels. Overlaps of Elav or Pros (red) with D-Pax2 or  $\beta$ -galactosidase of E7-2-36 (blue) are purple ((E), (F) and (H)). Genotypes of embryos are as described in the legend to Fig. 5. For a detailed description of clones, see text.

md neuron, as it expressed the E7-2-36 marker (Fig. 5E and F). Thus, while all transformed kölbchen of  $Poxn^{\Delta M22}$  mutants had three support cells and an md neuron, they also showed a reduced number of one or two bipolar neurons.

To analyze the pattern of cell divisions in the mutant kölbchen lineage, clones were labeled at 4-5 h AEL. Two types of clones that did not label the entire lineage were observed (Table S3 in Supporting information). One consisted of socket cell and shaft cell, based on their position within the lineage and the expression of D-Pax2 (Fig. 5G), respectively. The other type of clone labeled the D-Pax2expressing sheath cell and two (Fig. 5H) or three neurons (data not shown). Cell identities in these clones were corroborated by staining for Pros, which was absent in the socket and the D-Pax2-expressing shaft cell of the first type of clone (data not shown), but was present in the D-Pax2-expressing sheath cell of the second type (Fig. 5I). The remaining two cells of this second type of clone were neurons, as they were located more ventrally and expressed neither D-Pax2 nor Pros (Fig. 5I). The same types of clones were revealed in the mutant lineage that produced six cells (data not shown). These results show that in the kölbchen lineage of  $Poxn^{\Delta M22}$  mutants the socket and shaft cell are daughters of the pIIa cell, while the pIIb cell generates the sheath cell and neurons (Fig. 7F).

The division pattern of the pIIb cell was further examined in clones induced at 5-6 h AEL. Three types of clones were informative (Table S3 in Supporting information). One type, which is present in both five- and six-cell lineages of mutant kölbchen, consisted of the sheath cell and one bipolar neuron, which is located dorsal to the md neuron (Fig. 5J); a second type consisted only of the most ventrally located md neuron, when the lineage produced five cells (Fig. 5K); and a third type consisted of a bipolar neuron and the md neuron, when the lineage produced six cells (Fig. 5L). Thus, Poxn mutant kölbchen appear in two forms which result from two similar lineages: (i) when the organ consists of six cells, its lineage resembles that of an m-es organ (Fig. 7E), in which the precursor of the md neuron undergoes an additional division to produce a second bipolar neuron (Fig. 7F); (ii) when the transformed organ includes only five cells (Fig. 7G), its lineage is identical to that of an m-es organ (Fig. 7E).

To determine whether the reduction in cell number in the absence of Poxn results from cell death, we examined the kölbchen lineage in homozygous  $Poxn^{\Delta M22}$  embryos for apoptotic cells. Thus, all p-es cells of stage 12-15 embryos were labeled with antibodies against CD2 (Fig. 2A) and stained for activated Casp-3. As the mutant kölbchen consisted of at least five cells, we focused on lineages consisting of at least six CD2-positive cells. However, we observed neither co-expression of activated Casp-3 with CD2 at any stage nor any mutant kölbchen lineages consisting of more than six cells (Fig. S5A and B in Supporting information, and data not shown). Consistent with this result, when UAS-p35 was expressed under the control of *Poxn-Gal4* in the kölbchen lineage of  $Poxn^{\Delta M22}$ embryos, we found the same types of lineages described above (Fig. 5A and B), consisting of five (18/26; Fig. S5C in Supporting information) or six cells (8/26; Fig. S5D in Supporting information) with a ratio (18/8) similar to that observed in embryos that did not express P35. These results suggested that the reduced number of cells in the kölbchen lineage of Poxn null mutant embryos resulted from a reduced number of cell divisions rather than from apoptosis.

# Transformation of abdominal p-es organ lineages to an m-es organ lineage in the absence of Poxn protein

We next analyzed the p6 lineage of  $Poxn^{\Delta M22}$  mutants. Early induced clones labeled the entire lineage and included the presumptive epidermal gland cells (bracket in Fig. 6A). The mutant p6 cells were located more dorsally and showed much more overlap with each other (Fig. 6A) than in wild-type embryos (Fig. 3A). The lineage generated five differentiating cells: a socket cell, two D-Pax2-expressing cells—the shaft and sheath, and two Elavexpressing neurons (Fig. 6B). When the entire lineage was labeled with CD2 and embryos were stained for Casp-3 during stages 13 to 15 (similar to wild-type p6 in Fig. 4A), no apoptosis was observed in the lineage. Therefore, the reduced number of five cells does not result from apoptosis, but rather from a reduced number of cell divisions. To determine the division pattern of the mutant p6 lineage, clones were induced after division of the SOP to label sublineages. Two types of clones were found: one consisted of two cells,



**Fig. 7.** Lineages of larval p-es organs and their alterations in *Poxn* mutants. (A) Lineage of wild-type kölbchen. (B) Lineage of wild-type papilla p6. (C) Lineage of wild-type hair h3. (D) Lineage previously proposed for wild-type papilla p6 (Brewster and Bodmer, 1995). (E) Lineage of larval m-es organs (Orgogozo et al., 2001). ((F) and (G)) Alternative kölbchen lineages ((F) and (G)) and h3 or p6 lineage (G) in *Poxn* null mutants. The neuron with a white center (F) corresponds to the neuron that would express Poxn in the wild-type kölbchen lineage (A). Different colors of cells derived from the same lineage are to indicate that the cells could be distinguished from each other by the expression of suitable markers: the socket, the most dorsal cell (except in h3) expresses Su(H) (assumed to be true also for h3), but not D-Pax2; shaft and sheath cells express D-Pax2, but not Su(H), while the sheath cell also expresses Pros (assumed to be true also for h3); all neurons express Elav, while the most ventral cell, the mole neuron, is the only cell that expresses the enhancer trap marker E7-2-36. The socket, shaft, and sheath cells, and neurons are each represented in the same colors to indicate same cell types in different lineages. The bipolar neuron, whose sibling is the md neuron and which is present only in the kölbchen lineage, is the only neuron that expresses Poxn. BarH1 is expressed in socket, shaft, and sheath cell but in none of the neurons of the kölbchen lineage. By contrast, BarH1 is expressed in one of the two bipolar neurons and in the presumptive sheath cell of the p6 and h3 lineages. For discussion, see text.

the socket cell and the D-Pax2-expressing shaft cell, neither of which expressed Elav (Fig. 6C); the other consisted of three cells, the D-Pax2-expressing sheath cell and two neurons (Fig. 6D), one of which was identified as the md neuron through its expression of the E7-2-36 marker (Fig. 6E). The identity of the sheath cell in three-cell clones was again confirmed by its expression of both Pros and D-Pax2 (Fig. 6F). These results suggest that in the p6 lineage of  $Poxn^{\Delta M22}$  mutants, the socket and shaft cell are produced by the plla cell, while the sheath cell and neurons are derived from the pllb cell (Fig. 7G). Two types of clones were observed in the pllb lineage: one consisted of the D-Pax2-expressing sheath cell and an Elav-expressing neuron (Fig. 6G); the other type consisted only of the md neuron, which is marked by E7-2-36 (Fig. 6H). Since no two-cell clone expressed the md neuron-specific marker, it follows that the

sheath cell and the bipolar neuron are derived from pIIIb. Therefore, in *Poxn* mutants the p6 lineage (Fig. 7G) is identical to that of wild-type m-es organs (Fig. 7E).

Finally, the lineage of the dorsal abdominal p-es organ, h3, was analyzed in  $Poxn^{\Delta M22}$  mutants. Early induced flip-out clones consisted of only five cells, instead of the six observed in the wild-type h3 lineage (cf. Fig. S2H with A in Supporting information). Again staining for Casp-3 in the mutant h3 lineage marked by CD2 revealed no apoptosis, like in the mutant lineages of p6 and kölbchen. Two of these five cells were neurons expressing Elav, two were shaft and sheath cells that express D-Pax2, and one expressed neither of these markers and was the socket cell, since it was located most dorsally (Fig. S2H in Supporting information). Like in the mutant p6 lineage, two types of two-cell mutant h3

clones were observed. One type consisted of two support cells because neither of them expressed Elav (Fig. S2I in Supporting information), while one of them expressed D-Pax2 (Fig. S2I in Supporting information) but not Pros (Fig. S2J in Supporting information). Hence, the D-Pax2-expressing cell was the shaft cell, and the other cell that expressed neither D-Pax2 nor Pros was the socket cell. The other type of two-cell mutant h3 clone consisted of a D-Pax2-expressing cell and an Elav-expressing cell, which thus are the sheath cell and a bipolar neuron, respectively (Fig. S2L in Supporting information), because none of the two-cell clones ever expressed the md neuron-specific marker E7-2-36. The only type of three-cell mutant h3 clone observed consisted of two Elavexpressing neurons and one D-Pax2-expressing cell, which thus was the sheath cell (Fig. S2K in Supporting information). The more ventral of the two neurons displayed multiple dendrites and thus is an md neuron (Fig. S2K in Supporting information). Thus in Poxn mutants, the lineage of h3, like that of p6, is transformed to that of m-es organs (Fig. 7G).

#### Discussion

The lineages of the larval es organs may appear simple, as they include only few cells. The simplest is the m-es organ lineage, which generates four cells, in addition to an md neuron (Orgogozo et al., 2001; Fig. 7E). In comparison, the larval p-es organ lineages are slightly more complex, such as that of the abdominal papilla p6, which has an additional bipolar neuron. Despite their simplicity, however, essential features of the p-es lineages determined here (Fig. 7A–C) deviate from those proposed (Bodmer et al., 1989) or determined by an earlier analysis (Brewster and Bodmer, 1995; Fig. 7D). For example, in contrast to the m-es lineage (Orgogozo et al., 2001; Fig. 7E) and the previously reported p6 lineage (Fig. 7D), we have demonstrated that the three support cells of all p-es lineages are in fact derived from one secondary precursor, while the other secondary precursor generates only neurons and thus is a purely neuronal precursor (Fig. 7A-C). Moreover, the primary precursor cell generates the entire p-es organ in only three (Fig. 7A–C) rather than the four rounds of cell division that were thought to be necessary (Brewster and Bodmer, 1995; Fig. 7D). In addition, we have shown that, different from all other p-es lineages, the p6 lineage involves apoptosis (Fig. 7B). Surprisingly, the p6 lineage also differs in one important aspect from that of its dorsal analog, the abdominal p-es organ, hair h3, even though the same types and number of cells are produced in both lineages (Fig. 7B and C). In the h3 lineage, the neuronal secondary precursor, pllb, divides to generate an md neuron and the precursor of two bipolar neurons (Fig. 7C). In the lineage of p6, however, pIIb produces two precursors: one that generates two bipolar neurons, and another that produces an md neuron and a cell determined to undergo apoptosis (Fig. 7B). Thus, in the former lineage the absence of one cell division is compensated by the absence of apoptosis, whereas in the latter lineage apoptosis annihilates the additional cell division. We have further shown that the lineage of the thoracic homologs of the abdominal p-es organs, the kölbchen, is identical to that of the p6 lineage, except that the neuronal secondary precursor in the kölbchen lineage produces three, instead of only two, bipolar neurons because apoptosis does not occur (Fig. 7A). Finally, analysis of the p-es organ lineages in the absence of Poxn has revealed the importance of Poxn functions at various levels of these lineages.

#### Differences and similarities between cell lineages of larval p-es organs

In contrast to the cell lineages of m-es organs that have been extensively studied in both larvae and adults (Bodmer et al., 1989; Hartenstein and Posakony, 1989; Brewster and Bodmer, 1995; Gho et al., 1999; Reddy and Rodrigues, 1999; Orgogozo et al., 2001; Fichelson and Gho, 2003), little was known about the development of p-es organs. In this study, we have described the cell lineages for all larval p-es organs, the thoracic kölbchen as well as their abdominal homologs, h3 and p6 (Fig. 7A–C). Interestingly, these lineages are quite distinct from any previously reported lineages of sensory organs in *Drosophila*.

All p-es organ lineages share one part that is identical, namely the lineage derived from one of the two secondary precursors, pIIa (Fig. 7A–C). This precursor generates the three support cells, the socket cell in a first division and the shaft and sheath cells in a subsequent division. On the other hand, the p-es lineages derived from the secondary precursor pIIb are similar but show clear differences. In the dorsal abdominal p-es organ, h3, pIIb divides to generate the md neuron and one tertiary precursor, pIIIb (Fig. 7C). However, in the thoracic kölbchen and the ventral abdominal homolog p6, pIIb produces two tertiary precursors, one of which, pIIIb1, divides to form two bipolar neurons, while the other, pIIIb2, gives rise to an md neuron and a third bipolar neuron in the kölbchen lineage (Fig. 7A) or to an md neuron and a cell that is eliminated by apoptosis in the p6 lineage (Fig. 7B). Therefore, the kölbchen has three bipolar neurons, whereas p6 has only two.

There are additional important differences between the lineages of p6 and kölbchen. First, the SOP of p6, pI, is not derived from a proneural cluster but from a precursor, p0 (Fig. 3D), that also generates a presumptive epidermal gland (Orgogozo and Schweisguth, 2004). This common precursor, p0, is selected from a proneural cluster by lateral inhibition and begins to express Poxn. Second, the sibling bipolar neuron of the md neuron in the kölbchen lineage is the only cell in all p-es organ lineages that continues to express Poxn throughout embryogenesis. Third, one of the bipolar neurons of p6 expresses *BarH1*, a gene that is not active in the bipolar neurons of kölbchen (data not shown).

It is unclear how an earlier analysis of the p6 lineage, in which the clones were labeled by flip-out in the act > Draf > nuclacZtransgene (Struhl and Basler, 1993) and neurons were identified by staining for 22C10 (Brewster and Bodmer, 1995), led to a model that is similar to the m-es lineage (Brewster and Bodmer, 1995; Fig. 7D). The analysis that led to this model differs from ours in at least one important aspect. In this earlier study, the transgene is expressed ubiquitously after flip-out. Thus one might argue that marked clones of epidermal cells adjacent to the p6 cells could have interfered with the analysis of the p6 clones. However, since the frequency of clones observed in the PNS was below three per embryo (Brewster and Bodmer, 1995), it seems improbable that such an effect could explain the 12 clones observed that were proposed to consist of the sheath cell and all neurons, or the 5 clones that were thought to consist of only the socket and shaft cells (Brewster and Bodmer, 1995). Neither could such interference explain that no clones were observed that consisted only of the three support cells (Brewster and Bodmer, 1995), which would have been expected to occur at a high frequency similar to that of clones including all neurons. Since these results were only documented by a table, it appears probable that this study was guided by adhering to a lineage previously proposed for larval p-es organs (Bodmer et al., 1989).

#### Multiple functions of Poxn in the lineages of p-es organs

In homozygous  $Poxn^{\Delta M22}$  larvae, p-es organs are transformed into papilla-like mono-innervated organs or hairs that, like all m-es hairs, lose their shaft after the first instar. Our analysis of all p-es organ lineages, in the wild type and *Poxn* mutants, shows that not only the phenotypes but also the lineages of all p-es organs are transformed (Fig. 7G) into that of an m-es organ (Fig. 7E). This is if we disregard a minority of a third of all kölbchen lineages that slightly deviates from the m-es lineage by forming a sibling of the md neuron, which is a second bipolar neuron (Fig. 7F). Thus, we may consider the formation of an m-es organ as the default developmental pathway in the absence of Poxn. In this case, one would expect that ectopic expression of Poxn in the m-es organ lineage is able to transform m-es into p-es organs. Although this question has not been addressed experimentally, a similar experiment is consistent with such a conclusion: high levels of ubiquitous Poxn expression for 15 min during stages 9 to 11 is able to produce ectopic kölbchen in the thorax (Dambly-Chaudière et al., 1992). Based on the sum of our results, our interpretation of this ubiquitous Poxn expression phenotype is more plausible than the alternative explanations that (i) ectopic Poxn recruits additional SOPs and thus gives rise to ectopic kölbchen, (ii) ectopic Poxn can transform internal sensory, i.e., chordotonal, organs into supernumerary kölbchen, or (iii) high levels of Poxn in the kölbchen lineage are able to duplicate kölbchens. Therefore, Poxn is not only necessary, as shown here, but may also be sufficient to discriminate between m-es and p-es development.

These considerations also argue that the differences between the lineages of kölbchen (Fig. 7A) and their abdominal homologs, p6 (Fig. 7B) and h3 (Fig. 7C), are determined by factors different from Poxn, such as those encoded by the bithorax complex which, if absent, transform the abdominal h3 and p6 at least partially into kölbchen (Dambly-Chaudière and Ghysen, 1987). Similarly, it is probable that the difference between the lineages of p6 (Fig. 7B) and h3 (Fig. 7C) result from factors expressed differentially along the dorsoventral axis of the embryo.

Keeping this in mind, it should be noted that the pIIa part of the p-es lineages is always changed in the same manner to the m-es type in *Poxn* mutants (Fig. 7E–G). In the wild-type p-es lineage, plla produces all support cells, including the sheath cell, whereas in the Poxn mutant lineages, pIIa generates only the socket and shaft cells, like in the m-es lineage. We conclude that Poxn increases the developmental potential of pIIa and pIIIa, and prevents differentiation by inducing mitosis in pIIIa. Analogous considerations further suggest that Poxn reduces the developmental potential of pIIb and its offspring, which is restricted to neural fates in the presence of Poxn. However, in the absence of Poxn, the developmental potential of pIIb and its offspring is expanded and similar or identical to those of pIIb and pIIIb of the m-es lineage, which are able to form a sheath cell in addition to neurons. In pIIIb2, which is not produced in h3 (Fig. 7C), Poxn inhibits differentiation and promotes mitosis, thereby generating an md neuron and a bipolar neuron in kölbchen (Fig. 7A) or an md neuron and an apoptotic cell in p6 (Fig. 7B). In the absence of Poxn, however, pIIIb2 is always absent in the transformed lineages of p6 and h3 (Fig. 7G) and is generated in only a third of the kölbchen (Fig. 7F). This shows that without Poxn pIIb can still produce pIIIb2 in a fraction of the kölbchen lineage, which is then able to promote mitosis and form a bipolar neuron and an md neuron. It follows that in this sublineage of the kölbchen Poxn acts with at least another factor to regulate its specification.

While the pIIIb2-generated bipolar neuron in the kölbchen lineage continues to express Poxn under wild-type conditions (Fig. 7A), the analogous cell in the p6 lineage does not, but instead undergoes apoptosis. One might therefore hypothesize that Poxn inhibits apoptosis of this neuron in the kölbchen lineage. However, the fact that this bipolar neuron does not undergo apoptosis when pIIIb2 is formed in a third of all mutant kölbchen that lack Poxn (Fig. 7F), argues strongly against such a hypothesis. Rather, we propose that the continued expression of Poxn in this bipolar neuron of the kölbchen lineage is necessary for its proper specification and differentiation. Interestingly, asymmetric cell divisions followed by apoptosis of the cell that is not destined to become an md neuron, as seen in the p6 lineage (Fig. 7B), have been previously reported in the md-solo lineage in the embryo (Orgogozo et al., 2002). In this case, the situation is more extreme, as the single md neuron is produced by two consecutive asymmetric cell divisions, each of which is accompanied by apoptosis.

Thus, the functions of Poxn in the cells of the p-es organ lineages occur at several levels. First, Poxn is necessary to specify the developmental potentials of the secondary precursors, plla and pllb. In its absence, the developmental potential of plla is reduced at the expense of that of pllb, such that the lineage is transformed to an m-es lineage. Second, Poxn is required to promote mitosis and inhibit differentiation in pllla and plllb2. Finally, Poxn presumably serves as a differentiation factor in the bipolar neuron that continues to express it in the kölbchen lineage.

However, Poxn is not required for the differentiation of the three support cells because these cells are also produced in the absence of Poxn, albeit with a different lineage. We further assume that, like in the m-es lineage, the specification of different cellular fates depends on asymmetric cell divisions mediated by N-signaling (Posakony, 1994).

Little is known about the biological functions of the larval p-es organs. It has been suggested that kölbchen are involved in chemosensory detection. For example, the *Gal4* transgene that is activated by the enhancer of one of the candidate gustatory receptors in *Drosophila*, Gr2B1, has been shown to be expressed in one of the bipolar neurons that innervates the ventral kölbchen, which is consistent with the idea that kölbchen play a role in larval taste perception (Scott et al., 2001). The late expression of *Poxn* in a single neuron might lead to a cell-specific expression of some chemosensory receptors, which allow this neuron to respond to certain environmental stimuli and process sensory information.

*Is the lineage of adult chemosensory bristles similar to that of larval p-es organs?* 

Poxn also plays an essential role during development of the chemosensory bristles on legs, wings, and labellum of adult Drosophila (Awasaki and Kimura, 1997; Boll and Noll, 2002; Krstic et al., 2009). Since the lineages of larval and adult m-es organs are very similar, they might be derived from one ancestral lineage (Lai and Orgogozo, 2004). Similarly, we might argue from an evolutionary point of view that the lineages of poly-innervated chemosensory bristles in adult flies are similar to those of larval p-es organs that we describe here. This possibility is further suggested by the alterations observed in adult taste bristles of Poxn null mutants, which are analogous to the changes observed here for larval p-es organs. Taste bristles on labellum, legs, and anterior wing margins are innervated by one mechanosensory and four chemosensory neurons (Ray et al., 1993; Lai and Orgogozo, 2004). Poxn-Gal4-13 UAS-GFP is expressed in clusters of four (sometimes of three or in only two, observed mainly in the labellum) chemosensory neurons per taste bristle on the legs, wing margins, and labellum (Boll and Noll, 2002). These Poxn-Gal4-expressing neurons are absent or, in a few cases, reduced to one neuron in Poxn null mutants (Boll and Noll, 2002). The cell lineage of taste bristles on the labellum has been analyzed previously by labeling replicating cells with BrdU (Ray et al., 1993). However, the proposed model is again similar to the m-es lineage, with a precursor for socket and shaft cells and another for the sheath cell and all neurons (Ray et al., 1993). Because of the technical limitations of BrdU incorporation and since no cellspecific markers were used in this earlier study, the cell lineage of taste bristles on the labellum may also need to be re-examined.

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#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.10.013.

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## Table S1

	clone types								
hours AEL	all cells	three support cells	four neurons	socket cell	shaft and sheath cell	bipolar neurons	bipolar and md neuron	others	
2-3	83	7	10	0	0	0	0	0	
3 – 4	42	24	31	0	0	0	0	0	
4 – 5	7	44	53	2	3	4	1	1	
5 - 6	0	10	13	10	41	33	30	6	

**Table S1.** Numbers of clones, marked after various time intervals by a 30-min heat shock at  $32^{\circ}$ C in the dorsal and lateral kölbchen lineages of *y* w hs-flp (homozygous or over *Y*); UAS>CD2 y<sup>+</sup>>CD8::GFP (homozygous or over *CyO*); Poxn-Gal4<sup>ups lf</sup> (homozygous or over *TM6B*) embryos. Clone types are indicated at the top and time intervals of heat shock on the left. Time intervals correspond to development at 25°C, but development before and after heat shock was at 18°C. The clones counted as 'others' are double clones induced in two different precursors of the lineage. No single-cell clones other than socket-cell clones were observed, presumably because these cells originated only after the heat-shock intervals used.

## Table S2

		clone types							
hours AEL	p6 cells and eg cells	eg cells	p6 cells	support cells	neurons	socket cell	shaft and sheath cell	two bipolar neurons	md neuron
2-3	94	10	16	0	0	0	0	0	0
3 – 4	56	23	28	0	0	0	0	0	0
4 – 5	4	33	32	2	1	0	0	0	0
5 - 6	0	n.d.	8	33	35	1	8	2	0
6 – 7	0	n.d.	0	8	6	16	49	36	15

**Table S2.** Numbers of clones induced after various time intervals by a 30-min heat shock at 32°C in the p6 and epidermal gland (eg) lineage of embryos. Clone types are indicated at the top and time intervals of heat shock on the left. Time intervals correspond to development at 25°C, but development before and after heat shock was at 18°C. n.d., not determined. Genotypes of embryos are *y* w hs-flp (homozygous or over *Y*); UAS>CD2 y<sup>+</sup>>CD8::GFP (homozygous or over CyO); Poxn-Gal4<sup>upslf</sup> (homozygous or over TM6B) and, in addition, *y* w hs-flp (homozygous or over *Y*); E7-2-36 UAS>CD2 y<sup>+</sup>>CD8::GFP (homozygous or over CyO); Poxn-Gal4<sup>upslf</sup> (homozygous or over TM6B) for clones induced after intervals of 5-6 and 6-7 hours AEL.

## Table S3

		clone types					
hours AEL	all cells	socket and shaft cell	sheath cell and neurons	socket cell	shaft cell	sheath cell and bipolar neuron	md neuron or md and bipolar neuron
3 – 4	37	11	15	0	0	0	0
4 – 5	11	20	28	0	0	0	0
5-6	2	12	13	6	4	9	9

**Table S3.** Numbers of clones marked after various time intervals by a 30-min heat shock at 32°C in the dorsal/lateral and ventral kölbchen lineage of *Poxn* null mutant embryos. Clone types are indicated at the top and time intervals of heat shock on the left. Time intervals correspond to development at 25°C, but development before and after heat shock was at 18°C. Genotypes of embryos are *y* w *hs-flp* (homozygous or over *Y*); *Poxn*<sup>ΔM22</sup> *UAS*>*CD2 y*<sup>+</sup>>*CD8*::*GFP*; *Poxn*-*Gal4*<sup>ups1f</sup> (homozygous or over *TM6B*).



**Fig. S1.** Clones of ventral kölbchen. (A-D) Clones of stage 16 embryos are shown that were generated at 3-4, 4-5, 5-6, or 6-7 hours AEL. Embryos were pooled and immunostained with antibodies directed against the proteins indicated in the corresponding colors below each panel. Genotypes of embryos are as described in the legend to Fig. 2. For a detailed description of clones, see text.



**Fig. S2.** Clones of hair 3 in the presence and absence of Poxn protein. (A-L) Clones of stage 16 embryos are shown that were generated at 3-4, 4-5, 5-6, or 6-7 hours AEL in the presence of wild-type Poxn protein (A-G) or in its absence (H-L). Embryos were pooled and immunostained with antibodies directed against the proteins indicated in the corresponding colors below each panel. The overlap of  $\beta$ -gal (red) with Elav (blue) is purple (D). Note that in (G) the more ventral bipolar neuron weakly expresses BarH1 (red), which is also expressed in the sheath cell of h3, adjacent and to the right of the clone (not labeled by GFP). There are several md neurons visible in (G), the most ventral of which, because of its position (D), is probably the one that belongs to h3. Genotypes of embryos are as described in the legend to Fig. 2 (in the presence of Poxn) and Fig. 5 (in the absence of Poxn). For a detailed description of clones, see text.



**Fig. S3.** No apoptosis in the wild-type lineage of hair 3. (A-D) None of the GFP-labeled cells of the wild-type h3 lineage stain for activated Casp-3 in stage 12 (A), stage 13 (B), stage 14 (C), and stage 15 (D) y w; UAS-CD8::GFP; Poxn-Gal4<sup>upslf</sup> (homozygous or over TM3) embryos. Note that in panel (A) the lineage shows only five cells, probably because the largest cell is still in the process of division.



**Fig. S4.** No apoptosis in the wild-type lineage of kölbchen. (A, B) None of the GFP-labeled kölbchen cells stain for activated Casp-3 in stage 13 (A) and stage 14 (B) y w; UAS-CD8::GFP; Poxn-Gal4<sup>ups1f</sup> (homozygous or over TM3) embryos. Asterisks indicate apoptotic cells in neighboring tissues that express activated Casp-3. (C) No supernumerary cells are observed among the CD2-labeled kölbchen cells when apoptosis is inhibited by the expression of P35 in a y w hs-flp/(w or Y); UAS>CD2 y<sup>+</sup>>CD8::GFP/UAS-p35; Poxn-Gal4<sup>ups1f/+</sup> embryo at stage 16.



**Fig. S5.** No apoptosis in the kölbchen lineage of Poxn null mutants. (A, B) None of the CD-2-labeled kölbchen cells stain for activated Casp-3 in stage 13 (A) and stage 14 (B) y w hs-flp;  $Poxn^{\Delta M22} UAS > CD2 y^+ > CD8$ .: *GFP*; Poxn-*Gal4<sup>upslf</sup>* (homozygous or over *TM6B*) embryos. Asterisks indicate apoptotic cells in neighboring tissues that express activated Casp-3. (C, D) No supernumerary cells are observed among the CD2-labeled kölbchen cells when apoptosis is inhibited by the expression of P35 in y w hs-flp;  $Poxn^{\Delta M22} UAS > CD2 y^+ > CD8$ .: *GFP*/ $Poxn^{\Delta M22} UAS > CD2 y^+ > CD8$ .: *GFP/Poxn^{\Delta M22} UAS > CD2 y^+ > CD8*.: *GFP/Poxn^{\Delta M22} UAS = P35*; *Poxn-Gal4<sup>upslf</sup>* (homozygous or over *TM6B*) embryos. The ratio of the number of lineages producing 5 (18; C) or 6 (8; D) cells remains the same as observed in embryos without P35 expression (Fig. 5A and B).