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# Spalt modifies EGFR-mediated induction of chordotonal precursors in the embryonic PNS of *Drosophila* promoting the development of oenocytes

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## SUMMARY

**Genes of the *spalt* family encode nuclear zinc finger proteins. In *Drosophila melanogaster*, they are necessary for the establishment of head/trunk identity, correct tracheal migration and patterning of the wing imaginal disc. Spalt proteins display a predominant pattern of expression in the nervous system, not only in *Drosophila* but also in species of fish, mouse, frog and human, suggesting an evolutionarily conserved role for these proteins in nervous system development. Here we show that Spalt works as a cell fate switch between two EGFR-induced cell types, the oenocytes and the precursors of the pentascolopodial organ in the embryonic peripheral nervous system. We show that removal of *spalt* increases the number of scolopodia, as a result of extra secondary recruitment of precursor cells at the expense of the oenocytes. In addition, the absence of *spalt* causes defects in the normal migration of the pentascolopodial organ. The dual function of *spalt* in the development of this organ, recruitment of precursors and migration, is reminiscent of its role in tracheal formation and of the role of a *spalt* homologue, *sem-4*, in the *Caenorhabditis elegans* nervous system.**

Key words: *Drosophila melanogaster*, *spalt*, Chordotonal organ, dEGFR, PNS, Cell migration, Oenocytes

## INTRODUCTION

The evolutionary conserved *spalt* genes encode for $C_2H_2$ zinc finger transcription factors involved in numerous developmental processes. In *Drosophila melanogaster*, two members of this family have been identified, *spalt* (*sal*) and *spalt-related* (*salr*), which are highly similar at the levels of sequence, regulation and function (Barrio et al., 1999; Barrio et al., 1996; Reuter et al., 1996). During tracheal development, *sal* represses tracheal placode formation, and ensures the correct migration and fusion of the dorsal tracheal trunk (Kühnlein and Schuh, 1996). In the wing imaginal disc, *sal* and *salr* are necessary for vein patterning in the pouch (de Celis and Barrio, 2000; de Celis et al., 1996; Lecuit et al., 1996; Nellen et al., 1996; Sturtevant et al., 1997), while in regions of the disc forming the thorax, they regulate bristle formation through the regulation of pro-neural gene expression (de Celis et al., 1999).

The conservation of their expression in nervous tissue of every species studied leads to the presumption that these proteins function in the development of the nervous system. Although the presence of *sal* members in the nervous system have also been shown in frogs, mice and fish (Buck et al., 2000; Hollemann et al., 1996; Köster et al., 1997; Ott and Schutz, 1996), their function in this tissue has only been studied in the nematode *C. elegans* and humans. In *C. elegans*, the *sal* homologue *sem-4* is required for correct development of neurons, vulva and mesoderm, including sensory organ cell lineages (Basson and Horvitz, 1996; Grant et al., 2000). Mutations in *sem-4* result in cell-fate transformations as well as failures in nuclear morphology, axonal outgrowth and cell migration. In humans, mutations in a *sal* homologue, *SALL1*, are associated with the Townes-Brock syndrome (Kohlhase et al., 1999b; Kohlhase et al., 1998). This genetic disorder leads to malformations in the anus and limbs, as well as sensoryneural hearing loss and mental retardation. In addition, the newly identified *SALL3* gene has been suggested to contribute to the 18q deletion syndrome (Kohlhase et al., 1999a), also characterised by malformations in the nervous system, mental retardation, hearing loss and facial and limb abnormalities.

In *Drosophila* nothing is known about whether these genes might function in the development of the embryonic sensory organs in the peripheral nervous system (PNS). The knowledge about the cell lineages, the existence of many molecular markers at the cellular level and known functional requirements for specific genes make the PNS an excellent choice for studying gene function during the development of the nervous system. We therefore chose to address the function of the *spalt* genes in the embryonic PNS. The *Drosophila* PNS comprises

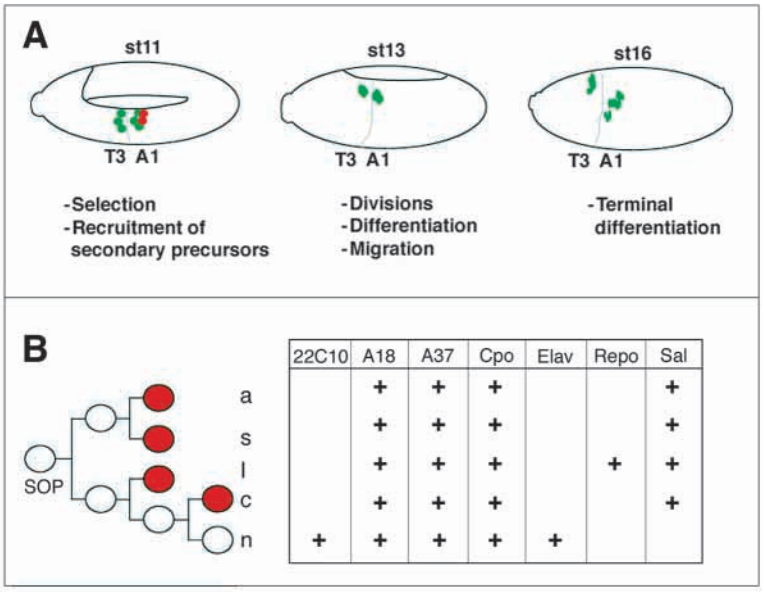


**Fig. 1.** Schematic representation of chordotonal organ development. (A) Development of the chordotonal organs dch3 (second and third thoracic segments, exemplified here by T3) and lch5 (abdominal segments A1-A7, exemplified by A1) in stage 11 to 16 embryos. At early stage 11, the three primary dch3 and lch5 SOPs are born in similar dorsoventral positions (green circles). EGFR signalling from the primary SOPs to the overlying ectoderm results in recruitment of two additional secondary SOPs only in abdominal segments (red circles). Soon after delamination, processes of cell division, migration and differentiation take place (shown in green). At stage 16, dch3 is located in a dorsal position with the dendrites pointing ventrally, while lch5 is located in a lateral position with dorsally pointing dendrites (see Fig. 3A). (B) Schematic representation of a SOP cell lineage that gives rise to one scolopodium (Brewster and Bodmer, 1995; Brewster and Bodmer, 1996). This consists of one neuron (n) and three support cells, the sheath (s), ligament (l) and cap (c) cells. Two of the five abdominal SOPs also give rise to one accessory cell (a). Cell-specific markers for the differentiated lch5 used in this study are shown. Note that *sal* is expressed in all the support and accessory cells of the SOP lineage (filled red circles), but not in the neuron. For additional markers in early steps of development see Fig. 5A.

| 22C10 | A18 | A37 | Cpo | Elav | Repo | Sal |
|---|---|---|---|---|---|---|
| | + | + | + | | | + |
| | + | + | + | | | + |
| | + | + | + | | + | + |
| | + | + | + | | | + |
| + | + | + | + | + | | |

approximately 600 neurons and 1200 associated cells, organised in a segment-specific pattern (Campos-Ortega and Hartenstein, 1997; Ghysen et al., 1986). Each abdominal hemisegment (A1-A7) contains 44 neurons organised in three clusters along the dorsoventral axis (ventral, lateral and dorsal). The PNS sensory organs can be divided into two types, external (es) and internal. The es organs are often mechanoreceptors in the cuticle. The internal receptors, called chordotonal organs (ch), are subepidermal stretch receptors consisting of one neuron (n), one ligament (l), one cap (c) and one sheath cell (s) belonging to the same cell lineage (Fig. 1B; Brewster and Bodmer, 1995; Brewster and Bodmer, 1996; Okabe and Okano, 1997). Eight chordotonal organs arise in each abdominal hemisegment. At a lateral position five associated scolopodia constitute a prominent compound structure, the pentascolopodial organ (lch5). In contrast, the corresponding organ in the thoracic segments consists of only three scolopodia (dch3) which, in this case, are located dorsally (Fig. 1A).

It was previously shown that the lch5 is generated in two distinct steps (Lage et al., 1997; Okabe and Okano, 1997). In the first step, the proneural gene *atonal* (*ato*) is required for the development of three primary sensory organ precursors (C1, C2 and C3; Fig. 1A, green circles; Jarman et al., 1994; Lage et al., 1997; Okabe and Okano, 1997). At the same time, the primary precursors for other chordotonal organs are also recruited (C4 for v′ch1 and C5 for vchA/B; Fig. 5A). In a second step, occurring only in abdominal segments, the primary SOPs express high levels of *rhomboid* (*rho*) and signal via *spitz* (*spi*) to the ectoderm through the EGFR receptor (dEGFR) to recruit secondary SOPs (Fig. 1A, red circles; Lage et al., 1997; Okabe and Okano, 1997; Price et al., 1989; Rutledge et al., 1992; Schejter and Shilo, 1989; Schweitzer et al., 1995). The ectodermal cells receiving the EGFR signal express several target genes including *pointed* (*pnt;* O'Neill et al., 1994), *argos* (*aos;* Freeman et al., 1992; Okano et al., 1992) and *sprouty* (*spry*; Kramer et al., 1999) but, interestingly, only two of these cells will become secondary SOPs for the lch5 and one of them for the vchA/B cluster. Conversely, in the thoracic segments the primary SOPs show a lower expression of *rhomboid* and do not signal at the same level to the ectoderm, thus secondary recruitment does not take place (Lage et al., 1997).

In this study we investigated the role of the *Drosophila sal* genes on lch5 development. While *salr* is not expressed in this organ, *sal* is present at two different stages of lch5 development. First, *sal* is expressed in a subset of the cells that surround the primary chordotonal SOPs and receive EGFR signalling. Later on, Sal appears in the support cells of the lch5, but not in the neurons. Through loss- and gain-of-function studies, we show that *sal* plays a dual role on lch5 development. On the one hand, it restricts the number of scolopodia to five per abdominal organ. On the other hand, it ensures the correct location of this chordotonal organ along the dorsoventral axis. Furthermore, through genetic analysis, we demonstrate that *sal* controls the number of lch5 scolopodia by restricting the capacity of EGFR-responsive ectodermal cells to become secondary SOPs. We present a model in which the extra scolopodia observed in the *sal* mutants develop at the expense of the neighbouring oenocytes.

## MATERIALS AND METHODS

### *Drosophila melanogaster* strains

Flies were raised on standard *Drosophila* medium at 25ºC. We used three *sal* alleles: $sal^{445}$, which expresses a truncated Spalt protein (Kühnlein et al., 1994), *DF(2L)32FP-5*, a small deletion which removes *sal* and *salr* (Barrio et al., 1999), and the hypomorphic allele *FCK-68*, which carries a translocation breakpoint between *sal* and *salr* and behaves as a *sal* hypomorph, probably because of the loss of regulatory regions (Barrio et al., 1999). The null alleles $spi^{1}$, $flbIK^{35}$ and $S^{IIN}$ were obtained from the Bloomington Stock Centre and $ato^{1}$

from A. Jarman (Jarman et al., 1994). The UAS-*sal* was described previously (de Celis et al., 1996). For misexpression experiments in the neuroectoderm, as well as early neuroblasts and SOPs, we used the driver-lines *scabrous*-GAL4 (*sca*-GAL4; Mlodzik et al., 1990) and *Krüppel*-GAL4 (*Kr*-GAL4; Castelli-Gair et al., 1994). For the misexpression experiments in the neural cells after delamination of SOPs we used the GAL4 driver-lines MZ1407-GAL4 (Sweeney et al., 1995), and *asense*-GAL4 (*ase*-GAL4; Hoch et al., 1994). The P[*lac,ry*$^{+}$]A18, and P[*lac,ry*$^{+}$]A37, referred to as A18 and A37 (Ghysen and O'Kane, 1989), label most cells, if not all, in the developing embryonic PNS. The RX-*drf-lacZ* insertion line reproduces *ventral veins lacking/drifter* (*vvl*/*drf*) pattern of expression in the oenocytes (Anderson et al., 1995). Information about strains not described in the text and balancer chromosomes have been described previously (Lindsley and Zimm, 1992).

### Immunohistochemistry

Immunohistochemistry was performed in whole-mount embryos using the following primary antibodies: anti-Sal rat and rabbit antisera (1:1000 and 1:250 dilution, respectively; de Celis et al., 1999); anti-Salr rat antiserum (1:250 dilution; Barrio et al., 1996); anti-Ato rabbit antiserum (1:1000 dilution; Jarman et al., 1994); rabbit anti-β-galactosidase (β-Gal) antiserum (1:5000 dilution; Cappel); anti-Couch potato (Cpo) rabbit antiserum (1:4000 dilution; Bellen et al., 1992); anti-dpERK (activated MAPK/Rolled) mouse monoclonal antibody (1:500 dilution; SIGMA; Gabay et al., 1997); anti-Reversed polarity (Repo) rabbit antiserum (1:300 dilution; Halter et al., 1995); anti-Embryonic lethal abnormal vision (Elav) monoclonal antibodies (9F8A9, 1:1000 dilution; (O'Neill et al., 1994) and 22C10 monoclonal antibody (1:20 dilution; Fujita et al., 1982) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Fluorescent Cy2- and Cy3-conjugated secondary antibodies (Jackson Immunoresearch) were used at 1:1000 dilution. HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies (Promega) were used at 1:250.

Embryos were staged according to Campos-Ortega and Hartenstein (Campos-Ortega and Hartenstein, 1997) and were fixed and processed for whole-mount antibody staining using standard techniques (Patel, 1994). Stained embryos were cleared in 80% glycerol, mounted and examined on a Zeiss Axiophot. Alternatively, fluorescent embryos were analysed by confocal microscopy using a Zeiss LSM 510 microscope.

### Electron microscopy

*Df(2L)32FP-5/sal*$^{445}$ and wild-type embryos were fixed in a double aldehyde mixture (2.5% glutaraldehyde, 4% paraformaldehyde) prepared in 0.1 M sodium cacodylate buffer, pH 7.4. The embryos were first dechorionated by hand and mounted in halocarbon oil on a microscope slide for determination of genotype and developmental stage under fluorescence and Nomarski optics. Embryos were fixed individually once they reached early stage 16. The halocarbon oil was cleaned off by gently rolling the embryo over a clean microscope slide and the fixation started by moving the embryo into a drop of fixative (50 μl). After 5 minutes, the embryo was moved out of the fixative, the vitelline membrane was ruptured with a needle and the embryo placed into 2 ml of ice-cold, fresh fixative for 5 hours. The aldehydes were washed out with a few changes in the same buffer before post-fixing for 1 hour in ice-cold 2% osmium tetroxide dissolved in distilled water. The embryos were then dehydrated in an ethanol series, embedded in EPON and polymerized for 48 hours at 60°C according to standard routines. Semithin sections in the transverse plane were cut at 2 μm and stained with boracic Toluidine Blue for localization of abdominal chordotonal organs. Ultrathin sections ('silver') were contrasted by serial incubation in lead citrate, uranyl acetate and lead citrate, and mounted on copper grids for examination on a JEOL 100 CX electron microscope operated at 60 mV. Serial sections of three different abdominal segments were examined in 4 embryos of each genotype.

## RESULTS

### Spalt and Spalt-related are expressed in a partially overlapping pattern during embryonic peripheral nervous system development

In order to understand the role of *sal* and *salr* during PNS development, we carried out a detailed analysis of their expression pattern in the trunk region during embryonic stages. We performed double immunostaining using anti-Sal antibodies together with different markers for the developing PNS in stage 16 embryos. To identify neuronal cells, we used the monoclonal antibody 22C10 that labels all PNS neurons (Fujita et al., 1982; Hummel et al., 2000; Roos et al., 2000). We also used antibodies specific for Elav, an RNA binding protein located in the nuclei of all neuronal cells (O'Neill et al., 1994). Finally, we used the A18 and A37 *lacZ* insertion lines, as well as anti-Cpo antibodies which are all markers for most if not all PNS cells (Bellen et al., 1992; Ghysen and O'Kane, 1989).

Sal-positive cells are located in the three abdominal PNS clusters, ventral, lateral and dorsal (Fig. 2A; nomenclature according to Brewster and Bodmer, 1995). In the lateral cluster *sal* is expressed in the sheath cell of the single chordotonal organ, v′ch1 (s′; Fig. 2C) as well as all the pentascolopodial support cells (s, l, c), but not the pentascolopodial neuron (n; Fig. 2B,D). Moreover, *sal* is also expressed in the two accessory cells associated with lch5 (a; Fig. 2C; Brewster and Bodmer, 1996; Ghysen and O'Kane, 1989). In the ventral cluster we identified neurons v′esA and v′esB as Sal positive (Fig. 2E,F), two unidentified cells in close proximity (u, Fig. 2E,F), as well as the sheath cells of the vchA and vchB chordotonal organs (data not shown). In the dorsal cluster *sal* is expressed in the dorsal bipolar neuron (dbp) and its associated glia (PG3), as well as another unidentified neuron (dn, Fig. 2B).

In addition to the PNS, other cells in the region stain prominently with anti-Sal antibodies. These cells are the oenocytes (oe), which are situated between the epidermis and lch5 in late embryos (Fig. 2A-F; Barrio et al., 1996; Kühnlein et al., 1994). Little is known about the development of these putative nephrocytes, except that they are located exclusively in abdominal segments and are of ectodermal origin. The analysis of a number of *lacZ* lines show that oenocytes originate in the epidermis of stage 11 embryos (Hartenstein et al., 1992).

Using the expression pattern of *sal* as reference, the expression of *salr* was analysed by in situ hybridisation (results not shown) and double immunostaining using anti-Sal and anti-Salr specific antisera (Fig. 2D-F). Salr was first detected at stage 13 in the oenocytes, where it colocalises with Sal and at stage 14 in some ventral cells. These are likely to be v′esA and v′esB since they are positive for Sal and Salr later in development. At stage 16, Salr is expressed in the oenocytes, the dbp neuron, v′esA and v′esB, but it is absent from other PNS organs in the abdomen (Fig. 2D-F). In summary, Sal and Salr are expressed in a partially overlapping pattern in the PNS. However, Sal and not Salr is expressed in distinct support cells of lch5, indicating that *salr* may not play an important role in the development of this organ.

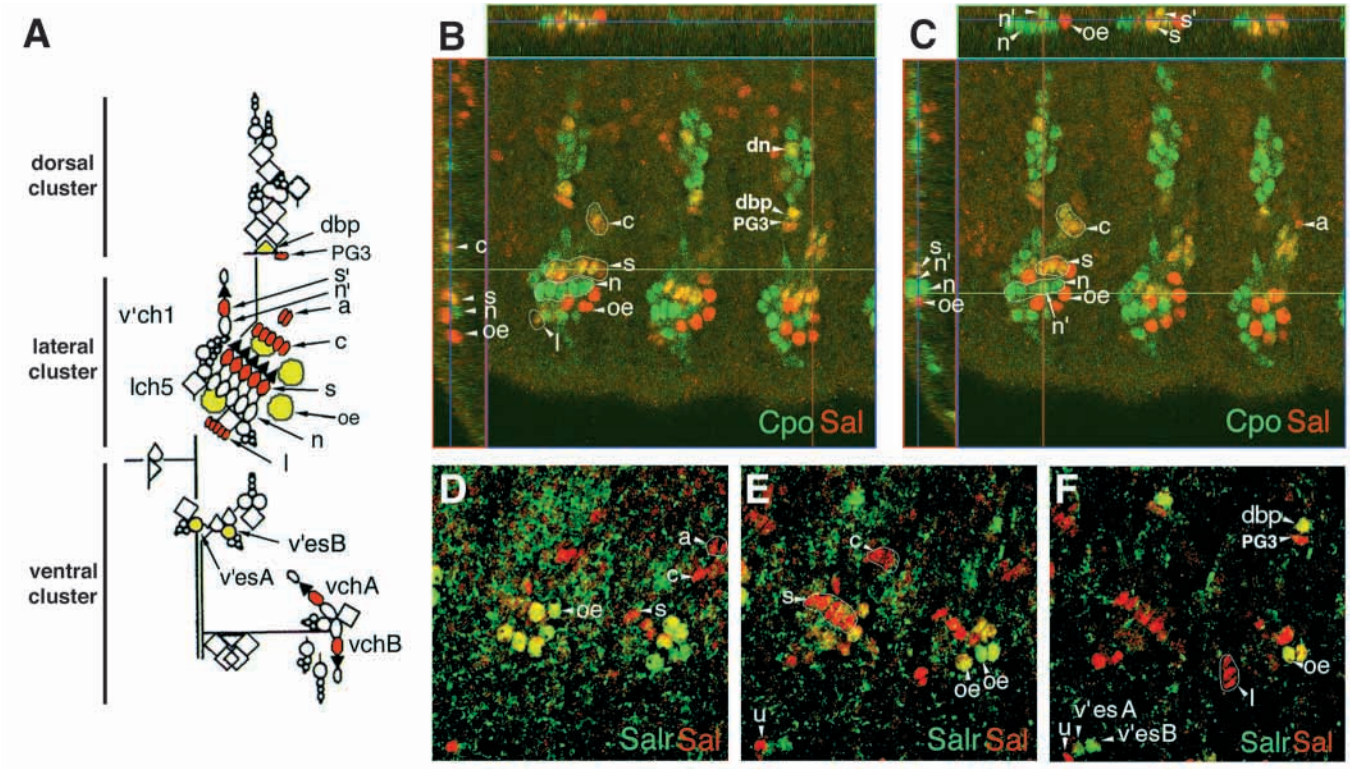


**Fig. 2.** *spalt* and *spalt related* are expressed in a subset of peripheral neurons and support cells in the embryo. (A) Summary of the expression pattern of Sal (red) or Sal and Salr (yellow). The cartoon represents one abdominal segment of stage a 16 embryo (adapted from Brewster and Bodmer, 1995). All the cells forming the ventral, lateral and dorsal clusters are depicted. In the dorsal cluster, the dorsal bipolar neuron (dbp) is positive for both Sal and Salr, while its associated glial cell (PG3) is only positive for Sal. In the lateral cluster, *sal* is expressed in two organs, v′ch1 and lch5. In vch′1 it appears only in the sheath cell (s′) but not in the neuron (n′). In lch5, Sal is detected in all the support cells: ligament (l), sheath (s), cap (c) and accessory epidermal cells (a) but, as in vch′1, not in the neurons (n). In close proximity to lch5, the oenocyte cells (oe) are positive for both factors. In the ventral cluster, the neurons of the v′esA and v′esB are Sal and Salr positive, and the sheath cells of the vchA and vchB organs are positive for Sal. (B-F) Single confocal sections of stage 16 embryos (anterior to the left and dorsal up) showing double immunostaining using anti-Sal (red) and either anti-Cpo (green in B and C) or anti-Salr antibodies (green in D-F) antibodies. Abbreviations are as in A. (B,C) Two consecutive single confocal sections show Cpo expression, which highlights all cells in the PNS. Stacked confocal sections were combined at the green and red lines generating orthogonal sections that are displayed at the top (green) and left (red) of each panel, respectively. The Sal-, but not Cpo-expressing cells (red) are oenocytes. Some clusters of neurons and support cells are outlined. In the dorsal cell cluster *sal* is also expressed in one unidentified dorsal neuron (dn). (D-F) Three consecutive single confocal sections show that Sal and Salr overlap in the dpb neuron and the oenocytes (yellow). Salr is not detected in the lch5. According to the relative positions shown by double staining with anti-Elav, 22C10 and anti-Cpo antibodies (data not shown) the cells in the ventral position are v′esA and v′esB, with 2 closely associated Sal-positive unidentified cells (u). In the latter, Salr is expressed at a significantly higher level than Sal.

## Loss of *spalt* increases the number of scolopodia in the lch5 organ

To investigate the role of *sal* in the development of the PNS, we performed immunostainings using the monoclonal antibody 22C10 (Fujita et al., 1982) in *Df(2L)32FP-5/Df(2L)32FP-5*, *FCK-68/Df(2L)32FP-5*, and *Df(2L)32FP-5/sal*$^{445}$ allelic combinations. These mutants show obvious malformations and changes in number of neurons in the lch5 organ. In wild-type embryos the lch5 scolopodia appear in a lateral position and are organised in an array with prominent dorsally pointing dendrites (Fig. 3A). In all mutant combinations analysed, the lch5 organs are mostly in a dorsal position (approx. 70% of the cases) and appear disorganised with ventrally pointing dendrites (Fig. 3B,C). Furthermore, the lch5 organ frequently has 1 to 3 supernumerary neurons (Fig. 3C,E) per hemisegment (Table 1). In the dorsal cluster the dbp neuron presents abnormally directed and shortened axonal projections (results not shown), but this could be a secondary consequence of other unrelated malformations seen in the mutants (e.g. failure of tracheal fusion). Otherwise, the dorsal and ventral clusters appear normal.

The increase in number of neurons could in theory be the result of cell fate transformation at the expense of support cells (such as ligament or cap cells), within the chordotonal lineage (Brewster and Bodmer, 1995; Brewster and Bodmer, 1996). Such a phenotype has been shown in *glial cells missing* (*gcm*) mutants, where the ligament cells are transformed into neurons (Jones et al., 1995), and in *numb* overexpression backgrounds where sheath cells are transformed into neurons (Chien et al., 1998). In the case of *sal* mutants, one of the support cells (normally expressing *sal*) could be transformed into one neuron (normally lacking *sal*). Therefore, we closely examined possible cell fate changes within the SOP lineage by electron microscopy, given the fact that each cell type of the scolopodium has characteristic microstructures (Carlson et al., 1997a; Carlson et al., 1997b; Hartenstein, 1988). In all the

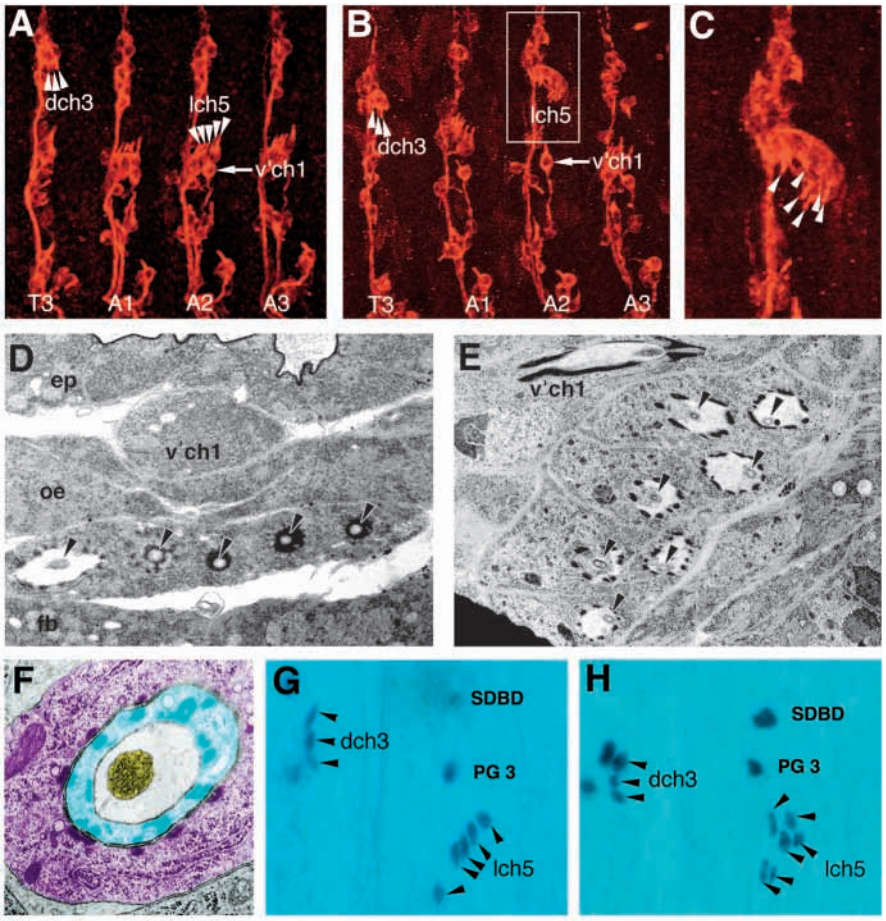


**Fig. 3.** Loss of *spalt* leads to supernumerary scolopodia in the sensory organ lch5. Immunostaining (A-C,G,H) and electron microscopy (D-F) analysis of the embryonic PNS in wild-type embryos (A,D,G) and transheterozygous *DF(2L)32FP-5/sal*$^{445}$ embryos (B,C,E,F,H). (A,B) Lateral view of stage 16 embryos with the neuronal marker 22C10 evident in segments T3-A3. Arrowheads point to the dendrites of the dorsally located dch3 organ in the thorax and of the laterally located abdominal lch5. The arrows indicate the v´ch1 organ in one abdominal segment. The lch5 of the *sal* mutant shows misplacement and supernumerary neurons, while thoracic dch3 does not show any detectable abnormality. (C) Enlargement of one mutant lch5 organ, boxed in B. Arrowheads indicate six ventrally pointing dendrites. (D-F) Electron microscopy analysis of abdominal segments reveals supernumerary and irregularly arranged lch5 scolopodia in stage 16 *sal* mutant embryos. The arrowheads indicate the ciliary dendrites. The five scolopodia of a wild-type embryo (D) are arranged in a linear array separated from the epidermis (ep) by the v′ch1 organ and the oenocytes (oe), and exterior to the mesodermally derived fat body (fb). In E, a mutant embryo shows up to seven irregularly arranged scolopodia (arrowheads). Each scolopodium presents correctly differentiated sheath and cap cells, distinguishable by their characteristic ultrastructural composition. (F) Ultrastructure of a chordotonal organ sectioned at the level of the dendrite. The color code indicates the different cell types; yellow marks the neuronal dendrite with its central cilium, blue the sheath cell, and purple the cap cell. (G,H) Lateral views of T3-A2 segments of stage 13 embryos showing staining for Repo in the ligament cells (arrowheads) of the dch3 and lch5 organs. Other Repo-positive glial cells (SDBD and PG3) are also detectable. In H, an increased number of ligament cells is shown in the mutant abdominal lch5, while the thoracic dch3 does not show differences.

cases examined, together with supernumerary neurons we could identify associated supernumerary sheath and cap cells (Fig. 3E). Moreover, we never observed more than one neuron inserted into a single sheath cell, as is the case in *gcm* mutants. Furthermore, all the cells in each scolopodium have normal ultrastructure, indicating that even the supernumerary scolopodia are fully differentiated (Fig. 3F).

The number of ligament cells is difficult to score by EM analysis at late stages of embryonic development. These cells, considered as glial cells, are located at different dorsoventral levels and their nuclei appear distant from the rest of the cells in the organ. Therefore, we performed immunostaining in stage 13 embryos using antibodies against the glial marker Repo (Campbell et al., 1994; Halter et al., 1995; Xiong et al., 1994). At this stage, all the cells of the chordotonal organs are already formed and are differentiating (Carlson, 1997; Carlson et al., 1997; Hartenstein, 1988), so they can be detected before peripheral and exit glial cells migrate out from the CNS and populate the PNS (Halter et al., 1995). In the *sal* mutant backgrounds we observed supernumerary glial cells in the position where the ligament cells are expected to be (Fig. 3H). These cells are clustered, supporting the idea that they are bona fide ligament cells. Furthermore, we never observed fewer than 5 ligament cells, excluding the possibility that supernumerary neurons derive from transformed ligament cells. Indeed, we frequently found clusters of 6 ligament cells (36 out of 96 hemisegments observed) or even 7 (8 out of 96), with an average of 5.4 ligament cells per cluster (Table 2).

**Table 1. Peripheral nervous system defects in various genetic backgrounds in late embryos**

| Genotype | Dorsalised lch5 | No. of neurons* | No. of hemisegments‡ |
|---|---|---|---|
| *sal*$^{445}$*/Df(2L)32FP-5* | + | 6.4±0.13 | 58 (6) |
| *sca*-Gal4;UAS-*sal*§ | – | 3±0.04 | 25 (2) |
| *kr*-Gal4;UAS-*sal*¶ | – | 2.9±0.11 | 9 (3) |
| *sal*$^{445}$*;S* | + | 3.05±0.20 | 76 (6) |
| *sal*$^{445}$*;spi* | + | 3±0.08 | 15 (4) |
| *aop*‡‡ | – | 5.3±0.10 | 42 (4) |
| *S*‡‡ | – | 3.04±0.2 | 76 (9) |
| *spi*‡ | – | 3.04±0.04 | 23 (2) |
| *dEGFR*‡‡ | – | 3.03±0.03 | 36 (3) |

*Number of neurons per lch5 organ and standard error.

‡Number of hemisegments analysed. Between brackets, number of embryos analysed.

§Other lateral and ventral clusters of neurons were affected. Sporadic mislocation of lch5 was observed (3 cases). v′ch1 is sometimes eliminated. dbp axons fail to extend anterior-posterior axonal projections.

¶Only abdominal segments A1-A3 were scored. Occasional loss of ventral and lateral neurons was observed.

‡‡Homozygous mutant embryos analysed.

**Table 2. Number of ligament cells in wild type and *sal* mutant embryos at stage 13 of development**

| Genotype | No. of ligament cells* | No. of hemisegments‡ |
|---|---|---|
| WT | 4.8±0.06 | 42 (3) |
| *Df(2L)32FP-5/Df(2L)32FP-5* | 5.5±0.12 | 35 (3) |
| *Df(2L)32FP-5/sal$^{445}$* | 5.4±0.11 | 61 (5) |

*Number of ligament cells per lch5 organ and standard error.
‡Number of hemisegments analysed. The number of embryos analysed is given in brackets.

Since we observed extra sheath, cap and ligament cells, rather than only extra neurons in each scolopodium, it seems unlikely that the supernumerary neurons represent a transformation within the SOP cell lineage. Furthermore, the extra scolopodia formed do not seem to result from fate transformations of neighbouring external sensory organs into chordotonal organs, as is known for *cut* mutant embryos (Blochlinger et al., 1990), since all sensory cells neighbouring the lch5 are still present in the *sal* mutants (data not shown). Consequently, it is plausible that the number of SOPs increases at earlier stages of development giving rise to a higher number of scolopodia. This phenomenon occurs only in abdominal segments while, in the thoracic ones, no defects of migration or number of cells was observed.

## Overexpression of Spalt causes a reduction in the number of scolopodia in the lch5 organ

The analysis of *sal* mutants revealed that *sal* restricts the number of developing scolopodia in the lch5. Thus, *sal* could inhibit neuronal development in the PNS as has been reported for the sensory precursor cells in the notum of the wing imaginal disc (de Celis et al., 1999). To further analyse this possibility, we misexpressed *sal* during the development of the lch5 organ using the UAS/GAL4 system (Brand and Perrimon, 1993). For this purpose, we analysed the β-Gal expression domains of four GAL4 drivers in combination with the UAS-*lacZ* line. Two of these drivers, *sca*-GAL4 and *Kr*-GAL4, direct early stage expression of β-Gal in the neuroectoderm, before the SOPs are singled out from the proneural clusters (PNC). This is the time when the definition of which ectodermal cells will become neuronal precursors is taking place. While *sca*-GAL4 drives β-Gal expression in the whole trunk, *Kr*-GAL4 does so only in segments T2-A3. The other two drivers, MZ1407-GAL4 and *ase*-GAL4 drive expression at later stages of development in the neural precursors at stages 11 and 12 and in the PNS neurons at later stages of development, when the SOPs are already recruited and have started their differentiation.

The effects of *sal* overexpression were monitored in allelic combinations of UAS-*sal* (de Celis et al., 1996) with the mentioned four GAL4 lines in stage 16 embryos, using the 22C10 antibody. Overexpression of *sal* in the ectoderm, as well as in the SOP lineage, using *Kr*-GAL4 and *sca*-GAL4 drivers reduces the number of neurons in the lch5 organ from five to three (Fig. 4E,F). With the *Kr*-GAL4 driver the phenotype is observed solely in segments A1-A3. By contrast, the ectopic expression of *sal* in the neuronal progenitors using MZ1407-GAL4 and *ase*-GAL4 drivers does not produce detectable defects in the PNS. No effects on lch5 cell numbers were observed in flies that carried the GAL4 drivers or the UAS-*sal* constructs alone. Interestingly, the reduction from five to three neurons is also reported to occur when EGFR signalling is compromised, as in *spi* mutants (Fig. 4D; Bier et al., 1990; Lage et al., 1997; Okabe and Okano, 1997).

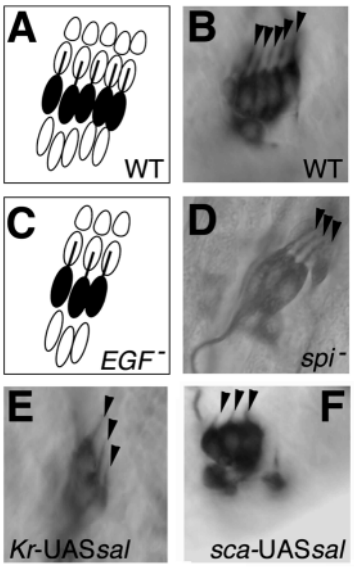


**Fig. 4.** *spalt* overexpression induces reduction of scolopodia number in the lch5 organ. (A,C) Representation of the scolopodia of one lch5 organ in a wild-type (A) and in a EGFR pathway mutant (C) stage 16 embryo. Neurons are drawn in black. When EGFR signalling is perturbed, the number of scolopodia is reduced to three. B,D,E,F. Close ups of an lch5 organ stained with 22C10 in stage 16 embryos. Arrowheads indicate the dendrites. While in the wild-type five dendrites are observed (B), in *spi* mutants, where the EGFR signalling is compromised, there is a reduction to three neurons per organ (D). The same effect is observed when *sal* is ectopically expressed in the neuroectoderm and early SOPs using the *Kr*-GAL4 (E) or the *sca*-GAL4 drivers (F).

The ectopic presence of Sal at early stages of development inhibits the formation of two scolopodia. However, after all the SOPs are recruited from the neuroectoderm, the ectopic expression of *sal* does not seem to interfere with the normal developmental pathways. This suggests that *sal* could interfere with the secondary recruitment of SOPs dependent on EGFR signalling.

## Spalt is expressed in early embryonic stages when the recruitment of sensory precursors takes place

The secondary recruitment of SOPs takes place at early stages of development and depends on EGFR signalling (Lage et al., 1997; Okabe and Okano, 1997). To test whether *sal* works at the level of secondary recruitment of SOPs, it was first necessary to analyse the expression of *sal* in relation to the pattern of a number of proteins involved in the development of lch5 during the early development/recruitment phase (Fig. 5).

At early stage 11, Sal is expressed in the dorsal ectoderm (Fig. 5). Using Ato as a marker for primary SOPs, we found that the most dorsal lch5 precursor, C1, delaminates from within the Sal-positive area and coexpresses Ato and Sal at this stage (Fig. 5B). Slightly later in development both Ato and Sal disappear from C1 (Fig. 5C), although this precursor can still be detected using anti-Cpo antibodies (Fig. 5D). We never observed coexpression of Ato and Sal in any other more ventrally located primary SOPs, (C2-C5) of the chordotonal organs, all believed to originate from the PNC ventral to the C1 precursor (Fig. 5B; Lage et al., 1997).

The DER signalling pathway involves the activation of the Rolled protein (Rl/Map kinase). We therefore used a monoclonal antibody against activated Rl (Gabay et al., 1997) to visualise candidate secondary SOPs. The expression pattern

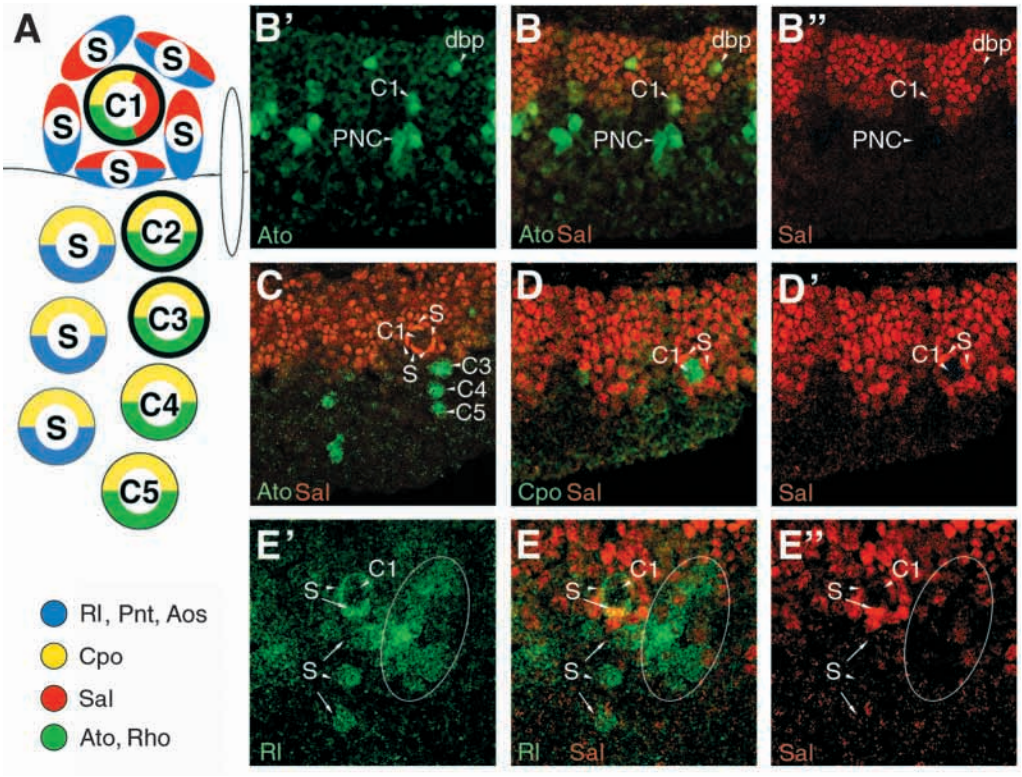


**Fig. 5.** *spalt* is expressed in the ectoderm during early lch5 development. A. The cartoon summarises the expression pattern of various markers in the different cell types during early lch5 development. C1-C5 are the primary chordotonal sensory organ precursors (Lage et al., 1997; Okabe and Okano, 1997), where C1-C3 (thick black outline) are thought to contribute to lch5, and C4 and C5 are thought to contribute to v′ch1 and one of the ventral vchA or vchB organs (Lage et al., 1997). The primary SOPs express proteins such as Ato, Rho and Cpo. The putative secondary precursor cells (S) transduce signals and express the target genes *pnt* and *aos*. Two of these cells will be recruited to form the lch5 and one to the vchA/B organs. The expression of *sal* is localised in the primary precursor C1 and in the cells that surround it (red). The horizontal line indicates the limit of *sal*-expressing cells in the ectoderm and the oval ring the tracheal pit. (B-E) Confocal sections stained with color-coded antibodies. B′, D′, E′ and B″, D″, E″ are single channel images of the same section. (B) Confocal sections of two consecutive segments in early stage 11 embryos showing the expression pattern of Ato (green). Ato is restricted to the C1 precursor of the lch5 and the SOP of the dbp, while it is still broadly expressed in the proneural cluster (PNC) that gives rise to the other SOPs of the chordotonal organs (C2-C5, compare with Fig. 2a in Lage et al., 1997). The C1 precursor delaminates within a Sal-positive ectodermal area (red), while the PNC arises ventral to the Sal domain. (C) Later on, at late stage 11, both Sal and Ato disappear from the C1 lineage (Lage et al., 1997). Ato is restricted to other chordotonal precursors (C3-C5, compare with Fig. 2b in Lage et al., 1997) where *sal* is not expressed. (D) A different late stage 11 embryo showing the C1 precursor expressing Cpo. Although Sal is not present in the C1 precursor, it is strongly expressed in the cells surrounding it, which have elongated nuclei. (E) Putative secondary chordotonal organ precursors showing active EGFR signalling visualised with anti-Rl antibodies (green). Sal and activated Rl coincide in the cells with elongated nuclei surrounding C1 (S). Activated Rl also labels three putative chordotonal precursors (S) ventral to the Sal domain, seemingly overlying the position of the primary signalling SOPs, as well as the tracheal pits (outlined).

of activated Rl corresponds well to the patterns reported for *aos* and *pnt* (Lage et al., 1997; Okabe and Okano, 1997; Okabe et al., 1996) and appears around the tracheal pits, in cells surrounding C1, and in cells overlying the more ventral C2-C5 SOPs (Fig. 5A,E). Only a ring of 4-5 cells with elongated nuclei around the C1 precursor coexpress Sal and activated Rl. Interestingly, Rl-positive cells in the ectoderm not expressing Sal overly the SOPs, C2-C5, and their number corresponds to the number of secondary SOPs recruited to the abdominal chordotonal organs (two for the lch5 and one for the vchA/B cluster; Fig. 5E). Later in development, Sal-positive cells surrounding C1 migrate ventrally and end up in a lateral position close to the lch5, strongly suggesting that they are the developing oenocytes. Later on, Sal expression can be detected at stage 13 in the lch5 lineage, at a time when all cell divisions have already occurred and differentiation and migration of the lch5 has initiated (Carlson et al., 1997a; Carlson et al., 1997b; Hartenstein, 1988). At stage 13, four additional Sal-positive cells in the lateral region are likely to include the v′esA and B neurons since these are Sal-positive in stage 16 embryos (Fig. 2D-F). In addition to the C1 precursor of lch5, some other SOPs were identified as Sal positive. These include the SOP of the dorsal bipolar neuron (Fig. 5B) and a precursor not identified (data not shown), which probably corresponds to the SOP of the Sal-positive neuron in the dorsal cluster of late embryos (dn in Fig. 2B). At stage 16 of embryogenesis the epidermal expression of Sal fades away.

Since EGFR signalling is involved in recruitment of secondary SOPs, an excess of potential secondary SOPs exist (Fig. 5A). We have shown that loss of *sal* generates more SOPs, and that among the candidates only those that surround the C1 express Sal. This leads to the hypothesis that the candidates overlying the C2-C5 and not expressing Sal, are the actual precursors of secondary SOPs. To prove this hypothesis it is necessary to show that the extra SOPs generated by loss of *sal* are dependent on EGFR signalling, and thus represent secondary SOPs.

### The supernumerary scolopodia observed in *spalt* mutants are dependent on EGFR signalling

The PNS phenotype displayed by *sal* mutants is reminiscent of that of the increased EGFR signalling observed in *aos* mutants (Freeman et al., 1992; Kretzschmar et al., 1992; Okano et al., 1992), *anterior open* (*aop*; Lai and Rubin,

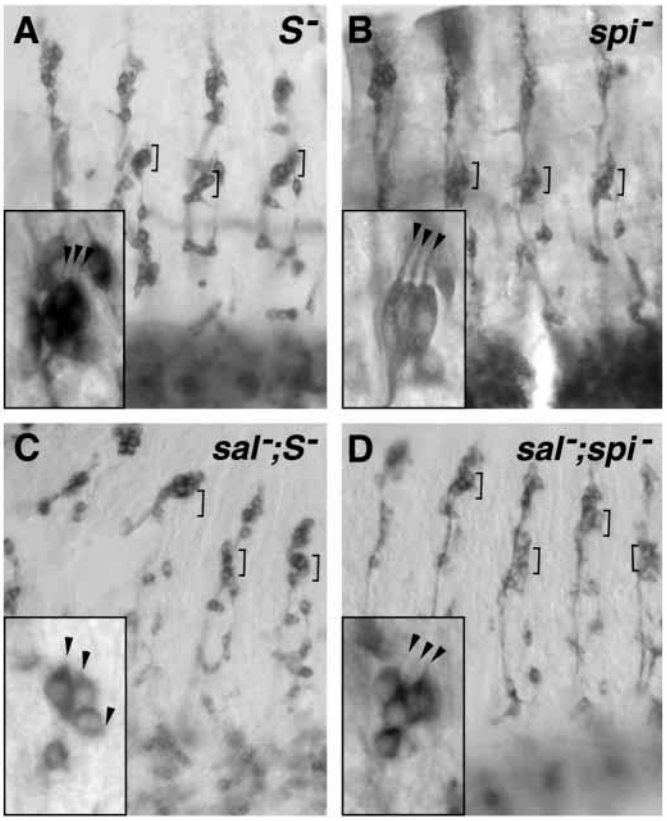


**Fig. 6.** *spalt* restricts EGFR signalling dependent recruitment of secondary sensory organ precursors. (A-D) Immunostaining using the neuronal marker 22C10 showing one thoracic and several abdominal segments of stage 16 embryos. Single homozygous mutants for *S* and *spi* null alleles of the EGFR signalling pathway (A,B) or combinations of those with $sal^{445}$ (C,D) are shown. For comparison with wild type or $sal^{445}$ single mutant embryos see Fig. 3. Embryos with mutations in the EGFR pathway show reduction from five to three neurons (arrowheads), resulting from failure of secondary SOPs recruitment (inserts in A,B). The same is true for the double mutant embryos in the combinations *sal*;*S* or *sal*;*spi* (inserts in C,D). However, while in the single EGFR mutants the lateral location of lch5 is not affected (brackets), this organ is retained dorsally in the double mutants, as observed in *sal* mutations (Fig. 3).

1992), *GTPase-activating protein 1* (*gap1*; Gaul et al., 1992), or *sprouty* (*spry;* Kramer et al., 1999). Conversely, loss of positive regulators of EGFR signalling such as *rho* (Bier et al., 1990), *S* (Star; Kolodkin et al., 1994), *spi* (Rutledge et al., 1992; Schweitzer et al., 1995), *EGFR* (Nüsslein-Volhard et al., 1984; Price et al., 1989; Schejter et al., 1989), *son of sevenless* (*sos*; Rogge et al., 1991), *pnt* (O'Neill et al., 1994), or *ras1* (Simon et al., 1991), results in reduction of scolopodia in the lch5 organ (Lage et al., 1997; Okabe and Okano, 1997; Okabe et al., 1996). Our results suggest that *sal* interferes with the secondary recruitment of SOPs from the cells surrounding the C1 primary precursor, probably preventing their response to the EGFR signalling to form secondary SOPs. In order to test this hypothesis, we performed an analysis of *sal;S* and *sal;spi* double mutant embryos. Two possible scenarios were envisaged. (i) *sal* restricts the number of EGFR-recruited secondary SOPs. In this case, inhibition of the EGFR pathway in the *sal* mutant background would produce three scolopodia. (ii) Alternatively, *sal* restricts the number of primary SOPs for the lch5. In this case double mutants where EGFR signalling is perturbed in the *sal* mutant background would produce more than 3 neurons or scolopodia in the lch5 organ.

As shown in Table 1, while the *sal* mutant embryos have an average of 6.4 neurons in the lch5 organ as scored by 22C10, *sal;spi* and *sal;S* double mutants have 3 and 3.05 neurons, respectively (Table 1; Fig. 6C,D). The double mutant values are similar to the ones obtained when single *S* (3.04), *spi* (3.04) or dEGFR (3.03) mutants were analysed (Table 1; Fig. 6A,B). Thus, this experiment demonstrates that the supernumerary precursors observed in the *sal* mutants require EGFR signalling and therefore are likely to represent supernumerary secondary SOPs. The *sal;spi* and *sal;S* double mutant embryos show misplacement of the lch5 organ along the dorsoventral axis comparable to the phenotype observed in single *sal* mutants, but the EGFR pathway mutants do not show dorsalisation of the organ (Table 1; Fig. 6A,B). It appears that secondary recruitment and migration of the organ are two distinct tasks that differ in terms of genetic regulation.

### The supernumerary scolopodia in *spalt* mutants develop at the expense of the oenocytes

Given the possibility that the Sal-positive cells surrounding C1 are the developing oenocytes, we hypothesised that the extra scolopodia observed in *sal* mutants would develop at the expense of these cells. To test this, we used the *dfr-lacZ* insertion line as a marker for oenocytes (Anderson et al., 1995). Double staining using anti-Spalt and anti-β-Gal antibodies confirmed the colocalisation of the two proteins in the cells surrounding the C1 precursor (Fig. 7A). Later in development these cells migrate ventrally in close association with the lch5 organ (Fig. 7B) and are finally located in the lateral position between the lch5 and the epidermis (Fig. 7C,G). This strongly suggests that the C1-surrounding cells are indeed the oenocytes. In concordance with our hypothesis, the cells surrounding the C1 precursors disappear in the *sal* mutants (Fig. 7D). At later stages of development, while the heterozygous *Df(2L)32FP-5/(drf-lacZ*) embryos and embryos only carrying the *dfr-lacZ* insertion have 5.9 ($n$=53) and 5.8 ($n$=53) oenocytes per hemisegment, respectively, *Df(2L)32FP-5,(drf-lacZ)/*$sal^{445}$ mutant embryos have an average of 0.4 oenocytes per hemisegment ($n$=84; Fig. 7G,H).

In analogy with the developing lch5, we then hypothesised that the oenocytes require EGFR signalling for proper development. We therefore analysed embryos mutants for *S* and *spi* at different stages of development. Interestingly, in stage 11 embryos the *sal* pattern of expression remains unaltered in the cells surrounding the C1 precursor, as well as in the epidermis (data not shown). However, later on, the development of the oenocytes is inhibited (Fig. 7E,F). These results indicate that *sal* regulation is independent of the EGFR pathway and that the oenocytes development depends on both *sal* and EGFR signalling activity.

Furthermore, if the signalling arises from the precursor C1, the formation of oenocytes would be restrained in the absence of SOPs. Indeed, in *ato* mutant embryos oenocytes originate only in the segments where remnant SOPs develop (Fig. 7I).

In conclusion, our results are consistent with a model where

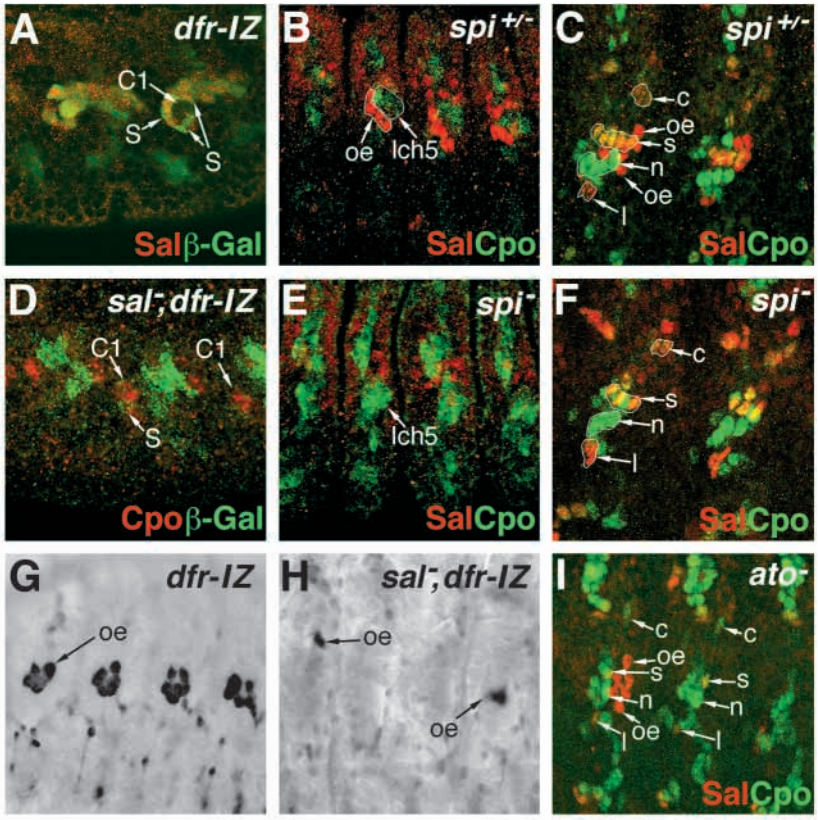


**Fig. 7.** The *spalt*-expressing cells surrounding C1 are the precursor of the oenocytes. (A-F,I) Immunostainings showing Sal (red) and β-Gal or Cpo (green) expression in embryonic stages 11 (A,D), 13 (B,E) and 16 (C,F,I). Abbreviations are as in Figs 1 and 2. A. The cells surrounding the precursor C1 express both *sal* and the *dfr-lacZ* transgene, and are the putative secondary sensory organ precursors (S) that receive the EGFR signal (Fig. 5). Later in development, these cells migrate ventrally together with lch5 (B) and at stage 16 they are located between the epidermis and the pentascolopodial organ, and continue expressing Sal and β-Gal (C). In *sal* mutants (D), these cells are not detectable at stage 11. In *spi* mutants, the cells that migrate together with lch5 are not longer visible (E), in correlation with the loss of oenocytes observed in the late embryo (F). (G,H) Enzymatic stainings using anti-β-Gal antibodies expressed by the *dfr-lacZ* transgene, reveal the loss of oenocytes in *sal* mutant background. Only a few cells are formed (arrows). Oenocytes do not form in homozygous *ato* mutants, where the formation of primary SOPs is compromised in most of the segments. In I, three consecutive hemisegments are shown. Sometimes, oenocytes (in red) develop in the hemisegments where a primary SOP has singled out, as revealed by the presence of the neuron, sheath, ligament and cap cells from one scolopodium (left segment). However, this is not always observed (middle segment), indicating that EGFR signalling to the ectoderm arises from some primary SOPs but not from others.

*sal* restricts the ability of C1-surrounding cells, receiving EGFR signalling, to adopt sensory organ precursor cell fate; these cells then develop as oenocytes rather than chordotonal organs (Fig. 8).

## DISCUSSION

The work presented here has documented the expression pattern of *sal* in the peripheral nervous system and associated cells in the *Drosophila* embryo. It also revealed the function of this gene in the formation of the pentascolopodial organs of the PNS and the associated oenocytes. It has shown that *sal* interacts with the EGFR signalling pathway, acting as a switch between the secondary SOP fate (which it restricts) and the oenocyte fate (which it promotes).

### Spalt restricts the EGFR mediated recruitment of SOPs in the developing lch5

The EGFR pathway is involved in a number of cellular processes such as cell survival, proliferation, patterning, migration, and cell fate decision (Dominguez et al., 1998). In particular, the EGFR pathway is implicated in the development of the chordotonal organs in *Drosophila melanogaster* (Lage et al., 1997; Okabe and Okano, 1997; Okabe et al., 1996). The pathway is necessary for the second step of recruitment of SOPs from ectodermal precursors, and for the consequent increase of number of scolopodia in the lch5 and in the vchA/B organs. Thus, during development of the lch5 organ, where two secondary SOPs are recruited, removal of positive EGFR pathway components like *rho*, *S*, *spi*, *pnt*, *sos*, *Drk*, or *DER*

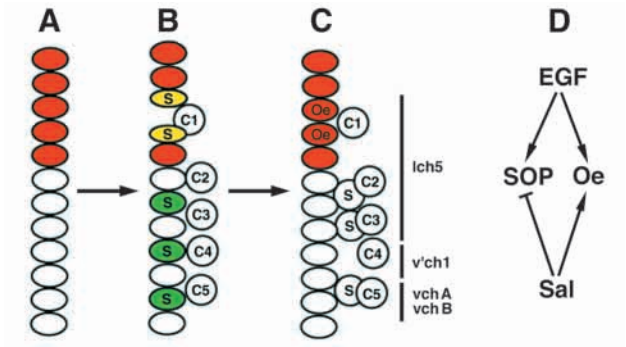


**Fig. 8.** Function of *spalt* in abdominal lateral chordotonal organ development. (A) In early stage 11 of embryogenesis, *Sal* is expressed in the dorsal ectodermal region (red) from which the C1 primary SOP is selected. (B) At late stage 11, the primary SOPs (C1-C5) delaminate and signal via the EGFR pathway to the overlying ectoderm. The receiving cells (S) respond to EGFR signalling, as shown by activated Rl staining. Only three are recruited as secondary SOPs (green). Thus, the presence of Sal in the other receiving cells (yellow) prevents them from becomimg sensory cells. C. The chordotonal organs are then formed: lch5 (C1-C3 plus two secondary SOPs), v′ch1 (C4; no secondary recruited SOPs), and the vchA/B organ (C5 plus one secondary SOP). The epidermal cells around C1 adopt the alternative oenocyte fate (Oe). D. Model in which EGFR signalling is necessary for the formation of both SOPs and oenocytes, while Sal acts as a switch restricting the sensory precursor in favour of the oenocyte fate.

itself, reduces the number of scolopodia in the lch5 from five to three. Conversely, mutations in negative regulators of EGFR signalling like *argos*, *gap1* or *spry* result in an increase of

secondary recruited SOPs in the thorax as well as in the abdominal segments.

Here, we show that the zinc finger transcription factor Sal plays a role in the formation of the lch5 in parallel with the EGFR signalling pathway: the absence of Sal generates supernumerary scolopodia, while the overexpression of Sal reduces the number of scolopodia from five to three. Our results are consistent with the model proposed in Fig. 8: that under wild-type conditions, Sal modifies the EGFR signalling output in the cells surrounding the primary precursor C1, which instead of becoming secondary SOPs adopt the oenocytes cell fate. Five lines of evidence support this idea. First, supernumerary support cells accompany the supernumerary neurons observed in *sal* mutants. Thus, the phenotype is not caused by cell fate transformation within the SOP lineage. Second, the C1-surrounding cells receive the EGFR signal (shown by the antibody staining for activated Rl/MAPK) and, therefore, are capable of becoming secondary precursors. These cells are Sal positive while the other potential secondary precursors, also showing activated Rl and overlying the more ventrally located C2-C5, are not. Given that the number of cells receiving the EGFR signal is larger than the number of cells that become secondary SOPs (two for lch5 and one for vchA/B), the output of the EGFR pathway must be modified in the rest of the cells receiving the signal. Third, the analysis of allelic combinations between *sal* and EGFR pathway mutants reveals that the supernumerary neuronal phenotype observed in the absence of Sal is EGFR dependent. Fourth, the oenocyte precursors depend on *sal* and EGFR signalling to develop. And fifth, in the absence of primary precursors oenocytes do not develop, as shown in *ato* mutants.

The effects of *sal* loss- and gain-of-function are similar, but not identical, to the ones exhibited by corresponding changes in negative regulators of EGFR signalling. There are at least two important differences between the role of these regulators and *sal*. First, *aos*, *pnt* and *spry* are expressed in all the cells receiving the EGFR signal from the primary SOPs, while *sal* is expressed only in a subset of them. Consistent with this, the loss of function of these regulators affects the secondary recruitment of SOPs to other chordotonal organs, like vchA/B and v′ch1, while *sal* seems to modify only lch5. Second, the increase of scolopodia numbers in lch5 is moderate in the *spry* and *aos* mutants, while in *sal* mutants we have observed up to eight scolopodia. In conclusion, differently from the EGFR pathway regulators that are involved in the development of all the chordotonal organs, *sal* is involved specifically in the formation of lch5.

According to our observations, the cells surrounding C1 migrate along the dorsoventral axis closely associated with the pentascolopodial organ. These cells are easy to recognise by the elongated shape of their nuclei and the strong *sal* expression that they display. These cells occupy the location of oenocytes in late embryonic stages. It is then likely that *sal* plays a role in deciding the fate of the EGFR responding cells surrounding the C1 precursor. In the presence of Sal these cells will become oenocytes while in the absence of Sal (as is true for the presumptive secondary precursors overlying C2, C3, C4 and C5), the cells will become sensory organ precursors. Since the putative precursors of the oenocyte cells need EGFR signalling to accomplish some aspects of their development, *sal* would act as a selector gene being necessary to direct them to their correct fate.

### The correct location of the lch5 organ is compromised in *spalt* mutant embryos

In addition to the extra recruitment phenotype, *sal* mutants have aberrantly located lch5 along the dorsoventral axis. In the wild type, lch5 precursors are recruited in a dorsal position and then migrate ventrally (Salzberg et al., 1994). In the mutant, the ventral migration does not seem to take place. The phenotype is similar, but not identical to that of *Homothorax*, *Abdominal-A* or *extradenticle* mutants, where the lch5 organ remains in a dorsal position and scolopodial numbers are reduced to three (Kurant et al., 1998). The involvement of *sal* in other migration process has been reported previously for tracheal development (Kühnlein and Schuh, 1996). There, in cells of the dorsal tracheal trunk, *sal* is required for anteroposterior migration and morphogenesis. Furthermore, it has been shown that *sal* is necessary for the correct location of some neurons in the CNS (T. E. R., R. C., J. U., G. T., F. C. K. and R. B., unpublished results). The molecular mechanisms involved in the specification of migration are largely unknown, and whether the same mechanism applies in the three mentioned cases remains unexplored.

The pleiotropic functions that Spalt proteins exert during development are remarkable. In *C. elegans*, *sem-4* phenotypes include cell fate changes, cell death, defects in axonal morphologies, extra cell divisions or migration. The same is true in *Drosophila*, where *sal* genes play a role in establishing homeotic identities in the blastoderm (Casanova, 1989; Jurgens, 1988), positioning the wing veins (de Celis and Barrio, 2000; de Celis et al., 1996), localising sensory organ clusters (de Celis et al., 1999) and affecting the migration of the dorsal tracheal trunk (Chen et al., 1998; Kühnlein and Schuh, 1996). It therefore appears that the Spalt proteins can function with different signalling pathways and act in combination with other transcription factors to serve diverse roles during development.

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