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Multiple roles for *u-turn*/*ventral veinless* in the development of *Drosophila* PNS

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SUMMARY

Most of the cells in the embryonic peripheral nervous system (PNS) of *Drosophila* are born in their final location. One known exception is the group of lateral chordotonal organs (lch5) whose precursors form in a dorsal position, yet the mature organs are located in the lateral PNS cluster. Mutations in the *u-turn* (*ut*) locus perturb the localization of lch5 neurons and result in a 'dorsal chordotonals' phenotype. We show that *ut* is allelic to *ventral veinless* (*vvl*), also known as *drifter*. VVL, a POU-domain transcription factor, has been shown to participate in the development of tracheae and CNS in the embryo, and in wing development in the adult; however, its role in PNS development has not been described. Characterization of the 'dorsal chordotonals' phenotype of *vvl* mutant embryos revealed that in the absence of VVL, cell fates within the lch5 lineage are determined properly and the entire organ is misplaced. Based on the positions of lch5 cells relative to each other in mutant embryos, and in normal embryos at different developmental stages, we propose a two-step model for lch5 localization. lch5 organs must first rotate to assume a correct polarity and are then stretched ventrally to their final position. In this process, VVL function is required in the ectoderm and possibly in the lch5 organs too.

VVL is also expressed in developing external sensory organs in the embryo and in the adult. In the embryo, loss of VVL function results in increased apoptosis in specific es organs. Analysis of *vvl* mutant clones in adults revealed a requirement for VVL in the control of cell number within the bristle lineage.

Key words: *vvl*, *ut*, PNS, Chordotonal, POU-domain, *Drosophila*

INTRODUCTION

The development of the peripheral nervous system (PNS) involves many basic developmental processes, such as cell differentiation, cell fate determination, cell-cell interactions and cell migration. The integration of these processes leads to the formation of a highly stereotypic array of sensory organs and pathways. There are two distinct types of multicellular sensory organs in the PNS of *Drosophila*: external sensory (es) organs, which function as mechanoreceptors and chemoreceptors, and chordotonal (ch) organs, which function as proprioceptors (reviewed by Jan and Jan, 1993). Although morphologically and functionally different, both types of organs are created in a similar fashion. In both cases, a single sensory organ precursor of ectodermal origin goes through several asymmetric cell divisions to produce the mature organ comprising at least four different cell types (Bodmer et al., 1989; Hartenstein and Posakony, 1989; Brewster and Bodmer, 1995). The mature es organ contains the internal neuron and sheath cell, and two outer support cells in the epidermal layer (Hartenstein, 1988). The mature ch organ is subepidermal and contains a neuron ensheathed by a scolopale cell, a ligament cell and a cap cell. The cap cells are connected to the ectoderm by attachment cells, which derive from the ch lineage (Matthews et al., 1990; Brewster and Bodmer, 1995).

In the embryonic PNS, abdominal segments A1-A7 share an identical pattern, consisting of a dorsal, a lateral and two ventral neuronal clusters. There are eight ch organs in each of these segments. The pentascolopidial ch organ (lch5) and the v'ch1 organ are located in the lateral cluster, and vchA and vchB reside in the ventral cluster (Fig. 3D) (Campos-Ortega and Hartenstein, 1985; Ghysen et al., 1986). In *Drosophila*, sensory organs are generally born in their final position. However, the precursors of the lch5 are born in the dorsal region (Bier et al., 1990), yet by an unknown mechanism, so far termed lch5 migration, the mature organs assume a lateral position. Genetic screens identified mutations that perturb this migration and result in a 'dorsal ch' phenotype (Salzberg et al., 1994; Salzberg et al., 1997; Kania et al., 1995). This phenotype is also characterized by an incorrect orientation of the affected neurons. When the lch5 neurons are located in a normal lateral position, their dendrites point dorsally; when they are abnormally located within the dorsal PNS cluster, their dendrites point ventrally or posteriorly. The correlation between the position of the neurons along the dorsoventral axis

and their orientation suggests that descending to the lateral cluster is accompanied by counter clockwise rotation of the lch5 neurons (Salzberg et al., 1994). The non-neuronal cells of the lch5 organs have not been characterized with respect to the lch5 migration process.

Few of the loci that are required for the correct positioning of the lch5 neurons have been characterized. Mutations in the Hox gene *abdominal-A* (*abd-A*) result in a transformation of lch5 to thoracic dorsal ch (dch3) (Heuer and Kaufman, 1992; Salzberg et al., 1994). Both localization and number of the lch5 neurons are affected in these mutants. A reminiscent phenotype is typical of embryos homozygous for a strong hypomorphic mutation in *homothorax* (*hth*), in which both a reduction in the number of lch5 neurons to three and abnormal dorsal localization of these neurons is observed (Salzberg et al., 1994; Kurant et al., 1998). Mutations in the *spalt* (*sal*) gene result in occasional dorsal localization of the lch5 neurons and an increased number of these organs (Rusten et al., 2001; Elstob et al., 2001). Although the pathway determining the number of lch5 organs is well characterized (Okabe and Okano, 1997; zur Lage et al., 1997), the process of their localization to the lateral cluster remains unclear. Loss-of-function mutations in the *u-turn* (*ut*) locus result in dorsal positioning of lch5 neurons without affecting their number (Salzberg et al., 1994).

We show that *ut* is allelic to *ventral veinless* (*vvl*), also known as *drifter*. VVL, a POU-domain transcription factor, has been shown to participate in the development of the embryonic tracheal system and CNS, and in wing formation in the adult (Anderson et al., 1995; de Celis et al., 1995). We find that VVL is also required for normal development of both embryonic and adult PNS. In *vvl* mutant embryos the lch5 organs fail to rotate and to stretch ventrally, the two steps required for achieving their lateral localization, as we suggest in a new model. The expression pattern of VVL and the results of rescue experiments suggest that VVL functions either non-autonomously or both autonomously and non-autonomously in this process. In addition, in the embryo, VVL is expressed in the developing es organs, and in its absence increased apoptosis is observed in these lineages. In the adult, VVL expression is observed in all cells of the developing bristles at early stages, and becomes restricted mainly to the socket cells at later stages of development. *vvl* mutant clones exhibit defects in bristle development, which are characterized by excessive numbers of cells and abnormal differentiation.

MATERIALS AND METHODS

Fly strains

Fly strains used in this study: ut^{H599}, ut^{H76} and ut^{M638} (Salzberg et al., 1994); vvl^{GA3} (de-Celis et al., 1995); 5′F:2.6 *atonal (ato)-lacZ* (Sun et al., 1998); *A1-2-29* (Bier et al., 1989); *A101 neuralized* (*neu*)*-lacZ* (Huang et al., 1991); *UAS-abd-A* (Greig and Akam, 1993), *armadillo* (*arm*)*-Gal4* vvl^{GA3}*/TM3, Sb ftz-lacZ* (this work); *ato-Gal4* vvl^{GA3}*/ TM6, Tb P[*w^+*, abd-A-lacZ]* (this work); *elav-Gal4*; ut^{H599}*/ TM6,* D^3 (this work); ut^{H599} *UAS-vvl/ TM6, Tb P[*w^+*, abd-A-lacZ]* (this work); *w*; ut^{H599} *FRT80B/ TM6, Tb P[*w^+*, abd-A-lacZ]* (this work); vvl^{GA3} *A101 neu-lacZ/ TM6, Tb P[*w^+*, abd-A-lacZ]* (this work); *A1-2-29;* ut^{H599}*/TM6, Tb P[*w^+*, abd-A-lacZ]* (this work); *UAS-lacZ, Df(3L)ZN47, Df(3L)v65c, Df(3L)CH12, In(3LR)282*; and y w *eyFLP2 glass-lacZ;* $M(3)RpS17^4$ w^+ *FRT80B/TM6B* y^+ (FlyBase, 1999).

Sequencing

The coding sequence of *vvl* was amplified by PCR from genomic DNA of heterozygous ut^{H599}, ut^{H76}, and ut^{M638} flies and controls. The PCR products were cleaned using the Qiaquick gel extraction kit (Qiagen, Valencia, CA) and sequenced using the Big Dye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA) and capillary electrophoresis on an ABI PRISM 310 automated sequencer. The presence of mutations identified in genomic DNA was verified by repeating PCR and sequencing on extracts from single homozygous embryos, following the protocol for PCR from single flies (Gloor et al., 1993).

Immunohistochemistry and TUNEL

Staining of whole-mount embryos was performed using standard techniques (Patel, 1994) with minor modifications. For the labeling of pupal tissues, pupae were aged at 25°C, dissected in PBS and fixed in 4% formaldehyde for 15-30 minutes. After several washes in PBT (PBS, 0.1% Tween 20), staining was performed as for embryos, except that incubation with antibodies was performed in PBT plus 5% normal goat serum. Primary antibodies were: mAb 22C10 (1/20) (Fujita et al., 1982); rat anti-VVL (1/300) (Llimargas and Casanova, 1997); mAb 21A6 (1/10; obtained from S. Benzer); mAb MR1A anti-Prospero (PROS) (1/5) (Spana and Doe, 1995); rabbit anti-REPO (1/200) (Halter et al., 1995); rabbit anti-α85E (1/50) (Matthews et al., 1990); mouse and rabbit anti-β-galactosidase (β-Gal) (1/1000; Promega and Cappel, respectively); rat anti-Suppressor of Hairless (Su(H)) (1/1000-1/2000) (Gho et al., 1996); rabbit anti-DPax2 (1:200) (Fu and Noll, 1997); mouse anti-ELAV and rat anti-ELAV (1/20 and 1/10, respectively) (O'Neill et al., 1994) as well as mAb8D12 anti-REPO (1/10), mAb BP102 (1/20) (Seeger et al., 1993) and mAb 2B10 anti-Cut (CT) (1/20) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Secondary antibodies for fluorescent staining were Cy3, FITC or Cy5-conjugated anti-mouse/rabbit/rat (Jackson). Secondary antibodies for non-fluorescent staining were biotinylated anti-mouse/rabbit/rat detected with Vecta-Stain Elite ABC-HRP kit (Vector Laboratories). Stained embryos and pupal tissues were viewed using bright field and confocal microscopy (Zeiss Axioskop and Radiance 2000, BioRad).

Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) reaction was carried out following fluorescent staining and proteinase K treatment, using the In Situ Cell Death Detection Kit, TMR red (Roche Molecular Biochemicals).

Cuticle preparations and scanning electron microscopy

Anesthetized adult flies were boiled in a 10% NaOH solution for 10 minutes. The cuticles were washed in water, dissected and mounted in Hoyer's. The preparations were examined on a Zeiss Axioskop. For electron microscopy adult flies were prepared following the HMDS method (Wolff, 2000). The flies were examined in a Jeol T-300 scanning electron microscope, operated at 25 kV.

RESULTS

ut is allelic to *ventral veinless* (*drifter*)

The *ut* gene was identified in a chemical mutagenesis screen for mutations affecting the development of the embryonic PNS. It was mapped by meiotic recombination to genetic position 3[26] on the left arm of the third chromosome. The approximate cytological location was determined to be 65A2;65E1 (Salzberg et al., 1994). Three embryonic lethal alleles of *ut* were generated in that screen: ut^{H599}, ut^{H76} and ut^{M638}.

To map the *ut* gene further, complementation tests were performed between the *ut* mutant strains and chromosomal

deficiencies in the 65A-E region. Two deficiency chromosomes failed to complement the *ut* mutant chromosomes: *Df(3L)ZN47* (64C2-64C10; 65C1-65D4) and *Df(3L)v65c* (64E1; 65C1-65D6). The *Df(3L)CH12* (65A11; 65C2) chromosome did complement the *ut* mutations. These results indicated that the *ut* locus resided either in the 64E1;65A11 or 65C2;65D4 genomic interval. One of the candidate genes in these genomic regions is *ventral veinless* (*vvl*), which maps to 65C5 (FlyBase, 1999). Two observations strongly suggested that *ut* and *vvl* could be allelic. First, a cross between *ut* alleles and the *In(3LR)282* chromosome, which has a breakpoint in the 65C-D region, yielded adult progeny that lacked the L4 wing veins (not shown). *vvl* is known to be required for wing vein formation (de Celis et al., 1995). Second, *vvl* mutant embryos exhibit a collapse of the anterior segments of the ventral nerve cord (VNC), a phenotype we observed also in *ut* mutant embryos (not shown). Complementation tests between the embryonic lethal allele vvl^{GA3} and *ut* alleles demonstrated that *ut* and *vvl* were indeed allelic.

To characterize the nature of the *ut* mutations, we amplified and sequenced the coding region of *vvl* from the ut^{H599}, ut^{H76} and ut^{M638} strains. The coding sequence of *vvl* from ut^{H599} flies revealed a point mutation converting tryptophan in position 138 to a stop codon. In ut^{H76} flies we identified a mutation changing tryptophan in position 351 into a stop codon. We were not able to identify any mutation in the coding sequence of the ut^{M638} allele, suggesting a possible mutation interfering with regulatory elements of the gene.

VVL is required for the correct positioning of lch5 organs

The most prominent defect in the PNS of *vvl* homozygous embryos is the frequent abnormal positioning of lch5 neurons (Salzberg et al., 1994) (this work). In wild-type embryos, these neurons are invariably located in the lateral cluster of abdominal segments A1-A7 (Fig. 1A). In 60-70% of the abdominal segments of ut^{H599} or vvl^{GA3} mutant embryos (n=138 and n=115 respectively), the lch5 neurons were situated in a more dorsal position than normally (Fig. 1B,C). As described previously (Salzberg et al., 1994), the orientation of the neurons differed depending on their location along the dorsoventral axis. When the lch5 neurons were situated high within the dorsal cluster, their dendrites pointed ventrally, similar to the dch3 in thoracic segments T2 and T3 (not shown). If the lch5 neurons were located just above or below the level of the dorsal bipolar dendrite (dbd) neuron, their dendrites pointed in a ventroposterior or a dorsoposterior direction, respectively (Fig. 1B,C). Only when these neurons were located near or within the lateral cluster, their dendrites pointed dorsally as in a normal embryo. The number of the lch5 neurons was not affected in these mutants. Of the three additional ch neurons present in each abdominal segment the v'ch1 neuron was invariably present in its correct position in *vvl* mutant embryos (Fig. 1B,C). The vchA and vchB neurons were always present too; however, in about 40% of the segments, either one or both vch neurons were abnormally oriented, more often the vchA neuron (not shown).

The dorsal localization of lch5 neurons in *vvl* mutant embryos reflects their inability to migrate to their correct position. This abnormal migration pattern could result from improper differentiation of the lch5 organs. We therefore examined the expression of several ch organ cell type-specific markers in ut^{H599} and vvl^{GA3} embryos. mAb 21A6 was used to label the scolopale (a structure surrounding the tip of the dendrite), and anti-PROS and anti-REPO were used to label the nuclei of scolopale and ligament cells respectively. Anti-α85E detects the 85E α-tubulin protein (Matthews et al., 1990), and was used to demonstrate the ligament and cap cells, as well as the attachment cells that anchor ch organs to the ectoderm. The expression of all these markers appeared to be normal in the dorsally positioned lch5 organs (Fig. 1E-G; data not shown), indicating that cell identities within the lch5 organs of *vvl* mutants are determined properly, and that cell numbers are normal. Staining with anti-α85E clearly demonstrated that the abnormal polarity of the mislocalized neurons reflects an abnormal polarity of the whole organ. When the lch5 dendrites pointed ventrally, the ligament cells were located in the most dorsal position within the organ as in the thoracic dch3 (Fig. 1G). When the dendrites pointed posteriorly, the organs were stalled in the ventral part of the dorsal cluster and appeared collapsed, with all the cells in approximately the same dorsoventral position (Fig. 1E,F). Once below the level of the dbd neuron, the organs began to assume their normal polarity and were rotated so that the ligament cells were the most ventral and the dendrites pointed dorsally (Fig. 1E,F). The location of the attachment cells, adjacent to the dbd neuron, was not affected in the mutant embryos (Fig. 1E,F). These data suggest that lch5 organs, which normally originate at the level of the dbd neuron (Hartenstein, 1987), are formed in a similar polarity to thoracic dch3. Later on they rotate to achieve both a reversed orientation and a more lateral localization, and are then stretched further ventrally, to reach their final position. The attachment cells seem to function as the hinge around which the lch5 organs rotate.

To verify this hypothesis we examined whether the dorsoventral location of the ligament cells, relative to the other cells of the organ, changes in the course of lch5 development in normal embryos. We double-stained *ato-lacZ* embryos, in which *lacZ* is expressed in all cells of the ch lineage, with anti-β-GAL and anti-REPO. Indeed, in stage 12 embryos the REPO-expressing ligament cells were often observed in a dorsal position within the organ. During stage 13, these cells acquired the most ventral position within the organ (Fig. 1H-J). These data suggest that the ligament cells migrate ventrally, possibly pulling the whole organ with them. We therefore conclude that the concept of lch5 neuronal migration is inaccurate, and refer to this process as lch5 lateral localization.

Where is VVL function required for lch5 lateral localization?

Lateral localization of the developing lch5 organs is probably directed by factors expressed within these organs and factors expressed in the surrounding environment. In embryos, VVL expression has been previously detected in the developing tracheae, midline glia, oenocytes and ectoderm (Anderson et al., 1995). This expression pattern suggests a non-autonomous function of VVL in lch5 localization. However, the ectodermal expression of VVL, which begins at stage 12, may mask concomitant expression in the developing PNS.

In order to determine whether VVL is expressed in the developing lch5 lineage, we double labeled *ato-lacZ* embryos with anti-VVL and anti-β-GAL. We found that VVL is not

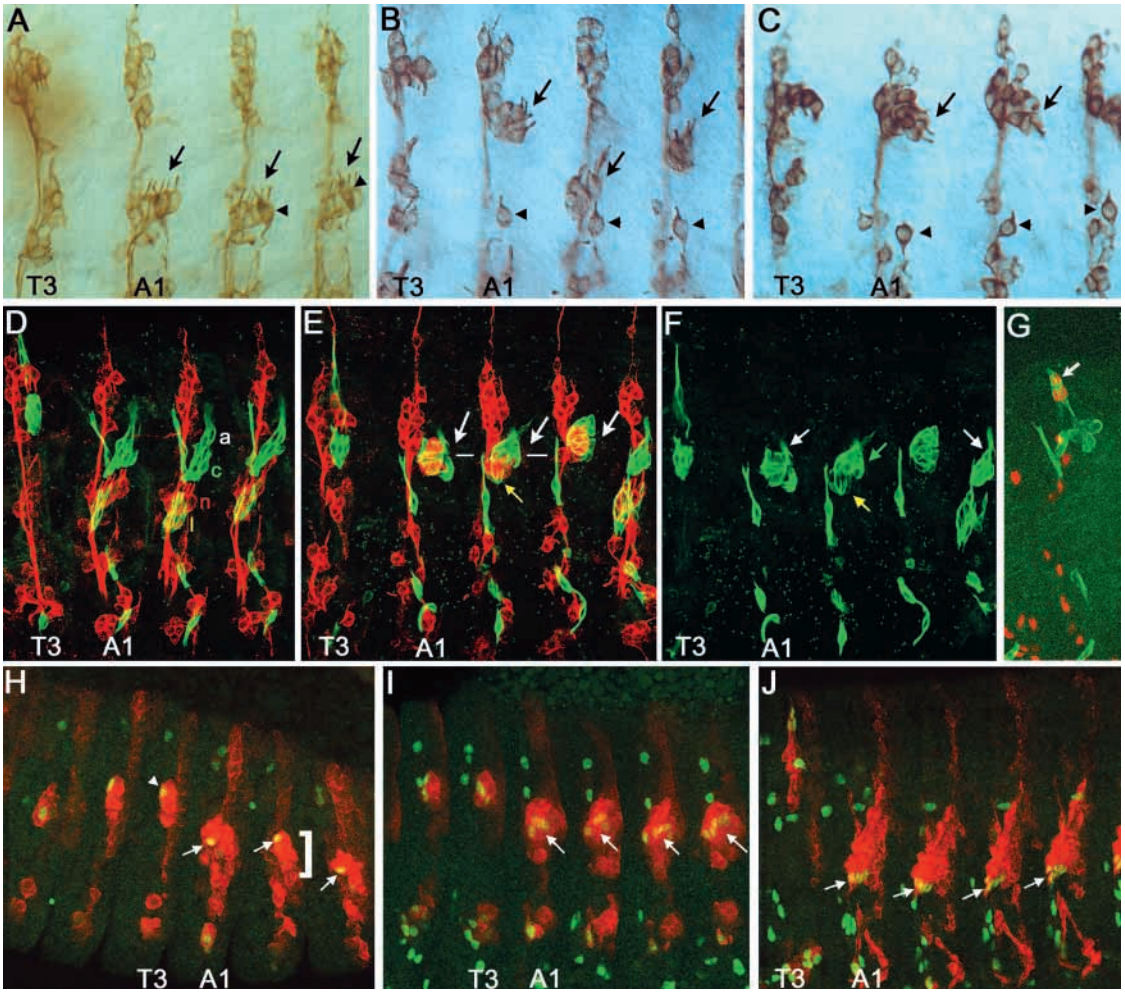

Fig. 1. Localization of lch5 cells in normal and *vvl* mutant embryos. (A-C) Dorsal and lateral PNS clusters visualized by mAb 22C10. Anterior is towards the left and dorsal is upwards in all panels. (A) The lch5 neurons (arrows) of a normal embryo are located in the lateral PNS cluster of abdominal segments A1-A7, and their dendrites point dorsally. The v'ch1 neurons can also be observed in these clusters (arrowheads). In ut^{H599} (B) and vvl^{GA3} (C) homozygous embryos, the lch5 neurons are often aberrantly positioned (arrows). When associated with the dorsal cluster, the dendrites of lch5 neurons point posteriorly or ventrally (compare A1 segments in A-C). The v'ch1 neuron remains in its normal position (arrowheads). (D-G) lch5 organs in normal (D) and ut^{H599} embryos (E-G). Neurons in D and E are labeled with mAb 22C10 (red); ligament, cap and attachment cells are labeled with anti-α85E (green). a, attachment cells; c, cap cells; n, neurons; l, ligament cells. Yellow labeling of the ligament cells (D,E) is due to the projection of several focal sections. (D) In a normal embryo, the organ is stretched with the ligament and attachment cells at its ventral and dorsal edges respectively. (E) White arrows indicate misplaced lch5 organs, associated with the ventral part of the dorsal cluster. The organs are collapsed. Note the correct orientation of the dendrites in A2, where the ligament cells (yellow arrow) have descended below the level of the dbd neuron (white line). (F) α85E staining of the segments shown in E. All support cells of the misplaced organs can be detected. The attachment cells are in their normal positions (white arrows). The ligament cells (yellow arrow) and cap cells (green arrow) fail to localize to their correct positions. (G) Occasionally the affected lch5 organs are oriented as thoracic dch3 (compare with T3 in D and F). The arrow indicates the five nuclei of the ligament cells, labeled with anti-REPO (red). (H-J) *ato-lacZ* embryos stained with anti-β-GAL (red) and anti-REPO (green), revealing the relative position of the ligament cells in developing lch5 organs. (H) Arrows and arrowhead indicate abdominal and thoracic ligament cells, respectively. Square bracket marks the lch5 cells. In A2 the ligament cells (yellow) are at the dorsal edge of the organ as in T3. In A1 and A3, these cells have already started descending (arrows). (I) An early stage 13 embryo. The ligament cells are assuming a more ventral position relative to the other cells of the organ (arrows). (J) At stage 16, the ligament cells are at their final position at the ventral edge of the lch5 organs (arrows).

expressed in the lch5 precursors in stage 11 (Fig. 2A). During stages 12-13, very low levels of VVL could be detected in some of the *lacZ*-expressing cells (Fig. 2B,C). Because, at these stages, all the cells of the developing lch5 organs are clustered together, we could not identify the VVL-expressing cells unambiguously. However, double labeling with anti-REPO and anti-VVL demonstrated that the ligament cells did not express VVL. From stage 14 onwards, a slightly stronger VVL expression was clearly detected in the lch5 neurons (Fig. 2D-F).

The expression of VVL in the developing lch5 organs could imply that it functions autonomously in the process of lch5 localization, as opposed to a non-autonomous function from the ectoderm. A third possibility is that VVL function is required both in the ectoderm and lch5 cells. To distinguish between these possibilities we attempted rescuing the 'dorsal ch' phenotype of ut^{H599}/vvl^{GA3} embryos by expressing a *UAS-vvl* transgene (Boube et al., 2000) using several drivers. To test the possibility of autonomous function, we used *ato-Gal4* (Hassan et al., 2000) and *elav-Gal4* (Lin and Goodman, 1994). The possible role of the ectodermal expression of VVL in lch5 positioning was examined using *arm-Gal4* (Sanson et al., 1996). To allow correct interpretation of the rescue experiments, we examined the expression pattern induced by these drivers by crossing them to *UAS-lacZ* flies and following

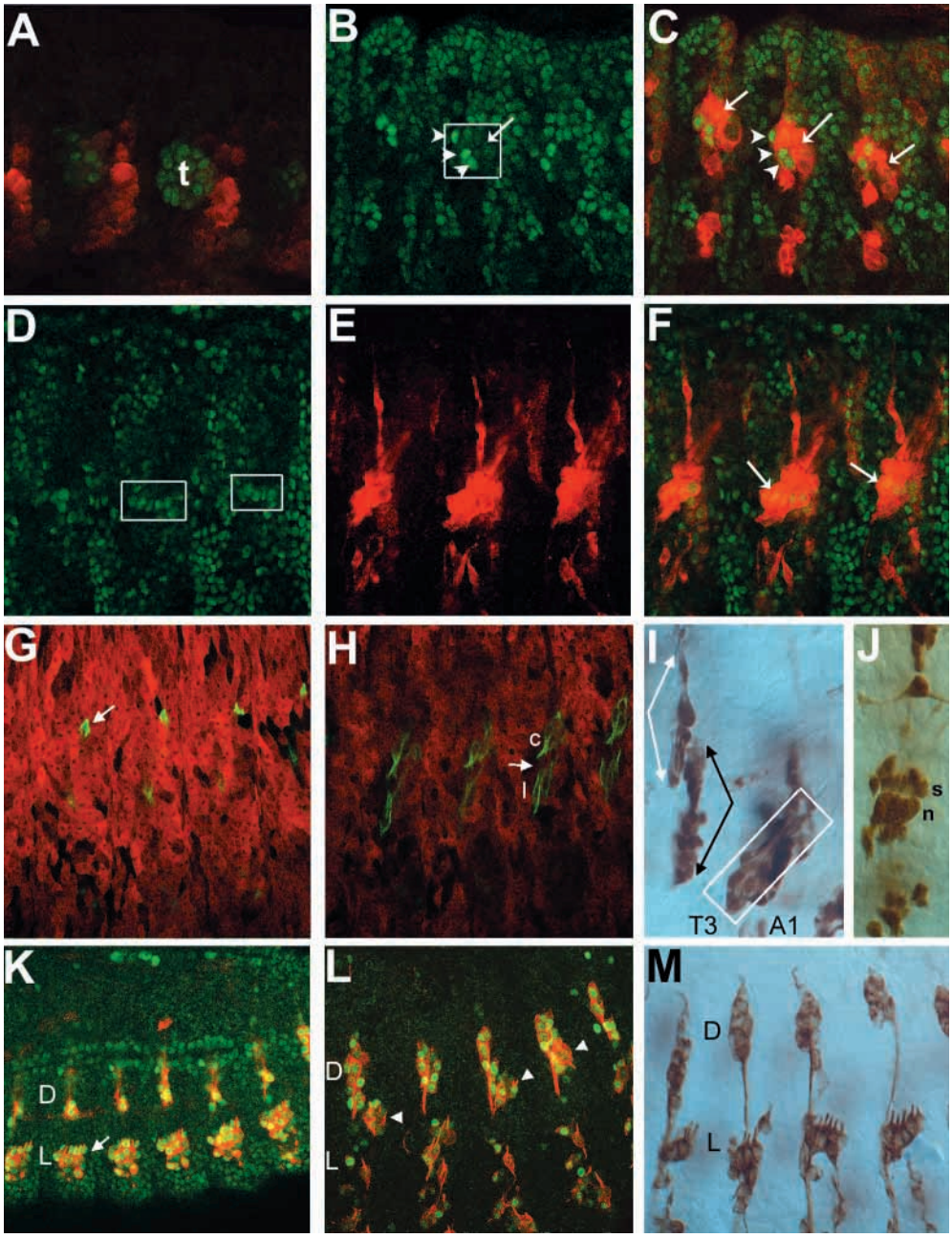

Fig. 2. VVL expression pattern in lch5 organs and rescue experiments. Anterior is towards the left and dorsal is upwards in all panels. (A-F) *ato-lacZ* embryos stained with anti-VVL (green) and anti-β-GAL (red). (A) An early stage 11 embryo showing VVL expression in the tracheal placodes (t). VVL is not expressed in the ch precursors. (B,C) Abdominal segments of a stage 12 embryo. The box in B marks VVL-positive cells that belong to the lch5 lineage (arrow) and the oenocytes (arrowheads). (C) A merge of anti-VVL (B) and anti-β-GAL staining. VVL-positive nuclei boxed in B colocalize with the *ato-lacZ* staining (arrows). The oenocytes are marked with arrowheads. (D-F) A stage 15 embryo. VVL expression is clearly detected in the nuclei of lch5 neurons (white box in D and arrows in F). (G-J) β-GAL expression under the regulation of *arm-Gal4* (G,H), *ato-Gal4* (I) and *elav-Gal4* (J). (G,H) Two focal planes of the same stage 14 embryo. *arm-Gal4* drives strong β-GAL expression (red) in the ectoderm (G), and much weaker expression in the PNS (H). Ectodermal and PNS focal planes were determined by staining with anti-α85E (green). The arrow in G indicates lch5 ectodermal attachment cells. The arrow in H indicates the position of neurons and sheath cells. c, cap cells; l, ligament cells. (I) *ato-Gal4* drives expression in the ch lineage and in a group of ectodermal cells that remains confined to the posterior compartment of each segment. lch5 in the A1 segment are boxed. White arrows in the T3 segment indicate the edges of dch3 organs, and black arrows indicate the cluster of the β-GAL-expressing ectodermal cells. (J) *lacZ* expression induced by *elav-Gal4* can be observed in the neurons (n) and sheath cells (s) of lch5 organs. (K-M) Rescue experiments with *elav-Gal4* and *arm-Gal4*. (K,L) Embryos are labeled with mAb 22C10 (red) and anti-VVL (green). (K) A normal embryo carrying both the *elav-Gal4* and *UAS-vvl* transgenes. The lch5 organs are positioned correctly in the lateral clusters in all abdominal segments (arrow). VVL expression is seen in the ectoderm and trachea (endogenous protein), and in neurons and sheath cells (induced protein). (L) A ut^{H599} homozygous embryo, carrying both transgenes as the embryo in K. VVL expression can be observed only in neurons and sheath cells. This expression is clearly insufficient for rescuing the abnormal localization of lch5 organs. Abnormally positioned lch5 neurons are marked with arrowheads. (M) Abdominal segments of a ut^{H599} homozygous embryo, carrying the *arm-Gal4* and *UAS-vvl* transgenes. Note the correct positioning of lch5 neurons. D, dorsal PNS cluster; L, lateral PNS cluster.

the pattern of β-GAL expression. *ato-Gal4* drives expression in the lch5 precursors and surrounding ectodermal cells from late stage 10 onwards (not shown). Until stage 13 all the β-GAL-expressing cells are clustered together. Only during stage 14, when the lch5 begin stretching in a ventroanterior direction, can they be observed separately from the ectodermal cells, which remain confined to the posterior segment boundary (Fig. 2I). *elav-Gal4* drives expression in the lch5 neurons as well as sheath cells (Fig. 2J). This expression begins at stage 12 and becomes pronounced from stage 13. No ectodermal expression is induced by this driver. The expression driven by *arm-Gal4* begins at the cellular blastoderm stage. This expression is mainly ectodermal, yet low levels of expression are also induced by this driver in the lch5 organs (Fig. 2G,H).

Expressing the *UAS-vvl* transgene under the regulation of *ato-Gal4* resulted in a 46% reduction in the number of misplaced lch5 organs, whereas expression of this transgene under the regulation of *elav-Gal4* did not result in any rescue at all (Fig. 2K,L; data not shown). The same experiment using *arm-Gal4* yielded an 80% decrease in the number of 'dorsal ch' (Fig. 2M). These results suggest that for the lateral localization of lch5 organs VVL functions either both autonomously and non-autonomously, or in a purely non-autonomous manner (see Discussion).

VVL is required for the normal development of embryonic es organs

Characterization of VVL expression in the embryonic PNS revealed it was expressed in developing es organs in stage 12 and older embryos (Fig. 3A-C). In accordance with this expression pattern, *vvl* mutant embryos stained with mAb 22C10 exhibited a mild loss of es neurons. Of the 15 es neurons normally present in each of the abdominal segments A1-A7 (Fig. 3D) [nomenclature according to Ghysen et al. and Bodmer et al. (Ghysen et al., 1986; Bodmer et al., 1989)],

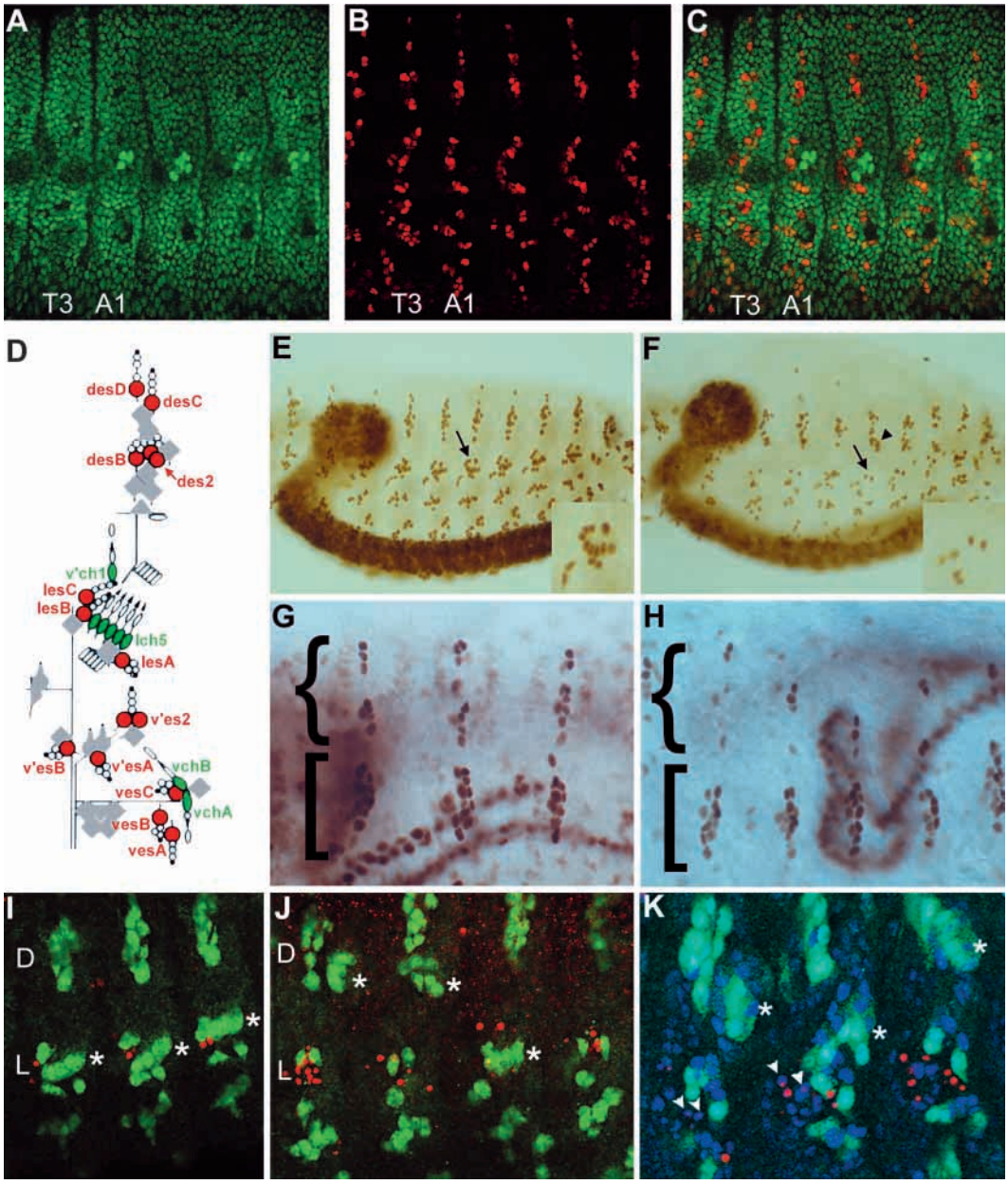

Fig. 3. VVL expression and loss-of-function effects in embryonic es organs. Anterior is towards the left and dorsal is upwards in all panels. (A-C) A wild-type embryo, stained with anti-VVL (A) and anti-CT (B). (C) A merge of A and B. Colocalization of CT and VVL is evident in many es cells (yellow). (D) The sensory organs of a single abdominal segment (A1-A7) in the embryonic PNS [adapted, with permission, from Bodmer et al. (Bodmer et al., 1989)]. es, ch and multiple dendritic neurons are depicted in red, green and gray, respectively. The nomenclature is according to Ghysen et al. and Bodmer et al. (Ghysen et al., 1986; Bodmer et al., 1989). (E,F) A normal embryo (E) and a vvl^{GA3} homozygous embryo (F) stained with anti-ELAV to demonstrate the nuclei of all neurons. In the lateral cluster of a normal embryo there are 13 neuronal nuclei (arrow and inset in E). In a lateral cluster from a *vvl* mutant embryo a reduced number of neurons, in this case only six, is evident (arrow and inset in F). The five nuclei of lch5 neurons can be detected near the dorsal cluster (arrowhead). (G,H) es organs of abdominal dorsal PNS clusters, demonstrated by anti-CT staining. (G) Normal embryo and (H) ut^{H599} homozygous embryo. Braces mark the position of desC and desD cells; square brackets mark the position of des2 and desB cells. A marked decrease in the number of cells in desC and desD is evident in the mutant embryo, whereas des2 and desB seem largely unaffected. (I-K) TUNEL analysis in normal (I) and *vvl* mutant embryos (J,K). Neuronal nuclei are demonstrated by ELAV staining (green) and apoptotic nuclei are labeled in red. The embryo in K carries also the *A101* enhancer trap and is stained with anti-β-GAL (blue). Asterisks mark the location of lch5. D and L, dorsal and lateral PNS clusters, respectively. (I) In the lateral cluster of a normal embryo only one or two apoptotic nuclei are typically observed. Many more apoptotic nuclei are evident in this cluster in ut^{H599} (J) and vvl^{GA3} (K) homozygous embryos. (K) Colocalization of the red (TUNEL) and blue (PNS) signals indicates that at least some of the apoptotic nuclei belong to PNS cells (arrowheads).

desC, desD, lesB, lesC, v'esA and vesC were often missing. All other es neurons seemed largely unaffected by the lack of VVL. The absence of these neurons was verified by anti-ELAV staining, which revealed accordingly reduced numbers of neuronal nuclei in all four neuronal clusters (Fig. 3E,F).

Such reduction in the number of es neurons could be the result of cell fate transformations within the lineage, or a failure of the entire organ to form. To distinguish between the two possibilities, we stained mutant embryos for several es organ cell type-specific markers. Sheath cell nuclei were detected by anti-PROS, and outer support cells were detected using anti-Su(H) and the *A1-2-29* enhancer trap. All of these markers showed a decreased number of cells when compared with wild-type embryos (not shown). For example, in the two dorsalmost es organs, desC and desD, the neurons were missing in 53-69% of the segments (n=45, desD and desC respectively). Sheath cells were missing in 24% (desD) or 57% (desC) of the segments (n=70). The two outer support cells were both missing in 18% (desD) or 61% (desC) of the segments (n=94), and only rarely there was only one cell present (not shown). Staining with anti-CT, which labels all cells of the es organ, demonstrated that it is not the whole organ that fails to form, rather, only some of the cells are lost (Fig. 3G,H). These results indicate that VVL is not required for precursor formation or for cell fate decisions within the es lineage.

Certain POU-domain containing proteins have been implicated in differentiation and survival of PNS cells (McEvilly et al., 1996; Xiang et al., 1998; Gan et al., 1999). In order to find whether VVL has a similar role in embryonic es organ development, we performed TUNEL analysis on *vvl* mutant embryos and examined the number of apoptotic nuclei in the PNS. We focused on the lateral cluster, in which one or two of the three es organs are often affected. In stage 14 ut^{H599}/+ embryos, very few apoptotic nuclei are present in the PNS of each abdominal segment. In the lateral cluster, typically one or two apoptotic nuclei are evident (Fig. 3I). In ut^{H599} homozygous embryos, a marked increase in the number of apoptotic nuclei in the lateral cluster is observed (Fig. 3J), indicating that lack of VVL expression results in enhanced death of cells in the developing PNS. To verify that the apoptotic nuclei belong to PNS cells, we performed TUNEL analysis on vvl^{GA3} embryos, which carry one copy of the *A101* transgene, stained with anti-β-GAL. This analysis confirmed that at least some of the increased apoptosis in *vvl* mutant embryos takes place in the PNS (Fig. 3K).

VVL is expressed in developing es organs of the adult

Although all four alleles of *vvl* used in this work are embryonic lethal when homozygous, the ut^{M638}/vvl^{GA3} and ut^{M638}/ut^{H76} allelic combinations resulted in a low percentage of pharate adults, presenting bristle defects (see below). This suggested that VVL functions in adult es organ development as well. We therefore looked for VVL expression in the developing adult PNS. Staining pupal heads and nota revealed that VVL is indeed expressed in the developing es organs. At 16 hours after puparium formation (APF), many of the es organ precursors have already divided once to generate the two daughter cells pIIa and pIIb. VVL expression was detected both in the precursors and in the two daughter cells (Fig. 4A). At 24 hours APF, when the division of pIIa and pIIb was completed, uniform expression of VVL could be observed in all four post-mitotic cells (Fig. 4B). In later stages this expression gradually decreased in three of the four cells (Fig. 4C), and by ~42 hours APF, strong VVL expression was detected in one large nucleus, which according to its position within the cluster belonged to the socket cell. A much weaker expression was occasionally observed in the adjacent large nucleus of the shaft cell (Fig. 4D). The identity of the VVL expressing nuclei was verified by double labeling with anti-VVL and with either anti-Su(H), which labels socket cells (Gho et al., 1996) (Fig. 4D), or anti-*Drosophila* Pax2, which labels shaft cell nuclei (Kavaler et al., 1999) (data not shown). Staining pupal retinas revealed that VVL was also expressed in the developing interommatidial bristles (Fig. 4E-G).

Adult phenotypes

ut^{M638}/vvl^{GA3} and ut^{M638}/ut^{H76} pharate adults presented abnormal bristles on the head, thorax and abdomen. All macrochaete and microchaete could be affected, and abnormalities in both shafts and sockets were observed. As the nature of the ut^{M638} allele is not clear, and in order to gain a better insight into the role of VVL in bristle development, we generated ut^{H599} somatic clones in adult flies using the FLP/FRT system (Xu and Rubin, 1993). The *eyFLP* strain was used to induce early vvl^{-} clones in the derivatives of the eye-antenna disc (Newsome et al., 2000). The mutant tissue included most of the eye as well as the head capsule, antennae and maxillary palps. Almost all bristles on these tissues were affected, presenting a series of defects resembling those observed in ut^{M638}/vvl^{GA3} and ut^{M638}/ut^{H76} flies. On the head cuticle, the defects included mainly shortened or distorted shafts, and splitting or duplication of shafts (Fig. 4I,J,L). Loss of the shaft occurred less frequently (Fig. 4J). Many of the shafts were associated with one to three supernumerary sockets, appearing either as fused cells or as abnormally flat sockets (Fig. 4I-K). In almost all organs with more than one socket, at least one shaft was present, indicating that the increased number of sockets did not form at the expense of shaft cells.

In the eyes, the phenotype was somewhat different and extremely consistent. All affected bristles had dramatically shortened shafts, but the number of shaft and socket cells appeared normal (Fig. 4M,N).

Loss of VVL results in supernumerary cells in adult es organs

If the increased numbers of shaft and socket cells were the result of cell fate transformations within the es lineage, then a reduced number of the internal cells, namely the neuron and sheath cell, is expected. However, no such reduction was observed and the number of internal cells was either normal or increased. Mutant clones stained with mAb 22C10, which labels the neuron and shaft cells, revealed that the number of neurons was mostly unchanged, whereas the number of shaft cells was often increased. Rarely, two neurons were observed in a single developing organ. Staining with anti-PROS demonstrated an occasional increase in the number of sheath cells to two or three per organ (not shown). These results suggest that no cell fate transformations occur in the mutant bristle lineages, rather extra cell divisions generate

supernumerary cells. This conclusion was corroborated by anti-CT staining which demonstrated many clusters with five or more CT-expressing nuclei, instead of only four in normal developing bristles (Fig. 4O,P). This conclusion, however, does not apply to interommatidial bristles, as we did not observe an increased number of cells in these organs.

DISCUSSION

A new model for the lateral localization of lch5 organs

The question of how lch5 organs reach from their dorsal place of origin to their final lateral position has not been answered yet. All existing data regarding the so called 'lch5 migration' come from descriptions of abnormally positioned (and abnormally oriented) lch5 neurons in different mutant backgrounds. Based on these data, it was previously suggested that concomitant with moving ventrally, the lch5 neurons turn approximately 145° counter clockwise (Salzberg et al., 1994).

By visualizing all the cells of lch5 organs located in a variety of positions between the dorsal and lateral clusters, we were able to determine that the polarity of the neurons reflects the polarity of the whole organ. When the neurons point ventrally, the ligament cells are the most dorsal cells of the organ and the cap cells are the most ventral, whereas for the dendrites to point dorsally, the ligament cells must be ventral and the cap cells dorsal. In normal stage 12 embryos, the ligament cells can be detected at the dorsal part of the organ, whereas in older embryos they migrate ventrally to become the ventralmost cells of the organ. Based on these observations, we propose a two-step model for the lateral localization of lch5 organs (Fig. 5A). In the first step, rotation of the organ takes place. The organ rotates around the attachment cells, which anchor it to the ectoderm and thus function as a pivot. The rotation results in both bringing the organ to its correct orientation and placing it in a more ventral position, closer to the lateral cluster. This step occurs during stage 12 and perhaps early stage 13. Once in their correct orientation the lch5 organs go through the second step, which involves ventral stretching into their final shape and position, as seen in stage 15 or older embryos. This model is further supported by the fact that when the thoracic dch3 are forced to descend to the lateral cluster by overexpressing *abd-A*, their orientation is reversed and the ligament cells are found at their ventral edge (Fig. 5B).

What makes the lch5 organs go through this process? It is possible that the ligament cells respond to a signal and migrate ventrally, thereby pulling with them all other cells of the organ. Interestingly, a similar change from a dorsal to ventral position has recently been shown for the PG3 cell (Umesono et al., 2002), which like the ligament cells expresses the glial marker REPO. This may suggest that a common mechanism governs the change in position of both types of REPO-expressing cells. Another possibility is that the rotation step depends on

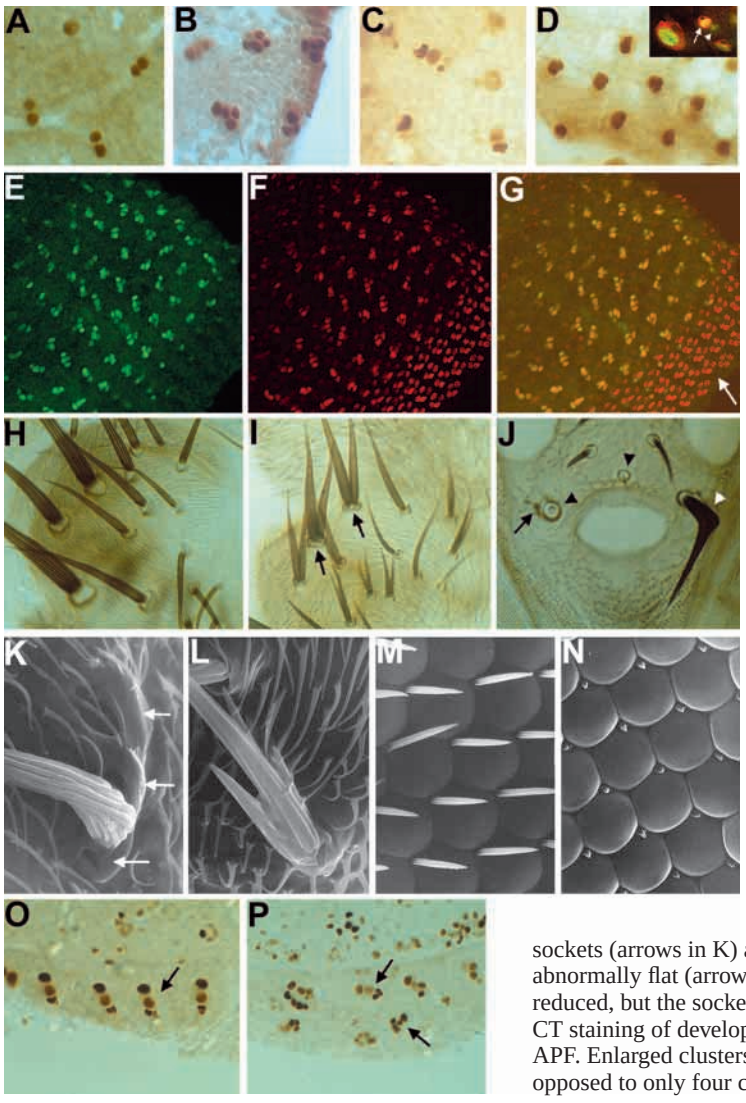

Fig. 4. VVL expression and loss-of-function effects in developing adult bristles. (A-D) Nuclear expression of VVL in developing bristles in pupal nota. (A) At 16 hours APF VVL expression is observed both in the precursors that have not divided yet and in the two daughter cells. (B) At 24 hours APF, VVL is evenly expressed in the nuclei of all four cells of the developing bristles. (C) By 37-40 hours APF, the expression of VVL decreases in three of the four cells of the organ. (D) At 42 hours APF strong VVL expression is evident in only one cell per organ; this is the socket cell, as indicated by colocalization of VVL (green) and Su(H) (red) (arrow in inset). Weaker expression is occasionally seen in the adjacent shaft cell nucleus (arrowhead). (E-G) Expression in a pupal retina (30 hours APF) of VVL (E), CT (F) and a merge panel (G). Colocalization of the two proteins can be observed in all four cells of the developing interommatidial bristles. Note the lack of VVL expression from the cone cells (arrow in G). (H-N) Cuticle preparations and scanning electron micrographs of wild type (H,M) and *vvl* mutant clones (I-L,N). (H,I) a2 antennal segments. Note shaft duplications accompanied by two fused sockets in the mutant (arrows). (J-L) Other bristle defects include reduced or missing shafts (black arrowheads in J), distorted shafts (white arrowhead in J), supernumerary sockets (arrows in K) and splitting of shafts (L). The supernumerary sockets can be abnormally flat (arrow in J). (M,N) In the mutant eye, the shafts are dramatically reduced, but the sockets and the distribution of the bristles appear normal. (O,P) Anti-CT staining of developing bristles in wild type (O) and *vvl* mutant heads (P) at 42 hours APF. Enlarged clusters, containing five or more cells are observed in the mutant, as opposed to only four cells normally (arrows in O and P).

the neurons and that the ligament cells are required only for stretching. As the rotation of lch5 is completed by early stage 13, when axonal outgrowth begins, it is possible that the growing axons serve as a guide for the ligament cells. However, in *vvl* mutant embryos, the lch5 organs often fail to stretch even when their axonal outgrowth seems largely normal. This suggests that although the axons may play a part in guiding the ligament cells, other factor/s are required as well. The morphogenetic movements occurring during dorsal closure in stage 14 are likely to affect the dorsoventral position of the lch5 ectodermal attachment cell. However, dorsal closure alone cannot account for the stretching of lch5, which is not completed before late stage 15, well after dorsal closure is completed.

The role of VVL in lch5 lateral positioning

Mutations in three loci, *abd-A*, *hth* and *sal*, have been shown previously to perturb the lateral localization of lch5 organs (Heuer and Kaufman, 1992; Kurant et al., 1998; Rusten et al., 2001). Mutations in these three loci result in both abnormal localization and abnormal number of lch5 organs. However, decisions of organ number and organ localization are not always coupled. For example, mutations in the EGFR pathway gene *rhomboid* and the EGFR pathway antagonist *argos*, affect the number of lch5 organs but only rarely affect their position (Bier et al., 1990; Okabe et al., 1996) (A.I and A.S., unpublished). *vvl* is the first gene that affects the localization of the lch5 organs without affecting their number. The abnormal localization of lch5 organs in *vvl* mutant embryos is similar to the abnormal localization of these organs in *hth* and *abd-A* mutant embryos, suggesting these genes are required in the same developmental pathway. However, epistasis experiments did not provide evidence for direct genetic interactions between *vvl* and *hth* or *vvl* and *abd-A* (A.I. and A.S., unpublished).

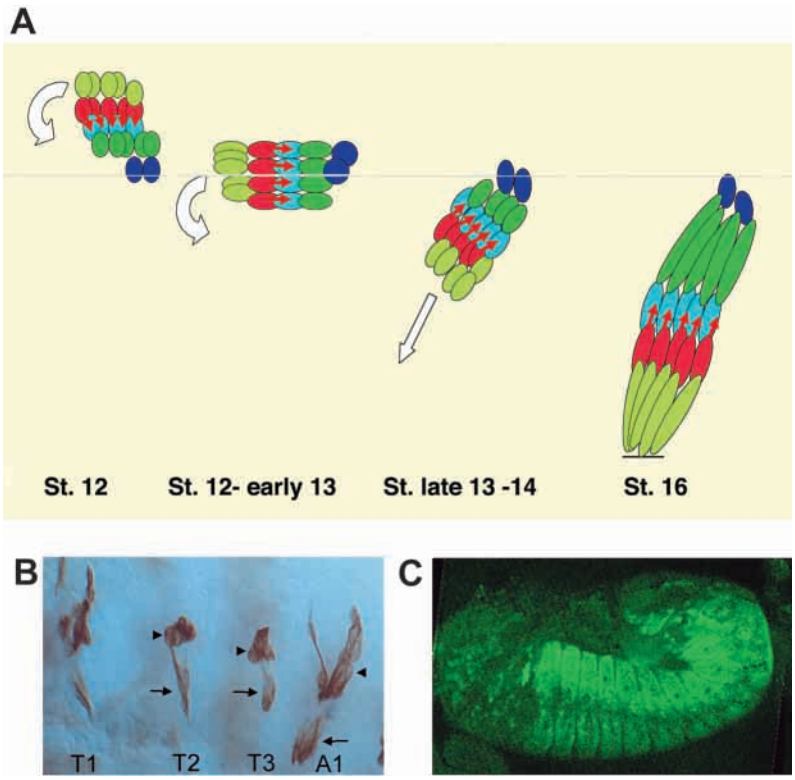

Fig. 5. A model for the lateral localization of lch5 organs. Anterior is towards the left and dorsal is upwards in all panels. lch5 organs in embryonic stages 12-16. The neurons are depicted in red, ligament cells in light green, scolopale cells in light blue, cap cells in dark green and the attachment cells in dark blue. In early stage 12 the ligament cells are located most dorsally within the developing organ. During stage 12 and early stage 13, they migrate ventrally, pulling with them the neuron, scolopale and cap cells. This leads to the rotation of the whole organ. The attachment cells do not change their position. Once the organs acquire the right orientation the ligament cells stretch ventrally and attach to the ectoderm in the lateral region. For simplicity, the lch5 cells in stages 12-13 are drawn next to each other, whereas in reality they are more tightly packed. (B) An embryo overexpressing *abd-A* under the regulation of *da-Gal4*, stained with anti α85E. dch3 organs in T2 and T3 segments have descended and reversed their orientation (compare with dch3 in figure 1F). Arrows and arrowheads indicate ligament and cap cells, respectively. (C) A stage 12 wild-type embryo stained with anti-VVL. Note the stronger VVL expression in the dorsal half of the embryo.

VVL, a class III POU-domain transcription factor, and its mammalian homologs, have been shown to be required for cell migration. Brn1 and Brn2, the mouse homologs of VVL, have a crucial role in the migration of cortical neurons (McEvilly et al., 2002; Sugitani et al., 2002). In the CNS of *Drosophila* embryos, VVL is required for the migration of midline glial cells (Anderson et al., 1995). In the embryo, VVL is also required for tracheal cell migration and in its absence the tracheal tree fails to form (Anderson et al., 1995; de Celis et al., 1995).

The mechanism by which VVL affects the lateral localization of lch5 organs is not clear. Cell migration requires the existence of signals from the environment and the ability of the migrating cell to receive and respond to these signals. Previous work has demonstrated that in tracheal development, VVL functions autonomously and it was suggested to regulate the expression of cell surface molecules necessary for the migration of tracheal cells (Anderson et al., 1995). In lch5 organs, VVL expression is detected in the neurons. However, expressing VVL under *elav-Gal4* regulation in *vvl* mutant background could not rescue the mutant phenotype. This result suggests that the neuronal expression of VVL is either not required or not sufficient for lch5 lateral localization. Driving VVL expression with *ato-Gal4* could rescue the mutant phenotype; however, in a much lower efficiency than when VVL was expressed under *arm-Gal4* regulation. The major differences between these two drivers are that while *ato-Gal4* drives strong expression in the lch5 lineage and in a small group of ectodermal cells, *arm-Gal4* induces strong expression throughout the ectoderm and only weak expression in lch5 organs. Thus, the results of these experiments cannot determine unambiguously where VVL is required during lch5 lateral localization, and suggest it could function in both lch5 organs and the surrounding ectoderm, or in the ectoderm alone. The more efficient rescue generated by *arm-Gal4* may indicate that the ectodermal expression of VVL is the main factor with

regard to lch5 positioning. In the ectoderm, VVL could be involved in the generation of a positional cue. Although the rescue of lch5 localization was achieved by ubiquitous expression of VVL in the ectoderm, it should be noted that the normal ectodermal expression of VVL during critical stages of lch5 positioning (stages 12 and early 13) is not uniform. VVL is more strongly expressed in a dorsal domain of the embryo (Fig. 5C), from the position of the lateral cluster dorsally. Later during stage 13, VVL expression becomes uniform throughout the ectoderm. It is not clear yet whether this differential expression is significant in the context of lch5 positioning. VVL has been shown to interact with other transcription factors in the CNS and trachea (Ma et al., 2000; Zelzer and Shilo, 2000). Thus, another possibility is that an unidentified partner of VVL confers a spatial specificity to its activity.

Two additional cell types in the vicinity of the developing lch5 organs express high levels of VVL: tracheal cells and oenocytes. The trachea is probably not involved in the process of lch5 localization, as *trachealess* mutants do not exhibit a 'dorsal ch' phenotype (A.S., unpublished). The possible role of oenocytes in lch5 migration is intriguing. Impaired lch5 localization is many times accompanied by partial or complete loss of oenocytes, as seen in embryos mutant for *abd-A* (Brodu et al., 2002), *sal* (Rusten et al., 2001), *hth* and *vvl* (A.I. and A.S., unpublished). However, *rhomboid* mutants lack oenocytes (Elstob et al., 2001), yet their lateral ch organs (which consist of three, instead of five, scolopidia) are almost always positioned properly. Thus, it seems more probable that lch5 organs and oenocytes are independently affected by the same mutations.

Apoptosis in the PNS in the absence of VVL – a common theme with mammalian POU-factors

In *Drosophila*, loss of es organ cells has been attributed to one of two reasons. Either the organ completely fails to form because of interference with the function of the proneural genes, or cell fate transformations occur between the cells comprising these organs. However, in *vvl* mutant embryos the decreased number of these cells is a result of increased apoptosis. Any of the cells of the organ could be affected, and the remaining cells expressed typical markers, suggesting that initial decisions of cell fates were not impaired. It is therefore possible that VVL is required for cell survival in the developing es lineages. Another possibility is that VVL is required for the differentiation of these organs, and that in its absence some of the cells fail to differentiate properly and go through apoptosis.

In mammals, POU-domain transcription factors were shown to play significant roles in survival of cells in the nervous system. Members of the class IV POU-factors are known to be essential for differentiation and survival of PNS cells (McEvilly et al., 1996; Xiang et al., 1998; Gan et al., 1999). The most interesting of those in the context of *Drosophila* es organ development is Brn3c, which is required for maturation and survival of the inner ear hair cells (Xiang et al., 1998). The vertebrate inner ear hair cells are mechanosensory organs, considered homologous to *Drosophila* bristles in many aspects. The parallelism between the two types of organs was shown at the levels of function, structure and the molecular mechanisms responsible for their development (Adam et al., 1998) (reviewed by Eddison et al., 2000). Mice deficient for Brn3c fail to develop inner ear hair cells and are completely deaf (McEvilly et al., 1996; Xiang et al., 1998). A mutation in the human homolog of this gene was shown to cause progressive hearing loss (Vahava et al., 1998). The defects seen in the development of the hair cells in *Brn3c*-null mice are limited to maturation and survival of these organs (Xiang et al., 1998).

Although there is not sufficient evidence to consider a functional homology between VVL, a class III POU-factor, and the mammalian class IV POU-factors, it will be interesting to determine whether the similarity of their loss-of-function phenotypes extends further at the molecular level.

The role of VVL in adult bristle development

vvl mutant clones in adult head tissue caused defects in bristle development, which typically resulted in supernumerary cells. One possible explanation for an increase in the number of bristle cells is that too many precursors were formed as a result of inefficient lateral inhibition. In such case, we would expect the appearance of complete ectopic organs. However, the supernumerary cells did not constitute separate organs, rather they increased the number of cells within a single es organ. This observation suggests that one or two extra cell divisions took place, resulting in the production of extra cells within the lineage. Thus, it is possible that VVL is required in these cells for exit from the cell cycle.

Many abnormalities were also observed in the structure of the external support cells of the mutant bristles, especially in the shaft. Whether the structural defects are secondary to the abnormal pattern of cell division, or they represent another independent role for VVL in the differentiation of these structures remains to be determined.

Loss of VVL function in the embryonic and adult es lineages results in what seem to be two very different phenotypes: loss of cells in the embryo as opposed to overproduction of cells in the adult. However, it is possible that both phenotypes reflect a failure of the es cells to commence differentiation. These cells may behave differently when unable to differentiate properly, depending on their developmental context.

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