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The *Caenorhabditis elegans* LIN-26 protein is required to specify and/or maintain all non-neuronal ectodermal cell fates

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SUMMARY

The *C. elegans* gene *lin-26*, which encodes a presumptive zinc-finger transcription factor, is required for hypodermal cells to acquire their proper fates. Here we show that *lin-26* is expressed not only in all hypodermal cells but also in all glial-like cells. During asymmetric cell divisions that generate a neuronal cell and a non-neuronal cell, LIN-26 protein is symmetrically segregated and then lost from the neuronal cell. Expression in glial-like cells (socket and sheath cells) is biologically important, as some of these neuronal support cells die or seem sometimes to be transformed to neuron-like cells in embryos homozygous for strong loss-of-function mutations. In addition, most of these glial-like cells are structurally and functionally defective in animals carrying the weak loss-of-function mutation *lin-26(n156)*. *lin-26* mutant phenotypes and expression patterns together suggest that *lin-26* is required to specify and/or maintain the fates not only of hypodermal cells but also of all other non-neuronal ectodermal cells in *C. elegans*. We speculate that *lin-26* acts by repressing the expression of neuronal-specific genes in non-neuronal cells.

Key words: *Caenorhabditis elegans*, hypodermis, glial cells, cell fate, asymmetric cell division, transcription factor, LIN-26

INTRODUCTION

How cell fates are specified is a basic problem in developmental biology. We recently proposed that, in *C. elegans*, the gene *lin-26* is required to specify the hypodermal (epidermal-like) cell fate (Labouesse et al., 1994). This gene was discovered because the *lin-26* mutation *n156* causes a specific set of hypodermal cells, the ventral hypodermal Pn.p cells, to express neuronal instead of hypodermal characteristics (Ferguson and Horvitz, 1985; Ferguson et al., 1987; Labouesse et al., 1994). Two observations indicate that *lin-26* acts not only to control Pn.p cell fates but also more generally to control all hypodermal cell fates (Labouesse et al., 1994). First, although *lin-26(n156)* homozygote animals are viable and healthy, animals heterozygous for the *lin-26(n156)* mutation and a chromosomal deficiency that deletes the *lin-26* locus arrest development as young larvae (Ferguson and Horvitz, 1985) and display defects in all classes of hypodermal cells (Labouesse et al., 1994). Second, alleles of *lin-26* that are stronger than *lin-26(n156)* result in embryonic lethality and cause hypodermal cells to die during embryogenesis (Labouesse et al., 1994). These findings suggest that *lin-26* function is essential for hypodermal cell development. The DNA sequence of the *lin-26* gene revealed that it encodes a presumptive transcription factor with two apparent zinc-fingers (Labouesse et al., 1994). Taken together, these observations led us to propose that one aspect of *lin-26* function is to act as a transcriptional regulator to specify the hypodermal cell fate.

To further our understanding of *lin-26* function, we examined expression patterns of the LIN-26 protein.

MATERIALS AND METHODS

Strains and general methods

Worms were maintained as described by Brenner (1974). Animals were raised at 20°C unless otherwise stated. *C. elegans* strain N2 (Brenner, 1974) was the wild-type strain. The following strains were used: MT156, *lin-26(n156)*; ML87, *dpy-2(e489) lin-26(mc4) unc-4(e120)/ mnC1*; ML93, *dpy-2(e489) lin-26(mc1) unc-4(e120)/ mnC1* (Ferguson and Horvitz, 1985; Labouesse et al., 1994).

Production of anti-LIN-26 antibodies

To raise antibodies, we used a fragment of the *lin-26* cDNA encoding the N-terminal 331 amino acids of the LIN-26 protein (Labouesse et al., 1994), referred to as the NLIN-26 protein. We cloned this fragment into the vectors pMALc (New England Biolabs; see Guan et al., 1987), pGEX-2T (Pharmacia Biotechnology; see Smith and Johnson, 1988) and pRSETB (InVitrogen) to generate a maltose-binding protein/NLIN-26 fusion (MBP/NLIN-26), a glutathione-S-transferase/NLIN-26 fusion (GST/NLIN-26) and a hexa-histidine/NLIN-26 fusion (His_6/NLIN-26), respectively. We purified recombinant proteins by affinity chromatography over maltose (New England Biolabs) or Ni^{2+} ions (Qiagen, see Anderson and Porarth, 1986) and SDS-polyacrylamide gel electrophoresis, or just SDS-polyacrylamide gel electrophoresis for the GST/NLIN-26 protein. We immunized three rabbits with more than 100 μg of the MBP/NLIN-26 fusion protein mixed with complete Freund's adjuvant and boosted

them with more than 100 μg of the GST/NLIN-26 fusion protein mixed with incomplete Freund's adjuvant. We performed subsequent studies with antibodies from one rabbit purified by chromatography over a column containing agarose beads (Biorad) covalently coupled to the His_6/NLIN-26 fusion (see Harlow and Lane, 1988). We adjusted this preparation to a 0.1 mg/ml final concentration and used it at a 200-fold dilution. Protein and antibodies were manipulated as described by Harlow and Lane (1988) and by the suppliers of the chromatography resins.

Specificity of anti-LIN-26 antibodies

Two experiments established that anti-LIN-26 antibodies were specific for LIN-26 protein. First, we tested anti-LIN-26 antibodies by indirect immunofluorescence (see below) against embryos homozygous for the deficiency *mnDf88*, which deletes *lin-26* (Labouesse et al., 1994), or for a recently isolated null allele of the gene, which generates a LIN-26 protein of only 45 amino acids (M. Labouesse and R. Plasterk, unpublished data). We did not detect any staining, although the same embryos showed staining with the monoclonal antibody MH27 (Priess and Hirsh, 1986; Waterston, 1988), indicating that they had been permeabilized successfully. Second, we performed competition experiments. Embryos, larvae at all stages and adults were stained by indirect immunofluorescence with anti-LIN-26 antibodies (see below) in the presence of varying concentrations of the MBP/NLIN-26 protein (varying from 0 to a 200-fold molar excess of this competitor protein). Cells in which staining was effectively competed were deduced to be cells that expressed *lin-26*, either steadily such as hypodermal cells, support cells and the gonad, or, transiently such as a few key neuroblasts - ALM/BDU, Pn.a and V5.paa - to which we paid particular attention. Staining was not competed by a MBP fusion protein made with an unrelated protein.

Immunofluorescence of whole-mount animals

To synchronize larvae, we treated N2 hermaphrodites with hypochlorite and allowed embryos to hatch in M9 buffer (see Wood et al., 1988); this procedure results in larvae arrested at the beginning of the L1 stage. We next added bacteria and removed samples every other hour. To synchronize embryos, we allowed N2 hermaphrodites to lay eggs for 30 minutes and then collected eggs at 30 minute intervals. Larvae and adults were fixed as described by Finney and Ruvkun (1990); embryos were fixed as described by Krause et al. (1990). Anti-LIN-26 purified antibodies were incubated with fixed animals overnight at room temperature and were revealed with FITC-coupled mouse anti-rabbit IgG antibodies (Cappel). Diamidinophenolindole (DAPI) at 2 μg/ml was included during the second antibody incubation (also overnight at room temperature) to visualize nuclei (Wood et al., 1988). Animals were mounted on a slide with phenylenediamine in 50% glycerol/50% PBS (8.4 mM $NaH_2PO_4 . 2H_2O$/ 16.6 mM Na_2HPO_4/ 7.3% NaCl) and observed with a Zeiss Axioplan microscope equipped with fluorescence capabilities.

Identification of stained nuclei in embryos

In 240-minute or 350-cell-stage embryos (see Sulston et al., 1983), when cells of the external body epithelium (seam cells, Pn cells, hyp4-hyp7, hyp11, XXX) are generated, we detected strong immunostaining only in these cells, their neuroblast sisters (see text) and the ectoblasts ABplpppap and ABprpppap. In embryos containing 28-350 cells, our identifications of cells that express LIN-26 as progenitors of the external body epithelium cells were indirect and based on the following considerations. (1) One cell division prior to the 240-minute stage, in embryos with approximately 190 nuclei, we detected staining in 39.9±6.1 cells (n=44). These cells, which did not stain as strongly as in 240-minute embryos, were located on the posterior and dorsal surface of the embryo. Because of their number and positions, we presume that they correspond to the 39 ectoblasts present at this stage that generate the cells of the external body epithelium (Sulston et al., 1983). (2) About two cell divisions prior to the 240-minute stage, in embryos with approximately 130 nuclei, we detected staining in 21.2±4.8 cells located dorsally and posteriorly (n=18). In embryos with 90-100 nuclei, we detected very weak staining in 11.4±5.4 similarly located cells (n=36). Based upon the number and positions of the stained cells in 130-cell embryos, we believe that these cells are the 23 ectoblasts (Sulston et al., 1983) that generate first the 39 ectoblasts mentioned above and subsequently the cells of the external body epithelium. We conclude that expression of LIN-26 protein commences in the AB and C cell lineages two cell divisions prior to the generation of the cells of the external body epithelium, for some ectoblasts (about 11) shortly after the sixth AB and third C division and for others (the remaining 12) slightly later. Prior to the 80-cell stage, we could detect LIN-26 protein only in the germline lineage (P_0-P_4, Z2 and Z3) and in the germline of gravid adults.

We could detect LIN-26 protein in the lineages that generate the rectal and tail hypodermal cells only after 240 minutes of development. We first observed staining of the tail ectoblasts ABplpppap and ABprpppap (see above). As these cells divided, we then detected staining of the ectoblasts ABplpppapa and ABprpppapa as well as of their sisters, which undergo programmed cell death, and subsequently of the ectoblasts that generate the rectal cells (K, K′, B, F, Y, U) and of the ectoblasts ABplpppppp and ABprpppppp (mothers of the hyp10 and tail-spike cells, which later express LIN-26 protein). We first detected LIN-26 protein in the excretory cell, excretory duct cell and G1, immediately after the generation of these cells. Similarly, we first detected LIN-26 protein in neural support cells and hypodermal cells of the head after or possibly immediately prior to the generation of these cells, that is after 290 minutes of development based on the pattern of interdigitation of the dorsal hyp7 nuclei and on the presence of the rectal cells, which are generated at about 285 minutes of development.

Nomarski microscopy

ML87 hermaphrodites were allowed to lay eggs. About 20 embryos that looked like lima bean embryos under the dissecting microscope were picked, mounted for microscopy on a 5% agar pad in M9 and covered with a coverslip, which was sealed. Embryos were superficially examined until the first two or three *lin-26(mc4)* could be distinguished, that is when embryos reached the comma to 1.5-fold stage of embryogenesis. Those mutant embryos were followed for the next 7 hours. Each embryo was examined at 30 minute intervals; the position and aspect of the hypodermal nuclei H2 and H1, of the amphid sheath nucleus and of the surrounding neuronal nuclei were drawn precisely. After 7 hours, non *lin-26(mc4)* siblings, which initially were at the lima bean stage as well, hatched, behaved normally, and had normal H2, H1 and amphid sheath nuclei, indicating that embryos had not been damaged by experimental conditions.

Staining sensory neurons with DiO

Animals were stained with 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) as described by Perkins et al. (1986) and Herman and Hedgecock (1990). Animals were examined with a Zeiss Axioplan microscope equipped with fluorescence capabilities (Zeiss FITC filter 487910, excitation range 450-490 nm).

Assay for ability to form dauer larva

We assayed the ability of animals to form dauer larvae on plates containing different quantities of dauer pheromone preparations as described by Golden and Riddle (1982), with the following modifications. Plates with dauer pheromone contained 1.7% agar, 0.3% NaCl and 5 μg/ml cholesterol. We placed 50 to 100 eggs in M9 buffer, which were obtained by treating adults with bleach (Wood et al., 1988), on Petri dishes containing the dauer pheromone and incubated the plates for 48 hours at 25°C in a humidified chamber.

Electron microscopy

Animals were prepared for electron microscopy as described by Bargmann et al. (1993).

RESULTS

LIN-26 protein is present in hypodermal cells and in neuronal support cells

We raised polyclonal antibodies against LIN-26 protein in rabbits (see Materials and Methods) and used these antibodies to examine the distribution of LIN-26 protein by immunofluorescence in whole mounts of wild-type *C. elegans*. We identified the cells that reacted with LIN-26 antibodies in embryos based on comparisons with existing cell maps (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston et al., 1983) and by using monoclonal antibody MH27, which recognizes an adherens junction protein surrounding hypodermal cells (Waterston, 1988). Because we stained larvae and embryos that had been synchronized, we could relate the observed staining patterns to specific cells at precise developmental stages. For early embryonic stages (28 to 350 cells), there are no published cell maps. For those stages, we identified cells expressing LIN-26 protein as the ectoblasts that generate the external body epithelium by correlating the positions and number of the stained cells (see Materials and Methods for details) with the known number of hypodermal precursor cells (Sulston et al., 1983) and their predicted positions.

In brief, we found LIN-26 protein in all hypodermal cells, in all neuron-associated support cells and in the somatic gonad. In all cases, antibodies against LIN-26 protein stained the nucleus, as appropriate for a transcription factor.

More specifically, we found LIN-26 protein to be continuously present throughout embryonic, larval and adult life in all hypodermal cells (Figs 1A,E,G,I, 2A,B) and in all of the glial-like socket and sheath cells associated with ciliated sensory organs (Figs 1A,J, 2C,D). LIN-26 protein was also present in the blast cells G1 (Fig. 1A), Q (data not shown) and W (Fig. 1A). These three cells have hypodermal features in young L1 larvae but subsequently divide and generate only neurons (Sulston and Horvitz, 1977; Sulston et al., 1983). Expression in all three of these cells ceased as they divided. In the somatic gonad, strong expression of LIN-26 protein was detected in the progenitor cells Z1 and Z4 in young L1 larvae (Fig. 1C); weak expression was detected in all cells in L2 larvae except the distal tip cells, and in the uterine cells of L4 larvae and adults (data not shown). All hypodermal nuclei were stained with about the same intensity. Socket and sheath nuclei (except the amphid sheath nuclei) were less intensely stained than were hypodermal nuclei.

As judged by the presence of LIN-26 protein, zygotic *lin-26* expression was initiated at distinct stages relative to the time of cell differentiation. Specifically, *lin-26* expression occurred in the AB and C lineages that generate hypodermal cells of the external body epithelium two cell divisions prior to the generation of these cells; in the cells that form the rectum and the tail hypodermis expression occurred one division prior to the generation of these cells; in the excretory cell, the excretory duct cell, the excretory pore cell, the neural support cells and cells of the head hypodermis, expression was found in the differentiating cells themselves (or possibly immediately prior to the formation of these cells) (Fig. 2). In the cells Z1 and Z4, which generate the somatic gonad, *lin-26* expression started approximately 3 hours after these cells were generated (data not shown).

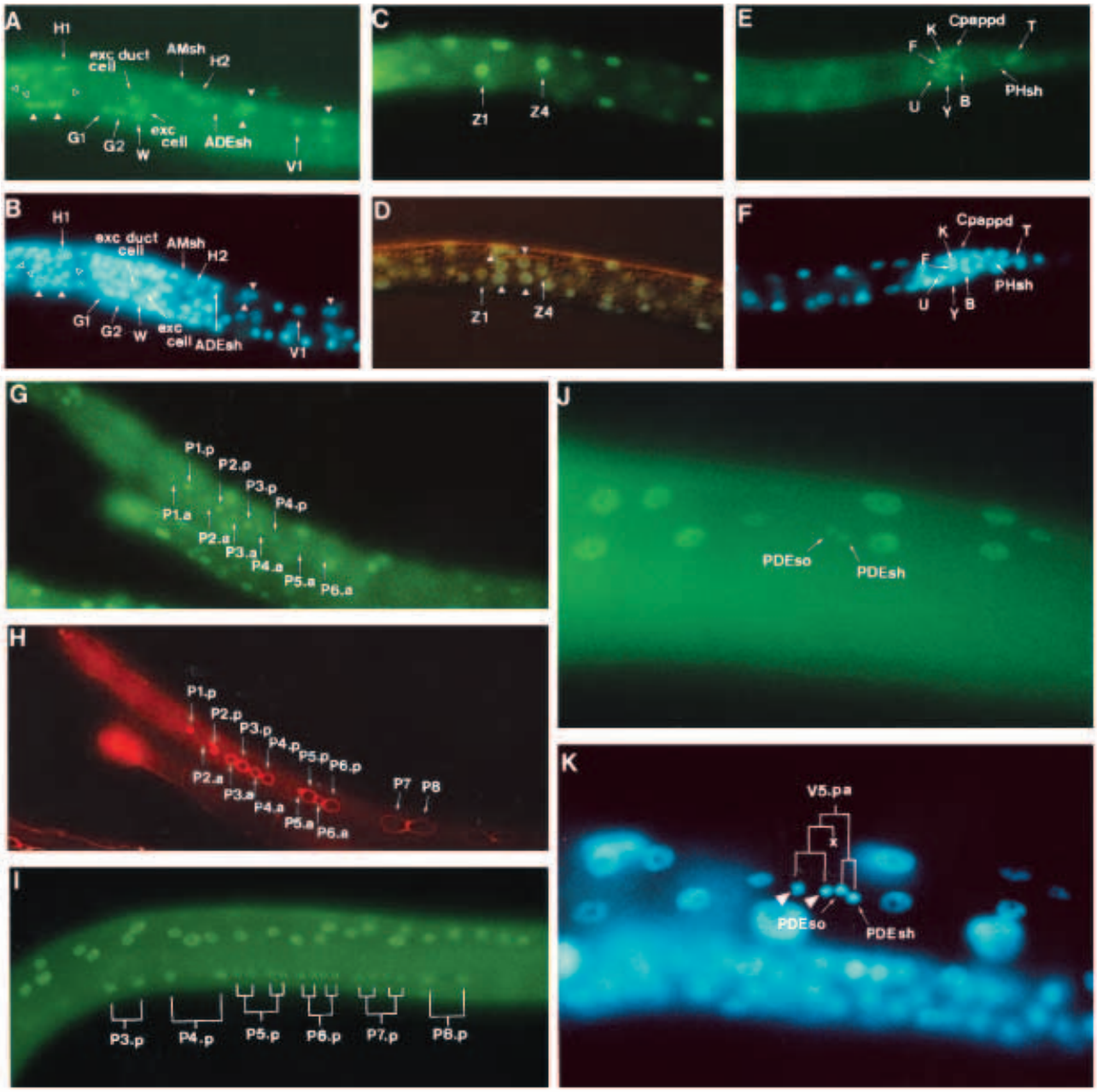
LIN-26 protein is symmetrically segregated during asymmetric cell divisions

We examined the fate of LIN-26 protein during the course of asymmetric cell divisions that generate one daughter cell which will express LIN-26 protein and one daughter cell which will not express LIN-26 protein. In each case studied, we found that LIN-26 protein was present in mother cells, subsequently present in both daughter cells, and then lost from the daughter cell that adopts the neuronal fate. For example, in embryos approximately 240 minutes of age, LIN-26 protein was transiently detected in the neuroblasts that generate the neurons ALM and BDU (Fig. 2A,B), the neuroblasts that are the grandmothers of the PLM and ALN neurons, and in the PVR neurons (data not shown). Later, LIN-26 protein was detected in neuroblasts, such as Pn.a (Fig. 1G,H), T.pp, K.p, V5.paa, and G2.a (data not shown). LIN-26 protein could be detected in those cells for only a short period (Fig. 1G,H), except in V5.paa, in which it could be detected very faintly until that cell divided (data not shown).

The mutation *lin-26(n156)* affects one of these asymmetric cell divisions: the Pn.p cells, which normally become hypodermoblasts, instead adopt neural fates in *lin-26(n156)* mutants (Ferguson et al., 1987; Labouesse et al., 1994). We examined the segregation of LIN-26 protein during P cell divisions in *lin-26(n156)* L1 larvae. We found that LIN-26 protein was segregated normally to the two P daughter cells (data not shown) but was subsequently lost from both the Pn.a and Pn.p cells, rather than from only the Pn.a cells. Specifically, in the ventral cords of *lin-26(n156)* larvae beyond the L1 stage, an average of only one cell expressed LIN-26 protein (0.9 stained Pn.p cells, n=69, in animals grown at 20°C; 1.2, n=140, in animals grown at 25°C; for comparison, in the ventral cords of wild-type L2 and young L3 larvae, we counted 12 stained Pn.p cells, n=37, at 20°C, and 11.9, n=83, at 25°C). These observations suggest that an abnormality in the activity or the stability of the LIN-26(n156) mutant protein rather than in its segregation that causes *lin-26(n156)* mutant phenotype.

Glial-like cells might die or change fates in *lin-26* loss-of-function mutants

To determine if *lin-26* expression in the glial-like socket and sheath cells is biologically important, we took advantage of the fact that staining with anti-LIN-26 antibodies remained strong in the mutants *lin-26(mc1)* and *lin-26(mc4)* (this observation is consistent with the fact that none of these mutations alters the region of the protein detected by anti-LIN-26 antibodies; see Labouesse et al., 1994). This staining allowed us to assess the number of LIN-26-positive glial-like cells in these mutants. As shown in Table 1 (4.5-7.0 hours after egg laying), shortly after the generation of hypodermal and glial-like cells, the number of cells expressing LIN-26 protein in *lin-26(mc1)* or *lin-26(mc4)* homozygous embryos was essentially normal. By contrast, by the end of the normal period of embryogenesis (9.5-12.0 hours after egg laying), this number was significantly reduced throughout *lin-26* embryos; for example, in *lin-26(mc4)* embryos, about 15% of the cells that normally express



LIN-26 protein could not be detected with anti-LIN-26 antibodies.

Several explanations could account for this observation. First, LIN-26-expressing cells could cease expressing LIN-26 protein without changing fates. Alternatively, they could cease expression because they are dying or changing fates. Using Nomarski microscopy, we previously observed that hypodermal cells die or, less frequently, become neurons in homozygous *lin-26(mc1)* and *lin-26(mc4)* embryos (Labouesse et al., 1994). Thus, for the body hypodermal cells, the data in Table 1 support our previous finding that these cells die.

We believe that glial-like cells also die or change fates in strong loss-of-function *lin-26* mutants. Using Nomarski microscopy, we focused on one particular support cell, the amphid sheath cell in the head, the nucleus of which has a distinctive size (almost as large as a hypodermal nucleus) and morphology (slightly grainy with a small but visible nucleolus). We followed 18 *lin-26(mc4)* homozygous embryos for approximately 7 hours starting at the comma stage and ending when normal embryos would hatch. The amphid sheath was initially present in each of these embryos. The morphology of the amphid sheath nucleus remained essentially unchanged in eight embryos; in two embryos its outline became almost invisible; in seven embryos its size had shrunk to the size of the largest neuronal nuclei and its morphology was becoming more neuronal-like; in one embryo, it could not be distinguished from neighboring neuronal nuclei (data not shown). Changes could be seen 4-5 hours after embryos had reached the comma stage. For comparison, hypodermal nuclei appeared similarly affected (for instance, in six embryos the H1 nucleus appeared unchanged; in seven embryos its outline was almost invisible; in three embryos it was severely

Fig. 1. Localization of LIN-26 protein in larvae and adults. Wild-type animals were stained with anti LIN-26 antibodies (A,C,E,G,I,J), the monoclonal antibody MH27 (H) or DAPI (B,D,F,K), as described in Materials and Methods. Anterior is to the left, and dorsal is up (except for the animals in C/D and G/H, which are ventral side up). (A) Ventral and left lateral anterior part of a young L1 animal (from the nerve ring to the V1 seam cell) showing LIN-26 protein in cells of the excretory system (excretory cell, excretory duct cell, G1, G2, W), the amphid sheath (AMsh, slightly out of focus) and the deirid sheath (ADEsh) cells, the seam cells H1, H2 and V1, the main hypodermal syncytium hyp7 (closed triangles), the OLL sheath, CEP socket and CEP sheath cells (open triangles from left to right, respectively). (B) DAPI staining of the animal in A (arrows and triangles point to nuclei stained in A). (C) 6-hour old L1 larva showing LIN-26 protein in Z1 and Z4, the somatic gonad precursor cells. (D) DAPI staining of the animal in C. This picture was taken using fluorescence and Nomarski optics to visualize nuclei and the gonad (arrows point at Z1 and Z4; triangles point at germline nuclei). (E) Middle focal plane of the posterior part of the L1 larva in A, showing LIN-26 protein in cells of the anus (B, F, and slightly out of focus Y, U), the rectum (K; its contralateral homolog K′ is not in this focal plane), the left seam cell T (slightly out of focus), the left phasmid sheath cell (PHsh; slightly out of focus) and in a nucleus of the main hypodermal syncytium hyp7 (Cpappd). (F) DAPI staining of the animal in E (arrows point to nuclei stained in E). (G) Ventral surface of an 8-10 hour old L1 larvae, showing LIN-26 protein both in the anterior neuroblasts Pn.a and in the posterior hypodermoblasts Pn.p. The 12 Pn cells divide mostly in sequence, with P1 first and P12 last (Sulston and Horvitz, 1977). In this animal, in which P1-P6 had divided, LIN-26 protein appeared to be as abundant in P4.a as it is in P4.p, but more abundant in P4.a than in P3.a, in P3.a than in P2.a, and in P2.a than in P1.a, in which it was barely detectable. Conversely, the amount of the LIN-26 protein seemed higher in P1.p than in P4.p. The LIN-26 protein can also be seen in P5.a and P6.a but not in P5.p and P6.p, which are slightly out of focus. (H) Same larva as in G stained with the anti-adherens junction monoclonal antibody MH27 (Waterston, 1988). An adherens junction surrounds the P cells and their daughters, the Pn.a and Pn.p cells. The adherens junction that surrounds the Pn.a cells rapidly disappears. The adherens junction that surrounds P1.p and P2.p also disappears when they fuse with the main hypodermal syncytium. (I) Ventral and left lateral central body region of an L3 larva, showing LIN-26 protein in cells derived from the Pn.p hypodermoblasts (identified by lineages), seam cells and nuclei of the main hypodermal syncytium hyp7 (all other nuclei). (J) Region surrounding the right postdeirid of a young adult showing LIN-26 protein in the postdeirid socket (PDEso) and sheath (PDEsh) cells and in the surrounding seam cells and hyp7 hypodermal syncytium (all other nuclei). (K) DAPI staining of the animal in J. The lineage of V5.pa that generates the postdeirid is shown. Note that the postdeirid neurons (arrowheads) are not stained.

shrunken; in two embryos it had a neuronal-like morphology), while neighboring neuronal nuclei did not appear to be affected. These results strongly indicate that the amphid sheath cell, like the H1 hypodermal cell, requires *lin-26* function during embryogenesis. Although we focused on only one class of neuron-associated support cell, we think that the results reported in Table 1 can be explained most simply by the hypothesis that, like hypodermal cells, many support cells die or perhaps are transformed into neurons in strong *lin-26* loss-of-function embryos (Labouesse et al., 1994). Our results suggest that the development or maintenance of glial-like cells requires *lin-26* activity.

The mutation *lin-26(n156)* affects the function of certain sensory organs

To assess further the requirement for *lin-26* in support cells,

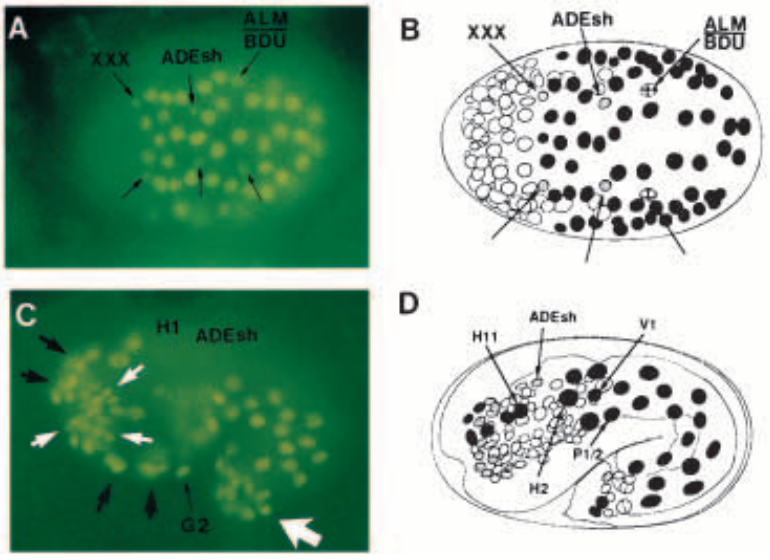


Fig. 2. Localization of LIN-26 protein in embryos. Wild-type embryos were fixed and incubated with anti-LIN-26 antibodies (A,C) and visualized as described in Materials and Methods. Anterior is to the left. (A) Dorsal surface of a 350-cell embryo showing strong LIN-26 staining in all newly generated hypodermal cells and in the deirid sheath cells (ADEsh). LIN-26 protein was also detectable in the neuroblasts ABarppaapp and ABarpppapp (designated as ALM/BDU, because they are the mothers of the neurons ALM and BDU), the sisters of the hypodermal cells ABarppaapa and ABarpppapa, respectively. This staining of the ALM/BDU mothers was transient. The six arrows point to the nuclei of the bilateral XXX ADEsh cells, embryonic hypodermal cells and ALM/BDU neuroblasts, which are smaller, show less intense staining and are easily recognizable landmarks. There was no expression in the anterior part of the embryo, where are located the precursors of the support cells and head hypodermal cells. (B) Schematic drawing adapted from Sulston et al. (1983) showing the positions of the dorsal hypodermal nuclei (black nuclei) in a 350-cell embryo; the XXX and ADEsh nuclei are shown in grey. The ALM/BDU nuclei are cross-hatched. (C) Lateral surface of a comma-stage embryo showing LIN-26 expression in hypodermal cells, glial-like support cells in the head (smaller nuclei; three white arrows) and hypodermal cells in the tail (single large white arrow). Other large nuclei in the head (four black arrows) and the body region (unlabeled nuclei) correspond to hypodermal cells generated at 240 minutes of development (the positions of the seam nucleus H1 and of the ADEsh nucleus are shown for orientation). (D) Schematic drawing adapted from Sulston et al. (1983) showing the positions of the lateral nuclei in a comma-stage embryo. Hypodermal nuclei (black), sheath and socket nuclei (grey). The embryo shown in C had been slightly flattened at mounting such that the hyp7 nucleus found immediately anterior to P1/2L, V1, H2 and the two hyp7 nuclei found dorsally to V1 and H2 were out of focus, while the G2 nucleus, six dorsal and six ventral head hypodermal nuclei (black arrows in panel C) and several additional tail hypodermal nuclei can be seen in C but are not indicated in the drawing shown in D.

Table 1. The number of LIN-26-expressing cells is reduced at the end of embryogenesis in strong *lin-26* mutant embryos

Time after egg laying (hours)	Genotype					
	wild-type		*lin-26(mc1)*		*lin-26(mc4)*	
	head	body	head	body	head	body
4.5-7.0	65.0±2.1	42.7±0.9	63.4±4.1	41.2±1.9	63.9±6.7	40.1±2.4
7.0-9.5	ND	ND	61.9±4.6	40.1±2.7	60.5±5.5	39.1±2.4
9.5-12.0	66.0±1.2*	40.7±1.3*	59.1±6.1	38.8±3.8	55.6±7.4	34.8±5.4

Gravid unstarved N2, ML87 or ML93 hermaphrodites were transferred to a Petri plate, allowed to lay eggs for approximately 150 minutes and then removed from the plate. After the amount of time indicated in the left column, embryos were collected and processed for staining with MH27 and anti-LIN-26 antibodies (see Materials and Methods). Normally, hermaphrodites lay eggs about 100 minutes after fertilization; thus eggs collected 4.5 to 7.0 hours after egg laying were lima-bean- to two-fold stage embryos; eggs collected 7.0 to 9.5 hours after egg laying were two-fold stage to young pretzel embryos, at the stage when pretzels begin to synthetize their cuticle; eggs collected 9.5 to 12.0 hours after egg laying were young pretzel to pretzel embryos ready to hatch (for embryonic stages, see Sulston et al., 1983). *lin-26(mc1)* and *lin-26(mc4)* embryos were distinguished from heterozygous and homozygous balancer siblings using the monoclonal antibody MH27: such *lin-26* embryos do not elongate beyond the 1.5-fold stage of embryogenesis and show abnormal head morphogenesis (Labouesse et al., 1994). When eggs were collected 7.0 hours after egg-laying, all non-*lin-26* siblings had developed beyond the 1.5-fold stage. When eggs were collected 4.5 to 7.0 hours after egg laying, a substantial fraction of normal siblings were still at the comma stage, making the distinction between normal and mutants embryos more difficult. In this first row, we counted only embryos with abnormal heads and have almost certainly disregarded mutant embryos because they had a normal head. For this reason, we believe that the staining defects reported in the row 4.5-7.0 hours, which are already statistically not different from wild-types for head counts, are likely to be even less severe. For cell counts, embryos were divided in three sectors: head (from the nose to the ADEsh nucleus), which contains more support cell nuclei than hypodermal nuclei; body (from the ADEsh nucleus to the V6 nucleus), which contains only hypodermal nuclei; and tail (from the V6 nucleus to the tip of the tail; data not shown). More than 35 embryos were counted for each time interval. The mean number of stained nuclei ± standard deviation is given. Using a *t*-test to compare distributions, we found that the number of nuclei in the heads of young *lin-26* embryos were not statistically different from the number in 4.5-7.0 hour N2 embryos; differences between old *lin-26* embryos and young N2 embryos or young N2 L1 larvae were statistically significant (P<0.001). Differences between young *lin-26* and old *lin-26* embryos were statistically significant (P<0.001).

*For wild-type, because in pretzel embryos it is more difficult to reliably determine if a given nucleus is in the body, the head or the tail, we chose to stain L1 larvae in which seam cells had not yet divided; 20 young L1 larvae were analyzed.

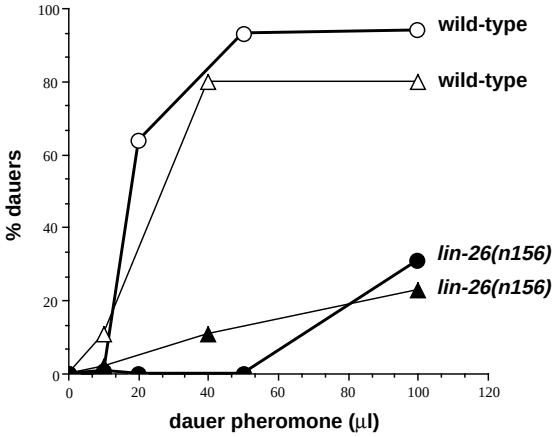


Fig. 3. *lin-26(n156)* mutants are defective in dauer-larva formation. The ability of wild-type and *lin-26(n156)* animals placed on a Petri plate containing a fixed amount of food and increasing amounts of a dauer pheromone preparation was assayed as described in Materials and Methods. Open symbols, wild-type animals (N2); closed symbols, *lin-26(n156)* animals. Triangles and circles correspond to two different dauer pheromone preparations; wild-type and *lin-26(n156)* animals were treated in parallel with each preparation.

we examined the functions of two classes of sensory organs, the amphids in the head and the phasmids in the tail, in the viable mutant *lin-26(n156)*. The amphids and phasmids are bilateral chemosensory organs (Ward et al., 1975; White et al., 1986; Bargmann and Horvitz, 1991b; Hall and Russell, 1991; Bargmann et al., 1993) that to function normally require the structural integrity of both neurons and their glial-like support cells (Perkins et al., 1986).

12 of the 24 amphid neurons and all 4 phasmid neurons can normally be stained by the carbocyanine dye DiO (Perkins et al., 1986; Herman and Hedgecock, 1990). In most *lin-26(n156)* adults, amphid and phasmid neurons failed to stain with DiO on one or both sides (Table 2A). This staining defect was caused by the *lin-26(n156)* mutation, since a plasmid containing the wild-type *lin-26* gene almost completely rescued the staining defect of the *lin-26(n156)* strain (Table 2A). DiO-staining defects became worse during development, particularly in amphids, as the staining of young *lin-26(n156)* larvae was almost normal (Table 2B). This last observation could reflect the fact that *lin-26(n156)* causes only a partial loss of *lin-26* function (Labouesse et al., 1994). Alternatively, *lin-26* might be necessary to maintain the fate of support cells.

Other mutants that fail to stain with DiO are known to be defective for aspects of development and behavior mediated by the amphid neurons (Perkins et al., 1986; Starich et al., 1995), such as dauer formation (Golden and Riddle, 1982; Bargmann and Horvitz, 1991a; Vowels and Thomas, 1992), osmotic avoidance (Culotti and Russell, 1978; Bargmann et al., 1990) and chemotaxis to soluble chemicals (Bargmann and Horvitz, 1991b). We tested *lin-26(n156)* animals for their ability to undergo dauer formation (Fig. 3) and to avoid high concentrations of glycerol (data not shown). Both were defective, suggesting that the functions of all exposed amphid neurons might be abnormal in *lin-26(n156)* animals. Fig. 3 shows that, at a given concentration of a dauer pheromone preparation, fewer *lin-26(n156)* animals formed dauer larvae than did wild-type animals exposed to the same conditions. The decision to form a dauer larva occurs during the late L1 and the L2 stages, yet L1 and L2 *lin-26(n156)* amphids and phasmids seemed normal by dye-filling criteria. These findings suggest that dauer formation is a more sensitive assay of amphid function than is dye filling.

The *lin-26(n156)* mutation affects the structure of most support cells

To correlate the sensory organ functional abnormalities described above with possible structural defects and more generally to examine sensory organs that we had not tested functionally, we examined the ultrastructure of seven of the eight classes of ciliated sensory organs (amphid, cephalic, OLQ outer labial, OLL outer labial, inner labial, deirid and

Table 2. *lin-26(n156)* mutants are defective in the staining of amphids and phasmids by DiO

A

Genotype	Amphid staining: no. cells	2-sides	1-side	0-side	Phasmid staining: no. cells	2-sides	1-side	0-side	n
wild-type	11.5	99%	1%		3.9	96%	4%		84
lin-26(n156)	6.6	46%	40%	14%	2.2	42%	28%	30%	90
lin-26(n156) and *lin-26(+)* transgene*	11.6	95%	5%		3.3	74%	20%	6%	86

B

	Number of cells stained									
	Amphid					Phasmid				
Genotype	L1	L2	L3	L4	Adult	L1	L2	L3	L4	Adult
wild-type	9.4	9.6	10.7	11.3	11.7	1.0	3.2	4	3.9	3.9
lin-26(n156)	8.5	8.2†	7.1†	8.4†	6.0†	1.8	3.4	3.3	3.4	2.9†

Animals were stained with DiO as described in Materials and Methods. Strains used were N2 (wild-type), *lin-26(n156)* and *lin-26(n156)* transformed with a *lin-26(+)* plasmid (pMLW006; Labouesse et al., 1994) together with the plasmid pRF4, which confers a dominant roller phenotype (Mello et al., 1992). In the latter case only roller, i.e. transgenic, animals were examined. Experiments presented in the two parts of this Table were performed with different batches of DiO, which is presumably the source of the small variations in the number of cells stained. (A) An amphid (six neurons can be stained on each side) or a phasmid (two neurons can be stained on each side) was considered to be stained if half or more of its neurons were stained. (B) The average number of amphidial and phasmidial neurons that were stained is presented for each developmental stage. At least 50 animals were examined for each stage. We reproducibly observed that wild-type L1, L2 and, to a lesser extent, L3 larvae took up DiO less efficiently than L4 larvae and adults. This difference might reflect the fact that certain neurons terminally differentiate late during postembryonic development.

*These animals were also scored for the presence of a vulva: 74 of the roller *lin-26(n156)* animals had a vulva, four had no vulva, and the remaining eight could not be scored because of their orientation on the slide. For comparison, the vulvaless phenotype caused by the *n156* mutation is 100% penetrant.

†Differences between wild-type and *lin-26(n156)* staining were significant at $P<0.01$ for L3, L4, and adult heads and for adult tails, at $P<0.02$ for L2 heads.

phasmid sensilla) in three *lin-26(n156)* animals (for descriptions of these organs in the wild-type, see Ward et al., 1975; White et al., 1986). We found that the structures of several classes of sensory organs were affected and that the amphids and the phasmids were more severely affected than were other sensory organs.

Specifically, we observed that ciliated endings failed to reach the environment in all six amphids (Fig. 4A,B), five phasmids (not shown), two of twelve CEP sensilla (Fig. 4F,G) and one of six OLL sensilla (not shown). These defects could well have resulted from abnormalities in the sheath and socket cells, since these cells were often observed to be abnormal. The socket and sheath cells showed an abnormal presence of electron-dense (Fig. 4B-D) material and sometimes failed to interconnect, causing sensory endings to end blindly within the sheath cell (Fig. 4C).

These ultrastructural abnormalities seem sufficient to account for the three sensory organ functional defects that we described above: (1) defects in dye filling; (2) defects in dauer-larva formation and (3) defects in the avoidance of high osmolarity solutions. These ultrastructural and functional defects of *lin-26(n156)* animals are very similar to those caused by mutations in the genes *daf-6* and *che-14*, which affect support cells (Perkins et al., 1986; Herman, 1987) .

DISCUSSION

Using antibodies against LIN-26 protein, we identified the cells in which this protein is expressed, determined the points in the lineage histories of those cells at which LIN-26 protein expression is initiated and established that, during certain asymmetric cell divisions, a LIN-26-containing mother cell generates two cells that transiently contain LIN-26 protein of which only one daughter cell continues to express LIN-26 protein. These studies revealed that LIN-26 protein is expressed not only in hypodermal cells but also in glial-like cells, the only other class of non-neuronal ectodermal cells in the animal, as well as in several cells of the somatic gonad. We showed that the expression of LIN-26 protein in glial-like cells is important for the development of those cells. Specifically, we found that in strong *lin-26* loss-of-function mutants, which lack most or all *lin-26* function and arrest development as embryos, one class of glial-like cell - the amphid sheath cells - were frequently abnormal in morphology, as if these cells were dying, becoming ambiguous in their cell identities or being transformed into neuron-like cells. Futhermore, other glial-like cells were also abnormal in strong *lin-26* loss-of-function mutants, as the number of these cells that could express LIN-26 protein was reduced. We also found that, in *lin-26(n156)* mutant animals, in which *lin-26* function is reduced but not eliminated, glial-like cells were both structurally and functionally abnormal. Based upon these observations, we conclude that one essential aspect of *lin-26* function is to ensure not only the normal development of hypodermal cells, as we reported earlier (Labouesse et al., 1994), but more generally the normal development of all non-neuronal ectodermal cells in *C. elegans*.

lin-26 is expressed both before the onset of differentiation and in differentiated cells

Our immunocytochemical studies indicate that the expression of *lin-26* in both hypodermal cells and glial-like cells is initiated at the times that these cells are generated or one to two cell divisions prior to their generation. *lin-26* expression in these cells is maintained throughout the lifetime of the animal. These kinetics of *lin-26* expression suggest that *lin-26* might act before hypodermal and glial-like cells express their fates and continue to act within these cells after they have differentiated. Similarly, in the somatic gonad, *lin-26* expression was detected until the L2 stage, when somatic gonadal cells begin to specialize, and later in uterine cells. (*lin-26* expression in the somatic gonad appears to be biologically important; B. den Boer and M. L., unpublished observations).

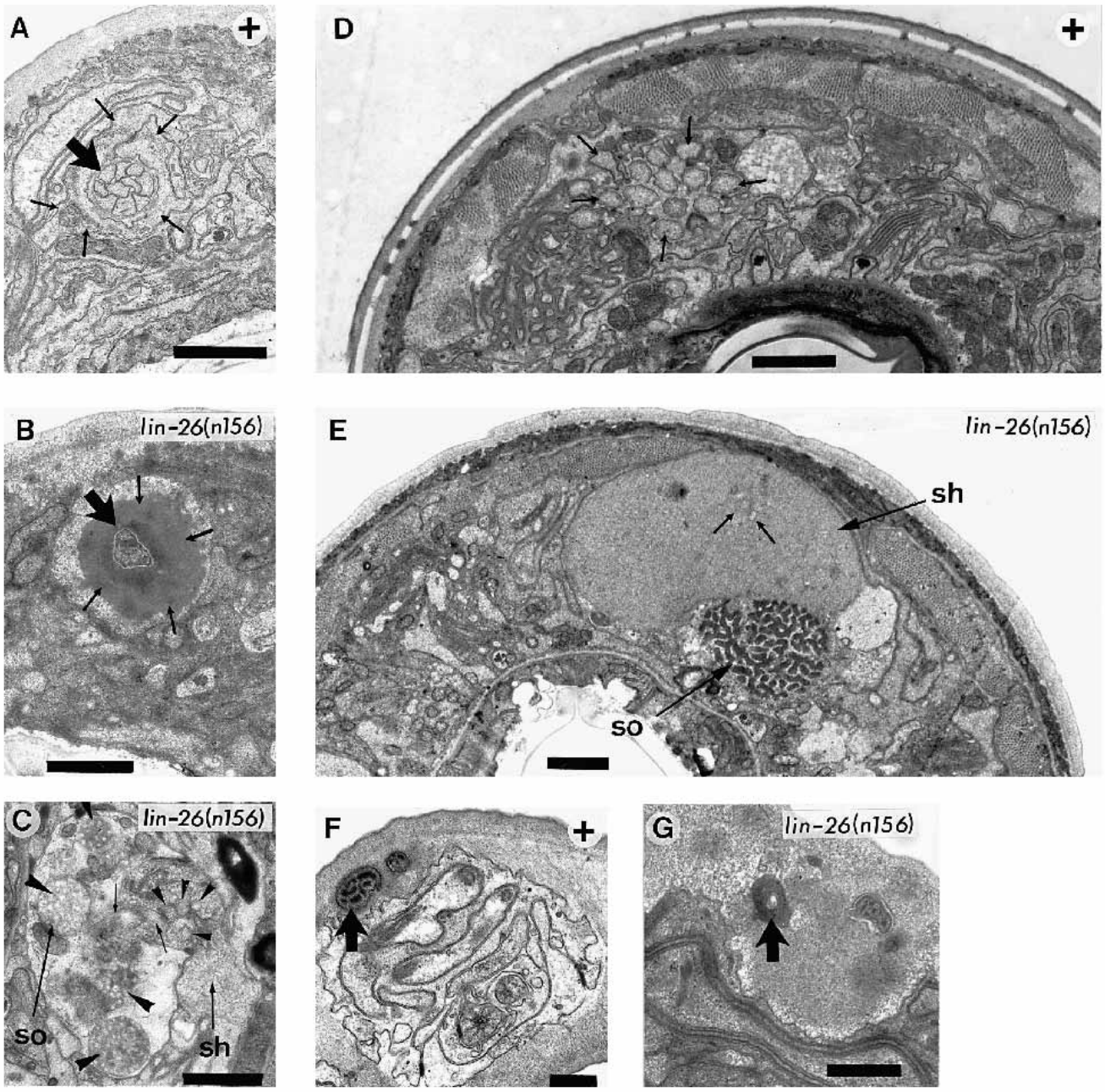


Fig. 4. The mutation *lin-26(n156)* affects the structures of the amphid socket and sheath cells. Electron micrographs of (A,D,F) a wild-type N2 adult grown at 20°C and (B,C,E,G) *lin-26(n156)* adults grown at 25°C (the amphid defect is more penetrant at 25°C than at 20°C). Scale bars, 1 μm (0.5 μm for F and G). The genotype of the sectioned animal is indicated in the upper right corner of each panel: +, wild-type animals. (A) Section of a wild-type animal 4 μm from the nose. At this level, the amphid socket cell (small arrows) forms a channel (large arrow) through which exposed sensory endings run. (B) Section of a *lin-26(n156)* animal at a level similar to the wild-type section in A. The amphid socket cell is completely dark (small arrows), and the amphid channel is empty (large arrow). (C) Section of another *lin-26(n156)* animal 5 μm from the nose at the level of the socket-sheath junction. The amphid socket (so) and sheath (sh) cells have failed to connect, causing some amphid neurons to be deflected laterally (small arrows). The socket cell was filled with round structures (large arrowheads). (D) Section of a wild-type animal posterior to the socket-sheath junction 6.5 μm from the nose. The exposed amphid neurons (small arrows) are beginning to spread within the amphid sheath cytoplasm. (E) Section of the same *lin-26(n156)* amphid as in B, at a level similar to the wild-type section in D. The amphid socket cytoplasm was filled with dark aggregates (so), while the amphid sheath contained a uniformly grey material (sh) that could be the matrix normally secreted by the sheath cell within the amphid channel. The development of most amphid cilia was affected, probably as an indirect consequence of the amphid support cells abnormalities: for instance, the cilia of the exposed amphid neurons (small arrows) are small in this section. (F) Section of a wild-type animal 1.6 μm from the nose. The CEP ending is shown (large arrow). (G) Section of a *lin-26(n156)* animal at a level similar to that in F. The CEP channel was empty (arrow).

lin-26 might control its own expression during asymmetric cell divisions

How sister cells can express different fates is a fundamental issue in developmental biology (reviewed by Horvitz and Herskowitz, 1992). *lin-26* appears to function in such asymmetric cell divisions, since, for example in the *lin-26(n156)* mutant two sister cells that normally express a neuronal and a non-neuronal fate instead both express the neuronal fate (Ferguson et al., 1987; Labouesse et al., 1994).

How might *lin-26* confer such asymmetry? We have found that during a number of asymmetric cell divisions LIN-26 protein is present in the mother cell, segregated symmetrically to both daughter cells, and subsequently lost from the cell that will adopt a neuronal fate. It seems likely that in these cases several classes of proteins could be responsible for generating sister cells, such as those that degrade LIN-26 protein, that repress *lin-26* gene expression in neuronal cells, or that maintain *lin-26* gene expression in non-neuronal cells. It is possible that LIN-26 protein itself plays an essential role in this asymmetry by maintaining its own expression during asymmetric cell divisions. Specifically, we found that, during the divisions of P cells in *lin-26(n156)* mutants, LIN-26 protein is segregated normally but, unlike in wild-type animals, is subsequently lost from both daughter cells. The *lin-26(n156)* mutation affects the presumptive DNA-binding domain of the LIN-26 protein (Labouesse et al., 1994). One hypothesis that could account for the *lin-26(n156)* phenotype is that the LIN-26(n156) mutant protein has acquired a novel function. For example, the LIN-26(n156) protein might bind in the Pn.p cells an element of the *lin-26* promoter capable of repressing *lin-26* expression. In this case, the mutant LIN-26(n156) protein would be responsible for inhibiting *lin-26* expression in the Pn.p cells, leading to the transformation of these cells into neuroblasts or neurons. The completely recessive phenotype of the *lin-26(n156)* mutants (Labouesse et al., 1994) weakly argues against this model. One alternative is that the *lin-26(n156)* mutation causes the mutant LIN-26 protein (or RNA) to be unstable in the Pn.p cells.

Another possibility, given the nature and position of the *lin-26(n156)* mutation, is that this mutation decreases the binding of the mutant LIN-26(n156) protein to its own promoter, thereby reducing *lin-26* expression in Pn.p cells. If so, LIN-26 protein would normally be involved in maintaining its own expression during asymmetric cell divisions. In this case, *lin-26* would be acting like several other autoregulatory transcription factors that control cell differentiation (see Way and Chalfie, 1989). However, such an autoregulatory role for *lin-26* in the maintenance of *lin-26* expression cannot be general throughout development, since LIN-26 protein was expressed at normal levels in embryos homozygous for the strong loss-of-function mutations *lin-26(mc1)* or *lin-26(mc4)*. Determination of the DNA sequences recognized by normal and mutant LIN-26 proteins might help distinguish among these hypotheses.

lin-26 may prevent non-neuronal ectodermal cells from expressing neuronal fates

lin-26 mutant phenotypes, the temporal and spatial patterns of *lin-26* expression and the fact that *lin-26* encodes a presumptive zinc-finger transcription factor together support the hypothesis that *lin-26* acts to specify and/or maintain cell fates. That *lin-26* in many cases is expressed prior to differentiation and that Pn.p hypodermoblasts adopt a neural fate in *lin-26(n156)* animals suggest that *lin-26* functions in the determination of cell fates. However, mutations in *lin-26* cause cells to degenerate rather than to be transformed in their fates: (1) direct observation with Nomarski optics as well as staining with antibodies against the LIN-26 protein (Labouesse et al., 1994 and this study) indicated that many hypodermal and glial-like cells degenerate in the strong *lin-26* loss-of-function mutants *mc1* and *mc4*; although the *lin-26(mc1)* and *lin-26(mc4)* alleles might not be null alleles, their genetic and phenotypic consequences are very similar to those of a recently isolated molecular null allele of *lin-26* (M. L. and R. Plasterk, unpublished observations); (2) the weak *lin-26* loss-of-function mutation *n156* when heterozygous with a deficiency for *lin-26* causes most hypodermal cells to degenerate (Labouesse et al., 1994), and when homozygous caused neuronal support cells to be structurally abnormal (this study); (3) these defects were more severe late compared to early during development, as both the number of cells missing from *lin-26(mc1)* and *lin-26(mc4)* embryos and the proportion of neurons failing to take up the dye DiO in *lin-26(n156)* homozygous larvae increased as development proceeded. These findings provide strong evidence supporting a role for *lin-26* in the maintenance of cell fates.

Hypodermal cells and glial-like support cells appear to have little in common other than the fact that they are both non-neuronal ectodermal cells. In fact, glial-like cells are considered to be more closely related by developmental history to neurons than to hypodermal/epidermal cells in nematodes (Sulston and Horvitz, 1977; Sulston et al., 1983), insects (see Klambdt et al., 1991) and mammals (see Turner and Cepko, 1987). These considerations suggest that one function of *lin-26* may be to prevent non-neuronal ectodermal cells from expressing or maintaining a neuronal fate. For example, *lin-26* might function in the *C. elegans* ectoderm as the human gene REST/NRSF has been proposed to function in human cells: by repressing the expression of neuron-specific genes in non-neural cells (Chong et al., 1995; Schoenherr and Anderson, 1995). Specifically, when *lin-26* is mutated, cells would fail to prevent the expression of neuron-specific characters but at the same time would continue to express hypodermal or glial-like characters, causing their degenerations and deaths. Our observation that a strong mutation in *lin-26* causes normally hypodermal or glial-like cells to adopt abnormal and often ambiguous cell fates supports this model. In addition to its role in maintaining cell fates, *lin-26* may or may not also act in establishing cell fates. If the *lin-26(n156)* allele, which affects the presumptive DNA-binding domain of the LIN-26 protein (Labouesse et al., 1994), simply reduces *lin-26* function, then *lin-26* function is needed for the Pn.p cells to express a hypodermal rather than a neuronal fate; on the contrary, if this mutation confers a novel function to the LIN-26 protein, then normal *lin-26* function may not be involved in this decision.

We have shown that *lin-26* is expressed not only in the non-neuronal ectoderm but also in the somatic gonad (this study) and very recently have obtained evidence indicating that *lin-26* is essential for the development of the somatic gonad (B. den Boer and M. L., unpublished observations). How might *lin-26* act in both the non-neuronal ectoderm and the somatic gonad? One possibility is that *lin-26* acts to establish and/or maintain the expression of specific differentiated characteristics common

to non-neuronal ectodermal cells and the somatic gonad. For example, hypodermal cells, glial-like support cells and the somatic gonad are all topologically open to the external environment. Perhaps *lin-26* controls the expression of a set of genes that function specifically in cells exposed to the environment. Alternatively, *lin-26* might act to control the development of hypodermal, glial-like and gonadal cells by interacting with a common set of co-regulators and hence acting as a component of a common regulatory pathway in all three cell types. The final fate of these different cell types could be controlled in part by such a common pathway and in part by other genes unique to hypodermal, glial-like or gonadal cells. By analogy, a common pathway that involves the *daughterless* gene of *Drosophila* controls neurogenesis and the development of ovarian follicle cells (Cummings and Cronmiller, 1994); *daughterless* also functions in sex determination by interacting with other genes. The identification of the targets of *lin-26* action and of the cell types in which each of these targets is normally expressed should help distinguish between these alternatives.

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