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**SUMMARY**

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1977; Singh and Sulston, 1978). Serial electron micrographic reconstructions show that several cells of the egg-laying apparatus, the dorsal uterine cell, vulval E cells, vulval muscles and the uterine seam cell (utse), form direct attachments to the overlying lateral seam on each side of the animal (Fig. 1B; Newman et al., 1996). In addition, the uterine-vulval cells (uv1 and uv2) connect the vulva to the uterus. At least a subset of these connections between the vulva, uterus and adult hypodermis is probably vital for the structural integrity of the adult egg-laying apparatus. Whether these connections require adult-specific characteristics of the lateral seam is not known. The role of lin-29 in vulval morphogenesis could be restricted to the lateral hypodermal seam cells, reflecting a structural role for the adult seam that is not provided by unfused larval-type seam cells. Alternatively, or in addition, there could be a direct requirement for lin-29 in some of the other vulva-associated cell types.

Consistent with the idea that lin-29 may play a broader role in development than specification of the adult hypodermis, we show that lin-29 protein accumulates in a variety of non-hypodermal cells with known roles in egg laying or vulva formation, including the anchor cell, the vulval cells, the uterine and vulval muscle cells (the sex muscles). We also describe a unique lin-29 allele that appears to affect vulval morphogenesis but not hypodermal terminal differentiation. Finally, we use mosaic analysis to assess the cell-type requirements for lin-29.

MATERIALS AND METHODS

Nematode strains and genetic analysis

Nematodes were cultured as described by Brenner (1974) and the wild-type strain was N2 var. Bristol. Alleles used were as follows and are described by Hodgkin (1997) unless otherwise noted. LGI: lin-29(n719); LGII: lin-29(ga93 and ga94) (gifts of D. Eisenmann and S. Kim, Stanford University), n333, n546, n836, sgt-1(sc13), unc-4(e120); LGIV: lin-3(e1417); LGV: him-5(1490); LGX: lin-4(mal135), mnCl(dpy-10(e128) unc-52(e4441)) is a LGII balancer chromosome that spans the lin-29 locus. ga93 and ga94 were isolated following ethyl methanesulfonate mutagenesis and mapped near the lin-29 locus (D. Eisenmann and S. Kim, personal communication). Unlike lin-29(0) mutants, adult ga94 animals have adult alae. However, approximately 73% of ga94 adults possess an abnormally everted vulva (the Evl phenotype) as do 100% of lin-29(0) animals. unc-4 ga94 hermaphrodites were crossed to sgt-1 lin-29(n333)/mnCl; him-5 males. All non-Unc non-Evl F1 animals segregated mnCl. The remainder of the non-Unc animals resembled ga94; they were Evl but their adult cuticles possessed alae. These animals segregated approximately 1/4 Sqt Lin and 1/4 Unc Evc animals indicating that ga94 fails to complement n333 for the Evl phenotype. Crosses with lin-29(n546) gave similar results. ga93 has a lin-29(0) phenotype and fails to complement lin-29(n333).

Microscopy

Animals were staged by examining the extent of gonad development, which is essentially unaffected by lin-14, lin-28 and lin-29 mutations. Vulval cell divisions were followed in lin-29(n546), n836 and ga94 animals from the L2-to-L3 molt through the L3-to-L4 molt. Vulva morphogenesis was observed at approximately 2 hour intervals from the L3-to-L4 molt until the early L5 or adult stage. Based upon the stage of gonad development, n546 and n836 animals are ‘adult’ following the fourth molt; however, they are referred to as ‘L5’ because they have larval-type cuticle and undergo additional molting cycles. Animals were anesthetized for photography by mounting on agar pads containing 5% sodium azide.

Indirect immunofluorescence of whole-mount worms using antibody to LIN-29 antibodies and the monoclonal MH27 (Francis and Waterston, 1991) was performed as described (Bettinger et al., 1996).

Molecular biology

Exons and intron/exon borders of the lin-29 alleles ga93 and ga94 were PCR amplified from genomic DNA, gel-purified and sequenced using the fmol® sequencing system (Promega). The following primer pairs were used to amplify and sequence the indicated exons, numbered according to Bettinger et al. (1996):

<table>
<thead>
<tr>
<th>Exons amplified</th>
<th>5′ Primer (5′-to-3′)</th>
<th>3′ Primer (5′-to-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AR49:GTCCTGATATGACAGTTGC</td>
<td>AR50:GAACCTTAAGTTTACACAGGTAG</td>
</tr>
<tr>
<td>2</td>
<td>AR47:AATCCCAAGTAGATGTTTADDG</td>
<td>AR48:CTATCTGTGAGATAGACAAAAATTG</td>
</tr>
<tr>
<td>4</td>
<td>AR45:GCTGTATATCCTTAAACCAC</td>
<td>AR46:CGAATCTGATTCTACTAGTTTC</td>
</tr>
<tr>
<td>5</td>
<td>AR37:GAACCACTGATACATTGAGAC</td>
<td>AR38:GCGCATATTGTCAATGTC</td>
</tr>
<tr>
<td>6</td>
<td>AR44:GCTAAGATGATTGTTATAGTAGAA</td>
<td>AR52:GCTGACAGTGACAGTTTGG</td>
</tr>
<tr>
<td>7</td>
<td>AR39:AGGAATGCGAGTACGAGG</td>
<td>AR40:GATTTTCTAGATCCTTAC</td>
</tr>
<tr>
<td>11</td>
<td>AR62:CCAAATACTAACCAGCTGGGACCC</td>
<td>AR17:GCGATACTTCGAGGGCTGA</td>
</tr>
</tbody>
</table>

A lin-29B minigene was constructed by fusing a 5.6 kb genomic fragment including lin-29B promoter sequences (Bettinger et al., 1996) in frame to a lin-29 cDNA at the EcoRI site of exon 6 relative to lin-29A. The 1.6 kb genomic EcoRI fragment from the 3′ end of lin-29 was added to provide sequences for 3′ end formation. Transgenic animals were generated by injecting plasmid p29BGM, containing the minigene, with pRF4 (rol-6(su1006d) Mello et al., 1991) as a transformation marker, into lin-29(n836)/mnCl animals. We obtained six independent lines and, from each, derived a strain homozygous for lin-29(n836) and bearing an extrachromosomal array. Complete rescue of the lin-29 mutant phenotype was observed in every line and dominant effects of the lin-29B minigene were not seen.

Mosaic analysis

Mosaic analysis was performed by scoring for mitotic loss of an extra-chromosomal array (Lackner et al., 1994; Miller et al., 1996) carrying lin-29(0)+. Fosmid H40L17 was identified and mapped to the lin-29 contig by the C. elegans sequencing consortium (S. Chissao, personal communication). We showed that this fosmid spans the lin-29 locus and fully rescues the lin-29 phenotype when present on a transgenic array (data not shown). Animals of the genotype lin-29(n836) II; ncl-1(e1865) unc-36(e251) III, carrying a transgenic array (veEx112) composed of fosmid H40L17, the ncl-1(+) cosmid C33C3 (Miller et al., 1996), and the unc-36(+) plasmid R1p16 (Herman et al., 1995), were used. The focus of unc-36 activity is among the AB.p descendent (Kenyon, 1986). Animals were scored for Ncl during the L3 stage and then scored for vulval morphology, egg laying and presence of alae in adults. The mitotic loss rate of veEx112 is approximately 6×10⁻³ losses per cell division.

RESULTS

Abnormal vulva formation in lin-29 mutants

lin-29 mutant animals are unable to lay eggs (Ambros and Horvitz, 1984). We observed vulval development in animals carrying null mutations in lin-29 [lin-29(0)] and found that vulval cell divisions occur at approximately the correct time and in the proper location and orientation, and that vulval invagination appears normal (data not shown). Morphogenesis continues to appear normal (Fig. 2C) until exit from the fourth molt, when eversion occurs abnormally and a ventral protrusion forms at the site of the vulva (Fig. 2F). Thus, the vulval
defect in lin-29 mutants occurs during a late step in vulval morphogenesis and results in an abnormally everted vulva (the Evl phenotype; Seydoux et al., 1993) that prevents egg laying.

LIN-29 accumulates in non-hypodermal cells

Previous work on lin-29 focused on the lateral hypodermal phenotype (Ambros, 1989; Ambros and Horvitz, 1984; Bettinger et al., 1996) and did not address the basis of the Evl phenotype. We showed previously that lin-29 protein is nuclear and accumulates throughout the hypodermis during the L4 stage, consistent with its control of seam cell terminal differentiation (Bettinger et al., 1996). Thus, normal vulva formation may require a component of the adult hypodermis (Ambros and Horvitz, 1984), which is lacking in lin-29 mutants. However, LIN-29 is restricted neither to the hypodermis nor to the L4 stage (Bettinger et al., 1996), suggesting that it may play a greater role in development than specification of adult hypodermis.

Many of the non-hypodermal cells that accumulate LIN-29 – the anchor cell, sex muscles and vulval cells – have roles in vulva formation or in other aspects of the egg-laying process. LIN-29 is detected in the anchor cell beginning at the L2 molt or early L3 stage (Bettinger et al., 1996; see Fig. 3A). Laser microsurgery experiments indicate that the anchor cell (AC) induces vulval cell fates during this time (Kimble, 1981). Anchor cell ablation slightly later, during the early to mid-L3 stage, can result in vulval invagination and eversion problems, despite formation of the correct number of vulval cells (Kimble, 1981; Seydoux et al., 1993). The final fate of the anchor cell is to fuse with the multinucleate utse cell during the L4 stage (Newman et al., 1996).

Midway through the L3 stage, LIN-29 accumulates in the progeny of the sex myoblasts (SMs) (Bettinger et al., 1996; see Fig 3C,F). The two bilaterally symmetric SMs are born in a posterior position during the late L1 stage and migrate to positions centered laterally over the gonad (Sulston and Horvitz, 1977). Each SM undergoes three rounds of division, beginning during the mid-L3 stage to generate four uterine muscles (ums) and four vulval muscles (vms) used for egg laying in the adult (Sulston and Horvitz, 1977). The eight vms attach the vulva to the gonad and are responsible for opening the vulva as eggs are laid, whereas the eight um cells produce uterine contractions during egg laying (White, 1988). LIN-29 is first detected in the SM lineage after the first round of division (in the four SM.x cells) and is ultimately present in all 16 of the uterine and vulval muscle cell nuclei (Fig. 3C,F).

The daughters of the vulval precursor cells P5.p-P7.p accumulate LIN-29 shortly after they are born (Fig. 3A,C). LIN-29 is detected in all 22 descendents of P5.p-P7.p (Fig. 3D-F) and persists in the adult stage.

Control experiments demonstrate that these staining patterns
with the adoption of vulval cell fates. In mutants bearing loss-of-function (lf) alleles of the heterochronic genes lin-14 or lin-28, the vulval cell fates are specified early and the VPCs divide precociously, during the L2 stage (Ambros and Horvitz, 1984; Euling and Ambros, 1996a). We examined LIN-29 accumulation in lin-14(ma135) (Fig. 3G) and lin-28(n719) mutants, and found that LIN-29 accumulates shortly after the precocious divisions of P5.p-P7.p in the L2 stage. Thus, LIN-29 accumulation in the vulva, as in the hypodermis, is temporally controlled by the heterochronic gene pathway.

In lin-3(lf) mutants, the anchor cell signal is missing (Hill and Sternberg, 1992) and vulval cell induction does not occur. Instead, P5.p-P7.p adopt the hypodermal cell fate characteristic of the three uninhibited VPCs; they divide once and fuse with the main body hypodermis (hyp7) late during the L4 stage. In lin-3(lf) animals, LIN-29 accumulation is not observed in any Pn.p descendants until they fuse with hyp7 (data not shown). These results establish LIN-29 as an early marker for the specification of vulval cell fates. In addition, they raise the question of whether lin-29 function is required in vulval cells for proper vulval morphology and egg laying.

Vulval cell fusions occur in lin-29(0) mutants

One defect in lin-29(0) animals is the failure of the lateral hypodermal seam cells to fuse during the L4-to-adult molt (Ambros, 1989; Bettinger et al., 1996). Since extensive cell fusions occur in the wild-type vulva during the L4 stage (Newman et al., 1996), we investigated whether these fusions occur in lin-29(0) animals. We assessed cell fusion by staining L4 stage lin-29(n836) animals with MH27, a monoclonal antibody that detects an antigen in adherens junctions (Waterston, 1988). Confocal microscopy of the stained animals showed that vulval cell fusion is unaffected by the loss of lin-29 function (data not shown).

A novel lin-29 mutant displays vulval defects but still executes lateral hypodermal terminal differentiation

lin-29(ga94) mutants are different in phenotype from all described lin-29 mutants: they have an apparently wild-type hypodermis, but 73% develop vulval protrusions and are

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### Table 1. Post-dauer suppression of lin-29(ga94)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Continuous dev.</th>
<th>Post-dauer dev.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bristol N2</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>lin-29(n836)</td>
<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>lin-29(ga94)</td>
<td>73%</td>
<td>11%</td>
</tr>
</tbody>
</table>

n = 200 for each sample.

1 Single, continuously developing, L3-staged animals were placed on individual plates and scored as adults for the Evl phenotype and the ability to lay eggs.

2 Dauer larvae were selected from three independently starved cultures by treatment with 1% SDS for 30 minutes. From each selection, single dauer larvae were placed on 67 plates and scored as adults.

3 We have not observed non-Evl egg-laying animals during continuous or post-dauer development in strains bearing the following lin-29 alleles: n333, n546, n836, n1368, n1440, ve5 and ga93.

4 Of the non-Evl worms, 5% formed a bag-of-worms prior to releasing progeny.

5 Of the non-Evl worms, 26% formed a bag-of-worms prior to releasing progeny.

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**Fig. 3.** Accumulation of LIN-29 in vulval and sex muscle cells. A-H are wild-type animals. (A) A mid-L3 stage animal in which P6.p has just divided and P5.p and P7.p are undivided. LIN-29 is detected in the anchor cell (AC) and the P6.p daughters. (B) Nuclei in the same focal plane stained with Hoechst. (C) A mid-L3 stage animal in which P5.p-P7.p have each divided once. LIN-29 is detected in all six P5.p-P7.p progeny, the AC and in the sex muscles which have divided once. The left pair of SM progeny is visible out of the plane of focus on either side of the AC. (D) An L3 stage animal in which P5.p-P7.p have divided twice generating 12 granddaughters. LIN-29 accumulates in these cells, the AC and the SM progeny (out of focus). (E) Ventral view of a late L3 stage animal. LIN-29 is detected in the 22 vulval cells arranged in a row and beginning to invaginate. (F) An L4 stage animal, ventral view. LIN-29 is detected in the 16 sex muscles and in the vulval cells, center ring. The cell division pattern of the left posterior SM daughter (SMlp) is shown. The vulval cells are in the process of invaginating. (G) An early-to-mid L3 stage lin-14(ma135) animal. LIN-29 is detected in the precociously divided vulva cells which are invaginating. (H) Hoechst staining of the animal in (G) to demonstrate the early L3 stage gonad in which the distal tip cell (DTC) has not reflexed. Scale bars, 10 μm.

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**Accumulation of LIN-29 in vulval cells correlates with cell fate specification**

The appearance of LIN-29 in P5.p-P7.p progeny correlates are specific to LIN-29. They are not observed in lin-29(0) animals and they are greatly reduced or eliminated when the LIN-29 antibody is preincubated with a LIN-29::glutathione S-transferase (GST) fusion protein but not with GST alone (data not shown).
unable to lay eggs (Fig. 2B,E; Table 1). As in wild-type worms, the lateral seam cells of ga94 animals cease dividing during the L4-to-adult molt; they then fuse and synthesize adult alae (Fig. 4B,E). In addition, lin-29-dependent transcription from a collagen promoter (Liu et al., 1995; Rougvie and Ambros, 1995) is detected in the hypodermis of adult lin-29(0) but not lin-29(0) animals (J. Abrahante and A. Rougvie, unpublished data). Finally, none of 19 ga94 animals analyzed executed supernumerary molts, which are characteristic of lin-29(0) animals.

The ga94 phenotype is suppressed by development through the dauer larva stage

The lin-29(0) Evl phenotype and the hypodermal phenotype are observed whether animals develop continuously or through the alternative dauer stage (Table 1). In contrast, 89% of post-dauer ga94 adults are non-Evl, and the majority are able to lay eggs (Table 1). The mechanism of post-dauer suppression of ga94 is unknown, but it is interesting to note that post-dauer suppression of precocious defects in both the lateral seam and the vulva is observed for loss-of-function lin-14 and lin-28 mutants (Liu and Ambros, 1991; Euling and Ambros, 1996b). LIN-29 levels are reduced in ga94 mutants (see below), but these lower levels are not altered by development through the dauer pathway. Thus, there could be a reduced requirement for LIN-29 during post-dauer development and ga94 might produce enough to fulfill this need.

lin-29(ga94) contains a premature stop codon

We sequenced the ga94 allele and identified a single point mutation that creates an amber stop codon in exon V of the larger of two predicted lin-29 transcripts, lin-29A (Fig. 4G) (Rougvie and Ambros, 1995). This lesion was not present in lin-29(ga93), which was isolated from the same genetic background (D. Eisenmann and S. Kim, personal communication). As a consequence of the ga94 mutation, the lin-29A open reading frame terminates prematurely, resulting in a 143 amino acid predicted protein that lacks zinc fingers (Fig. 4G). The mutated codon is flanked by the first two in-frame AUGs in the lin-29B transcript (Fig. 4G). LIN-29B translation could initiate at the second of these AUGs to produce a zinc finger protein just two amino acids shorter than wild-type LIN-29B. Indeed, antibody staining experiments show that LIN-29 accumulates in ga94 mutants (see below). Thus, the ga94 lesion likely eliminates LIN-29A function but only slightly affects LIN-29B. The ga94 phenotype could reflect tissue-specific functions of LIN-29A and LIN-29B; LIN-29B could direct hypodermal terminal differentiation whereas LIN-29A could program vulval morphogenesis. Alternatively, LIN-29B could provide both functions and the ga94 lesion could act by lowering the overall level of LIN-29B below that required for vulval morphogenesis. To address these possibilities, we examined LIN-29 tissue distribution in ga94 mutants and we...
tested the ability of a lin-29B cDNA to rescue the vulval and hypodermal defects of lin-29(0) animals.

We used antibodies that recognize a C-terminal LIN-29 domain (Bettinger et al., 1996) to detect LIN-29 in ga94 animals. This antibody should detect only the LIN-29B protein in ga94 mutants. We found that the spatial pattern of LIN-29 accumulation is not noticeably altered in ga94 mutants but that there is a reduction in LIN-29 levels relative to wild type in all cell types.

We also generated a minigene construct in which the lin-29B transcript is expressed from its own promoter (see Materials and Methods). Transgenic animals bearing this construct were fully rescued for the lin-29(0) hypodermal and vulval defects (data not shown). Thus, the lin-29A product is not absolutely required for vulval morphogenesis.

The protein accumulation and cDNA rescue experiments are consistent with a model whereby ga94 acts primarily by reducing overall LIN-29 levels. We suggest that a threshold level of LIN-29 is required for function, and that this threshold is higher for vulva formation than for lateral hypodermal cell terminal differentiation. In ga94 mutants, LIN-29 levels would be above the threshold for hypodermal development, and below, but near, the threshold for vulva formation. A level of LIN-29 near the threshold for proper vulva formation would explain the incomplete penetrance of the Evl phenotype in lin-29(ga94) animals (Table 1). Although these experiments suggest that different levels of LIN-29 are needed for different developmental events, they do not address which cells must express lin-29 to execute these events.

**Analysis of animals mosaic for lin-29**

We performed mosaic analysis to investigate where lin-29 function is required for proper vulva formation and egg laying. We also tested whether lin-29 function in lateral hypodermal terminal differentiation is cell autonomous.

To generate lin-29 mosaics, we made lin-29; ncl-1 unc-36 animals transgenic for an extrachromosomal array that carries wild-type copies of lin-29, ncl-1 and unc-36 (see Materials and Methods). Since the extrachromosomal array is mitotically unstable, it is subject to loss at each cell division, which generates clones of mutant cells. The ncl-1 mutation causes enlarged nucleoli (the Ncl phenotype; see Fig. 5) in a cell-autonomous fashion and is scoreable in most cells (Hedgecock and Herman, 1995). The Ncl phenotype was used to identify cells that had lost the array and thus were lin-29(-).

**lin-29 function is cell autonomous in the seam with respect to cell division and alae synthesis but is cell nonautonomous for cell fusion**

We found a strict correlation between the presence of the array in lateral hypodermal seam cells and the ability of seam cells to execute two aspects of the terminal differentiation program, cell cycle exit and alae synthesis. We scored seam cells for the Ncl phenotype in 16 early L4 stage non-Unc animals that were mosaic in the lateral seam. We subsequently scored the ability of each seam cell to terminally differentiate during the L4-to-

![Fig. 5. Photomicrographs of mosaic adult animals obtained from lin-29; ncl-1 unc-36; veEx112.](A,C,E) View of left lateral seam in mosaic animals. Open arrowheads indicate Ncl seam nuclei [lin-29(-)]. Filled arrows indicate non-Ncl seam nuclei [lin-29(+)]. (B,D) Views of the cuticle overlaying the lateral seam shown in A and C, respectively. Adult alae overlies only the non-Ncl seam cells. (F) Different focal plane of the animal shown in E, demonstrating fusion of the two non-Ncl seam cells (black arrow). The white arrow indicates cell border between the unfused Ncl and non-Ncl cells.
adult molt as judged by absence of cell division and synthesis of adult alae on the overlying cuticle. Every seam cell that lacked the array (n=55) failed to terminally differentiate; they continued to divide and synthesized a larval type cuticle during the fourth molt. In contrast, every seam cell that contained the array (n=129) terminally differentiated by these criteria.

A lin-29(+) seam cell adjacent to a lin-29(–) seam cell is incapable of performing one aspect of the terminal differentiation program, cell fusion. We found 13 examples of animals containing a single non-Ncl seam cell in an otherwise mutant seam (Fig. 5A,B). These lin-29(+) cells terminally differentiated by cell division and adult alae criteria, but they failed to fuse to their lin-29(–) neighbors within the seam. We also identified 14 isolated lin-29(–) seam cells in otherwise lin-29(+) seams (Fig. 5C,D). These isolated seam cells remained unfused and continued to divide and synthesize larval cuticle, even though they were flanked by lin-29(+) cells. Taken together, these results demonstrate that lin-29 functions cell autonomously in the seam for cell division and adult alae synthesis and that two adjacent seam cells must both be lin-29(+) in order to fuse (Fig. 5E,F).

**Vulva formation requires lin-29 function in a subset of the lateral seam cells**

We identified four animals that lost the array in AB while maintaining it in P1 (Fig. 6A). These animals were Evl, indicating a requirement for lin-29(+) in the AB lineage for proper vulval eversion. As shown in Fig. 6A, the LIN-29-accumulating cells that are derived from the AB blastomere include all of the lateral seam cells, the 22 vulval cells and the vast majority of the hyp7 nuclei.

We examined mosaic animals that lost the array at later cell divisions to narrow the focus of lin-29(+) activity within the AB lineage relative to vulval formation. Four animals that lost the array in AB.a were wild type for egg laying. Thus, the 24 seam cells born in the AB.a lineage (H01R and the progeny of H1R, H2L/R, V1L/R, V2L/R, V4L/R and V6L/R; Fig. 1A), which do not immediately flank the vulva, do not require lin-29(+) for proper vulva formation. In addition, the loss of the array in the 72 hyp7 nuclei derived from AB.a is tolerated.

Three mosaic animals with array loss in AB.p were Evl (Fig. 6A). AB.p progeny cells of particular interest are the vulval cells and the seam cells produced by V3L/R, V5L/R and TL/R. Focus on these cells is intensified by a complex mosaic with a specific double loss (from AB.plp and AB.prp) (Figs 1, 6). It was not technically feasible to identify a mosaic animal that lacks the array from the four V3 seam cells but maintains it in P5.p-P7.p progeny, because V3L and V3R arise as sister cells to the cells that generate either P7.p or P8.p (Fig. 6B). Such a mosaic would be rare, requiring a specific double loss (from AB.prapp and AB.plpp). The converse mosaic animal with losses in the P5.p-P7.p progeny but maintenance in the four V3-derived seam cells would be rarer. However, within the vulva, it is the vulE cells that attach to the lateral seam (Fig. 1B), and the four nuclei of vulE are derived from P6.p. We identified two mosaic animals with P6.p losses that were lin-29(+) in at least three of the four V3-derived seam cells. These animals were wild type for vulva formation and egg laying, suggesting that lin-29 is not required in the vulE cells. These two mosaics lacked the array in both P6.p and P7.p progeny. One of these animals was lin-29(+) throughout the seam while the other had lost the array from one V3-derived seam cell (V3R.p). These mosaic animals suggest that the focus of lin-29 within AB.p is not in the vulval cells.

We have analyzed 21 mosaics in which the array is absent from subsets of the V3 descendem seam cells and found a...
Table 2. Analysis of mosaic animals with losses in V3L/R

<table>
<thead>
<tr>
<th>Mosaic Class</th>
<th>n</th>
<th>V3L pappp</th>
<th>V3L pappp</th>
<th>V3R pappp</th>
<th>V3R pappp</th>
<th>Phenotype</th>
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<tr>
<td>1</td>
<td>14</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>WT</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>4WT; 1Egl</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>3WT; 2Egl</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>2WT; 1Egl</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>WT</td>
</tr>
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<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>2Evl; 1Egl</td>
</tr>
<tr>
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<td>1</td>
<td>+</td>
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<td>-</td>
<td>+</td>
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<td>+</td>
<td>Egl</td>
</tr>
</tbody>
</table>

In the absence of the array, lin-29(n83t) animals are Egl (see Table 1). + or − represents the presence or absence, respectively, of the array in the indicated V3 progeny.

requirement for lin-29(+) in three of these four cells for vulval morphogenesis (Table 2). If lin-29(+) is present in zero, one or two of the four V3-derived seam cells, an Egl phenotype usually results. Animals lin-29(+) in three of these cells are usually (10/14) wild type for egg laying. The vulval phenotype is observed with increasing severity and frequency as the number of lin-29(+) V3-derived seam cells decreases from four to zero. These results indicate a requirement for a lin-29-dependent component of the adult lateral seam, specifically in the V3-derived seam cells, for the formation or maintenance of vulva and/or uterus structure during eversion.

lin-29(+) function is not restricted to the AB lineage

If the sole requirement for lin-29 in vulval morphogenesis is within the V3-derived seam cells, then animals that lose the array in only P1 should appear wild type. We identified such mosaic animals; four of five were egg-laying defective demonstrating that lin-29 also functions within the P1 lineage (Fig. 6A).

Of the P1 mosaics, two were Egl and two had properly formed vulvae but were nonetheless Egl as judged by rates of egg laying and ages of eggs laid. These animals released no, or a few abnormally late-stage, progeny and ultimately formed a bag of worms. Each of these animals was confirmed to be lin-29(+) in all four V3-derived seam cells in the adult stage. As a control, we examined transgenic animals that maintained the array stably and found them to be non-Egl when manipulated through a mock mosaic analysis (n=25). Thus, the observed Egl phenotypes are due to loss of lin-29(+) from the P1 lineage.

The focus of lin-29(+) within the P1 lineage was narrowed by examination of additional mosaic animals. Four of four mosaic animals with losses in P2 were wild type for vulva formation and egg laying, suggesting that the focus of lin-29(+) is within the EMS lineage. These mosaics, and an additional four with losses in C, demonstrate that lin-29(+) is not required in the 12 hyp7 nuclei produced in the C lineage. A loss at EMS cannot be distinguished from a loss at MS, because the Ncl phenotype cannot be scored in the E lineage (Hedgecock and Herman, 1995). However, the E lineage is not a likely focus of lin-29 since it gives rise only to gut cells, which do not accumulate LIN-29. Thus, we focused our analysis on the MS lineage. Four of five animals with losses in MS were egg-laying defective (two were Egl, two were Egl). Three animals with losses in MS.p were wild type for egg laying while two of three MS.a losses were Egl, indicating that a focus of lin-29 activity is within the MS.a lineage.

The MS.a lineage produces many cells that accumulate LIN-29: the eight vulval and eight uterine muscle cells, one distal tip cell and the anchor cell (see Fig. 6A). Of these, the anchor cell and the sex muscle cells have known roles in generation or function of the egg-laying apparatus. In 7/7 animals lacking the array from the anchor cell lineage, vulva formation and egg laying appeared wild type. Thus, lin-29(+) is not required in the anchor cell and the contribution of LIN-29 to the utse cell from AC fusion is also not by itself an important factor in vulval morphogenesis or egg laying. We favor a role for lin-29 in the sex muscle cells; they are derived from MS.a, accumulate LIN-29, have a known role in egg laying and a subset of them make contact with the lateral seam. Alternatively, lin-29 could play a role in MS.a derivatives where its expression levels are below detection with our antibody. The requirement for lin-29(+) within the P1 lineage was not absolute, as two of ten animals with losses at P1 or MS were non-Egl. These animals could reflect a partial redundancy for lin-29 function in these cells.

DISCUSSION

Cell autonomy of lin-29 within the lateral seam

We demonstrate through mosaic analysis that a hypodermal seam cell must be lin-29(+) in order to execute two aspects of the terminal differentiation program (Fig. 5). lin-29(+) seam cells exit the cell cycle and synthesize alae during the L4-to-adult molt, whereas lin-29(−) cells continue to execute the larval program of cell division and larval-type cuticle synthesis. The behavior of seam cells in lin-29 mosaics differs with respect to a third aspect of the terminal differentiation program, that of cell fusion. We found that lin-29(+) is insufficient to promote fusion of a seam cell to a lin-29(−) neighbor. Rather, two adjacent seam cells must be lin-29(+) to fuse. This result implies that there is a lin-29-dependent recognition step in the fusion process. One possibility is that LIN-29 promotes expression of a cell surface protein that mediates seam cell fusion through selfrecognition of molecules on adjacent cells.

Component(s) of the adult lateral seam are critical for vulval morphogenesis

We demonstrate that a terminally differentiated adult lateral seam is required for maintenance of vulval morphology during the eversion step; a larval-type lateral hypodermis will not substitute. When a mosaic animal has larval-type undifferentiated seam cells (V3L/R progeny; see Fig. 1A and Table 2) overlying the vulva, due to lack of lin-29(+), eversion fails and a protruding vulva results. The Egl phenotype is probably due to the failure of one or more cell types to make structural connections to the lateral seam and, consequently, the structural integrity of the vulva is unable to withstand the forces associated with vulval eversion. This hypothesis is supported by electron microscopic studies that describe connections between the egg-laying apparatus and the lateral hypodermis and predict a structural role for these connections in maintaining vulval integrity (Newman et al., 1996; White et al., 1986). In agreement with
a seam cell structural role, as the number of lin-29(+) V3-derived seam cells increases from zero to four, the severity of the vulval defect decreases and the proportion of animals with normal vulvae increases (Table 2).

We do not know which of the cells with adult seam attachments are critical for maintenance of vulval integrity during eversion, but the multicellular utse cell, which connects the uterus to the seam, is thought to play a major role (Newman et al., 1996). When adult hermaphrodites are viewed by Nomarski microscopy, a thin line is visible at a position ventral to the seam, which is thought to reflect the utse-seam attachment (E. Hedgecock, personal communication; Newman et al., 1996). This line is not observed in lin-29(0) mutants (A. Antebi and E. Hedgecock, personal communication; our work), nor have we observed it in ga94 animals that are Evl. In mosaic animals that are wild type for egg laying, but are lin-29(+) in just three of the four V3-derived seam cells, the portion of the line corresponding to the lin-29(−) seam cell is absent. These results demonstrate the dependence of the observed line, and by extension the utse connection, upon the lin-29-dependent adult seam. Furthermore, they indicate that a half-connection, on one lateral side of the animal, can be tolerated. Interestingly, in lin-29 mutants, the uterine cells often appear aberrant in morphology, position and organization during the late L4 stage. The origin of these abnormalities is unknown, but they correlate with the time during which the utse connection is thought to be formed. Thus, the disorganized appearance of the uterus may reflect failure of the utse-seam attachments.

In addition to the utse, the vulE, um1 and vm1 cells make contact with the adult seam during the L4-to-adult molt (Fig. 1B; Newman et al., 1996; White et al., 1986). We have shown that these cells accumulate LIN-29, and that LIN-29 is present in the anchor cell prior to and at the time it fuses with the utse. Thus, it is possible that vulva formation requires lin-29(+) not only in the lateral seam, but also in the cells that contact the seam. However, mosaics that lack lin-29(+) in the vulE cells lay eggs normally, indicating that they are not required to be lin-29(+) for proper vulval morphology. Similarly, egg laying is normal in animals that lack lin-29(+) in the anchor cell (Fig. 6A), indicating that the anchor cell contribution of LIN-29 to the utse is not required for vulval morphogenesis. In contrast, egg-laying defects are observed in animals that lack lin-29(+) in the lineage that gives rise to um1 and vm1.

**lin-29(+) is required in the P1 lineage for wild-type egg laying**

Although a major factor in the Evl phenotype of lin-29 mutants is the lack of an adult lateral seam, we have identified many mosaic animals that are lin-29(+) in the four V3 seam progeny that span the vulva yet retain the Evl defect or are Egl (Fig. 6A). These mosaic animals suggest that at least one component of the lin-29(0) egg-laying defect is separable from the lateral seam. The focus of this component is likely to be in the P1 lineage since mosaic animals that lack lin-29(+) in P1, while maintaining it in AB, were egg-laying defective. We further traced this focus to the MS.a lineage (Fig. 6A), a lineage that does not produce hypodermal cells. Thus, lin-29 contributes to vulval morphogenesis and function through effects on multiple cell types.

The MS.a lineage gives rise largely to pharyngeal, body wall and sex muscles, and to somatic gonad cells including the anchor cell, and half of the dorsal and ventral uterine cells (including the utse). We have not detected LIN-29 in the dorsal and ventral uterine cells and, since half of them are derived from MS.p which does not require lin-29(+) (Fig. 6A), they are not likely candidates for a focus of lin-29. We suggest that, among the MS.a progeny, the likely focus of lin-29 is within the sex muscles. These muscles are known to be required in the adult for egg laying (Sulston and Horvitz, 1977), they accumulate LIN-29 (Fig. 3) and some of them make connections to the lateral seam (Fig. 1B).

The Evl phenotype of lin-29 mosaics with losses within the P1 lineage could reflect a structural requirement for sex muscle attachment to the hypodermis. However, several P1 losses had defects restricted to egg laying (Fig. 6A), consistent with a role for lin-29 in muscle function. Elucidation of the precise role of LIN-29 in these cells awaits a detailed structural and functional analysis of sex muscles in lin-29 mutants and the identification of genes regulated by the LIN-29 transcription factor in these cells.

**Are there other functions of lin-29?**

We have demonstrated that LIN-29 accumulates in a variety of cell types (Fig. 3; Bettinger et al., 1996); however, our mosaic analysis did not reveal roles for lin-29 in all of them. The vulE cells connect to the lateral seam, yet mosaics lacking lin-29(+) in the vulE cells were wild type. Since lin-29 has been shown to control expression of specific collagen genes (Liu et al., 1995; Rougvie and Ambros, 1995), it may play a role in cuticle synthesis by these cells. Consistent with this notion, the vulE and vulF cells (P6.p progeny) are responsible for synthesis of the internal cuticle of the vulva (White, 1988). Similarly, the P5.p and P7.p progeny could contribute to external cuticle synthesis in a lin-29-dependent manner.

We have also not detected an obvious phenotype associated with lack of lin-29 in the anchor cell. lin-29 may play a redundant role in this cell, participating in a process directed by multiple players. If so, a lin-29-dependent phenotype may be detected in animals only when the other components are reduced or absent.

LIN-29 also appears abundant in the distal tip cells (Bettinger et al., 1996), which lead migration of the gonad arms (Kimble and White, 1981). We have observed gonad migration defects in lin-29(0) animals; however, they occur at low penetrance perhaps reflecting a largely redundant function for lin-29 in this distal-tip-cell-directed process. Further work will assess LIN-29 molecular function in these stage-specific morphogenetic events.

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