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The role of *lin-22*, a *hairy/Enhancer of split* homolog, in patterning the peripheral nervous system of *C. elegans*

Lisa A. Wrischnik* and Cynthia J. Kenyon‡

Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA 94143-0554, USA

*Present address: Department of Molecular and Cell Biology, 401 Barker Hall, University of California, Berkeley, CA 94720, USA
‡Author for correspondence

SUMMARY

In *C. elegans,* six lateral epidermal stem cells, the seam cells V1-V6, are located in a row along the anterior-posterior (A/P) body axis. Anterior seam cells (V1-V4) undergo a fairly simple sequence of stem cell divisions and generate only epidermal cells. Posterior seam cells (V5 and V6) undergo a more complicated sequence of cell divisions that include additional rounds of stem cell proliferation and the production of neural as well as epidermal cells. In the wild type, activity of the gene *lin-22* allows V1-V4 to generate their normal epidermal lineages rather than V5-like lineages. *lin-22* activity is also required to prevent additional neurons from being produced by one branch of the V5 lineage. We find that the *lin-22* gene exhibits homology to the *Drosophila* gene *hairy,* and that *lin-22* activity represses neural development within the V5 lineage by blocking expression of the posterior-specific Hox gene *mab-5* in specific cells. In addition, in order to prevent anterior V cells from generating V5-like lineages, wild-type *lin*-22 gene activity must inhibit (directly or indirectly) at least five downstream regulatory gene activities. In anterior body regions, *lin-22(+)* inhibits expression of the Hox gene *mab-5*. It also inhibits the activity of the *achaete-scute* homolog *lin-32* and an unidentified gene that we postulate regulates stem cell division. Each of these three genes is required for the expression of a different piece of the ectopic V5-like lineages generated in *lin-22* mutants. In addition, *lin-22* activity prevents two other Hox genes, *lin-39* and *egl-5,* from acquiring new activities within their normal domains of function along the A/P body axis. Some, but not all, of the patterning activities of *lin-22* in *C. elegans* resemble those of *hairy* in *Drosophila.*

Key words: *lin-22*, *hairy*, *E(spl)*, *C. elegans*, A/P patterning, neurogenesis

INTRODUCTION

Understanding how precise patterns of neural and epidermal cells are generated along the A/P body axis is a central problem in developmental biology. In the nematode *C. elegans*, a diverse array of epidermal and neural cell types and structures is generated within the lateral ectoderm. At hatching, the lateral ectoderm consists of a row of six cells, the V1-V6 seam cells, that extends from the head to the tail. The V cells are stem cells that divide repeatedly to generate copies of themselves as well as specialized epidermal cells and neuroblasts (Figs 1 and 2). The lineages of the different V cells differ from one another, especially in the male. As a consequence, the V lineages generate considerable position-specific A/P body pattern.

We have chosen to analyze the development of the V cells because they provide a means to investigate how stem cell fates are specified during development and also how A/P patterns arise along the body axis. In wild-type animals, the anteriorly located V1-V4 cells all develop in the same way. These cells undergo repeated rounds of stem cell division, in most cases producing a posterior daughter that becomes another stem cell, and an anterior daughter that fuses with the large epidermal syncytium that covers much of the body. At the final cell division, all of the posterior daughters terminally differentiate into cells that generate cuticular ridges called alae, which run in lateral lines along the A/P body axis. The posterior V5 and V6 cells also undergo repeated stem cell divisions; however, many of these divisions give rise to cells whose fates differ from homologous cells in the V(1-4) lineages. The V5 lineage differs in three ways from the anterior V(1-4) lineages: first, during the second larval stage, the anterior daughter of the stem cell becomes a neuroblast called a postdeirid instead of an epidermal cell; second, during the third larval stage, a stem cell divides to produce two stem cells instead of just one (we refer to this as the L3 proliferative division); and third, late in the third larval stage, one stem cell divides to generate a ray precursor cell instead of an epidermal cell. This ray precursor cell, in turn, generates an epidermal cell and a sensory ray (a male mating structure). The V6 lineage is similar to that of V5, except that no postdeirid is produced and additional ray precursor cells are generated (Fig. 2A,B).

To learn how these complex V cell lineages are specified, we and others have been asking how specific cell fates within these lineages are influenced by developmental control genes. So far, three types of gene activities have been shown to influence the V cell lineages. First, the Hox gene *mab-5*, an *Antennapedia* homolog, specifies the fates of numerous cells in the posterior body region, including many of the cells that

arise within the male V5 and V6 lineages (Kenyon, 1986; Costa et al., 1988). In males lacking *mab-5*(+) activity, the V5 cell still generates the postdeirid, but the remainder of the cells in the V5 and V6 cells lineages develop like their homologs in the V1-V4 lineages. *mab-5* is expressed only in the posterior body region. Within the V5 lineage, *mab-5* is expressed in a complex, dynamic pattern, and acts multiple times at specific points in the lineage to regulate different cell fate decisions, including the stem cell proliferation event and the production of ray precursor cells (Salser and Kenyon, 1996). In V6, *mab-5* is expressed in a uniform manner, and this expression is activated by the *C. elegans caudal* homolog *pal-1* (Waring and Kenyon, 1990, 1991; Salser and Kenyon, 1996; Hunter and Kenyon, unpublished observations). The second set of genes important for V cell development is the set of heterochronic genes, which are required for temporal control of cell fate specification. In heterochronic mutants, stem cells within the V cell lineages undergo temporal transformations, dividing in a manner characteristic of a different developmental stage (Ambros and Horvitz, 1984; reviewed by Ambros and Moss, 1994). Third, a different type of gene required for V cell development is the *achaete-scute* homolog *lin-32*. The *lin-32*(+) gene is required for the formation of many different neuroblasts in *C. elegans* (Zhao and Emmons, 1995), including the postdeirid produced by V5 and the ray neuroblasts produced by the ray precursor cells within the V5 and V6 lineages. Several additional genes have been found to influence the development of the rays themselves; they will not be discussed here.

In this study, we have characterized an additional gene that also regulates pattern formation in the V cells. This gene, *lin-22*, has two different functions in patterning the V cell lineages. First, it is required for correct cell fate specification within one branch of the V5 cell lineage. Loss-of-function mutations in *lin-22* cause one of the stem cells in the V5 lineage to produce a ray precursor cell rather than an epidermal cell. Thus, V5 makes two rays instead of one. Second, *lin-22* plays a major role in the generation of A/P body pattern. In *lin-22(–)* mutants, the anterior V cells do not execute their normal lineages, but instead generate copies of the novel V5-like lineage (Figs 1, 2; Horvitz et al., 1983; Fixsen, 1985).

We find that *lin-22* encodes a basic helix-loop-helix protein similar to the transcriptional regulator *hairy* and the *Enhancer of split* complex [*E(spl)*-C] genes of *Drosophila melanogaster*. In addition, we find that different aspects of the *lin-22* mutant phenotype can be accounted for by changes in the activities of different downstream regulatory genes. Several aspects of the mutant phenotype arise because the *C. elegans Antennapedia* homolog *mab-5* is expressed in inappropriate locations, and because other Hox genes function in inappropriate ways within their normal domains of function. Other aspects of the phenotype arise because the function of *lin-32*, an *achaete-scute* homolog, is activated inappropriately. Finally, we postulate that one aspect of the *lin-22* phenotype arises because of altered activity of an unidentified gene that regulates stem cell proliferation. In flies, the *hairy* and *Enhancer of split* complex [*E(spl)*-C] genes regulate multiple aspects of A/P patterning and neural development. Some, but not all, of the activities of *lin-22* are reminiscent of the activities of *hairy* in *Drosophila.*

MATERIALS AND METHODS

General procedures and strains

Methods for routine culturing, nomenclature, strain construction, and genetic analysis were described by Brenner (1974) and Wood et al. (1988). Cell lineage analysis was carried out as described by Sulston and Horvitz (1977). All analyses were performed at 20°C. All strains contained the *him-5* (*e1490*) mutation, which increases the frequency of male self-progeny (Hodgkin et al., 1979).

Analysis of the *lin-22* phenotype

The isolation and characterization of *lin-22* alleles *mu2* and *mu5* is described by Waring et al. (1992). All strains analyzed in this work contained the *n372* allele of *lin-22*, unless otherwise noted. The complete V cell lineages of a single hermaphrodite, the V1-V5 lineages of two males, and the V6 lineage of a third male were carried out. Late L4/early adult males were also scored for gaps in alae production along the A/P axis (n=37). The location and extent of each gap was estimated by its position relative to body structures and cells along the A/P axis: the posterior end of the pharynx, the BDU cell, the anterior end of the gonad, the ALM cell, the dorsal to ventral bend in the gonad, the start of the fan, and the T rays.

In addition to the V cell phenotype, the *n372* allele was seen to affect the ventral ectodermal cells P(3-8).p (Fixsen, 1985). In wild-type males, these cells fuse with the syncytial hypodermis during L1. In *n372* males, these cells persist until L3, where they divide once. In wild-type hermaphrodites, P3.p adopts the 3° cell fate only 50% of the time, whereas in *n372* hermaphrodites, P3.p always adopts the 3° cell fate (G. Jongeward and P. Sternberg, personal communication). There is a low-penetrance Pvul (protruding vulva) phenotype (<5%) seen in adult hermaphrodites, and *lin-22* mutant males are somewhat uncoordinated and long.

Analysis of strains containing mutations in the *C. elegans* Hox genes

The following alleles were used in strain construction unless otherwise noted: *mab-5*(*e1239*), *lin-39*(*n1760*), and *egl-5*(*n945*). All three alleles appear to be null based on genetic and/or molecular criteria (Chisholm, 1991; Wang et al., 1993; Clark et al., 1993). The *lin-39 mab-5*; *lin-22* strain was made by first creating artificial vulvas in 10 young adult *lin-39 mab-5* hermaphrodites, and then mating these animals with >25 late L4 *e1490* males. The 'vulvas' were holes in the worm that were made with a microinjection needle filled with water, which was moved animatedly in both the horizontal and vertical directions in the appropriate position in the worm, under conditions used for transformation. Resulting *lin-39 mab-5*/++ hermaphrodites were then crossed with *lin-22* males, and from their progeny a strain homozygous for all three mutations was generated. The *lin-39 mab-5 egl-5*; *lin-22* strain was made by mating *lin-39 mab-5 egl-5*/*sma-3(e491) mab-5 egl-5* hermaphrodites (from A. Chisholm) to *lin-22* males. Individual hermaphrodite progeny from this cross were allowed to self-fertilize, and their progeny were screened for the presence of the *lin-39* mutation. Hermaphrodite progeny of these *lin-39* homozygotes were then scored for the presence of the Mab-5, Egl-5 and Lin-22 mutant phenotypes.

The *mab-5* and *mab-5*; *lin-22* V cell lineages were from Kenyon (1986). Lineages of the lateral ectoderm of *lin-39*; *lin-22* males (n=2) and *lin-39 mab-5*; *lin-22* males (n=3) were observed. Postdeirid formation was scored according to Waring et al. (1992). The presence of L3 proliferative divisions in the V cells of strains not lineaged was scored as follows: newly hatched L1 animals were collected at 2-hour intervals using 13 μm Nitex screen (Lewis and Fleming, 1995), allowed to develop for >27 hours, and the V cell progeny were examined. This technique was used to score *mab-5 egl-5*; *lin-22* males (n=11) and additional *mab-5*; *lin-22* males (n=8).

Late L4/early adult males were scored for gaps in alae production

along the A/P axis as above. The formation and location of tail seam cells in *mab-5*(*e2088*) (*n*=12) and *mab-5*; *lin-22* (*n*=12) mutants was analyzed during L4 lethargus (Sulston et al., 1980).

Analysis of strains containing mutations in *lin-32*

lin-32(*e1926*) (*n*=12) and *lin-22*; *lin-32*(*e1926*) (*n*=2) were lineaged. Postdeirid production in *lin-22*; *lin-32*(*e1926*) (*n*=60) was scored according Waring et al. (1992). Animals were staged as above and L3 proliferative divisions were specifically lineaged for *lin-32*(*u282*) (*n*=3) and *lin-22*; *lin-32*(*u282*) (*n*=2). Males in L4 lethargus were scored for the presence or absence of tail seam cells in *lin-32*(*u282*), *n*=16; *lin-22*, *n*=13; *lin-22*; *lin-32*(*u282*), *n*=12; *mab-5*; *lin-22*; *lin-32*(*u282*), *n*=15. Late L4/early adult males were scored for alae production (see above) in the following strains: *lin-32*(*u282*), *n*=36; *lin-22*; *lin-32*(*u282*), *n*=38; *mab-5*; *lin-22*; *lin-32*(*u282*), *n*=26; and *mab-5*; *lin-22*; *lin-32*(*e1926*), *n*=35.

Immunofluorescence using the α-MAB-5 antibody

Animals were staged and stained using rabbit polyclonal antisera to the MAB-5 protein following the protocol described by Salser and Kenyon (1996). Animals at stages showing weak or nonexistent anterior staining (i.e. hermaphrodites from 21-23 hours or later) were first selected on the basis of good morphology and strong V6 staining. Males and hermaphrodites were distinguished by the presence/absence of the HSN neuron during L2, and on the basis of their tail morphology and the number of tail cells (as assessed using DAPI) in L3.

Molecular cloning of *lin-22*

Unless otherwise noted, all procedures used are from Sambrook et al. (1989) or Church and Gilbert (1984). We identified and cloned a *Tc1* transposon genetically linked to the *lin-22* gene according to the methods described by Costa et al. (1988); Ruvkun et al. (1989). The approx. 1.5 kilobase (kb) of genomic DNA flanking this *Tc1* was subcloned, and a 450 bp subfragment lacking repetitive DNA was isolated and used to identify 8 overlapping Yeast Artificial Chromosomes (YACs) generated by the *C. elegans* genome center in St. Louis (Coulson et al., 1988). DNA was made from the 8 YAC-containing yeast strains (Frazer, 1993), and the YACs themselves were identified using PFGE and Southern blotting (pYAC4 vector probe). Whole YAC DNA was gel purified (using β-agarase 1, NEB; or GeneClean, Bio 101) and injected independently (approx. 3-5 ng/μl) into the germline of *lin-22*(*n372*) hermaphrodites (along with the dominant cotransformation marker *rol-6*; Mello et al., 1991). Rescue of the Lin-22 phenotype was observed for only a single YAC, Y68H6. *lin-22* rescue was never seen in F_1 progeny, and was only found in F_2 animals containing stable arrays (lines) of the injected DNA (at a frequency of 14-25% transformed Rol-6 lines).

Purified, radiolabeled Y68H6 (240 kb) was hybridized at 65˚C with unlabeled total genomic DNA to 'remove' labeled repetitive DNA (Frazer, 1993), and then used as a probe against an unamplified *C. elegans* genomic lambda library (a marvelous gift from A. Coulson) plated on *E. coli* strain CES200. 28 hybridizing clones were identified and isolated, and the DNA used to arrange the clones into overlapping sets where possible. DNA from 4-6 clones was then pooled (at approx. 10 ng/μl each, with *rol-6* DNA) before being injected into *n372* hermaphrodites to look for rescue of the mutant phenotype. This led to the identification of a single clone (λ#10) containing approx. 15 kb of genomic DNA that rescued the *lin-22* loss-of-alae phenotype (this clone could also rescue the *mu2* allele). Several restriction fragments of this clone were gel purified (Geneclean, Bio 101) and used to transform *lin-22* hermaphrodites. The smallest fragment found to rescue the *n372* alae phenotype was a single 12 kb *Xho*I-*Spe*I subfragment which lacked 3 kb from the right-hand end of the clone (2/7 lines, see Fig. 4).

Identification of *lin-22* sequence

Approximately 8 kb from subclones of λ#10 were sequenced (Sequenase, USB), and a region homologous to members of the *hairy/E(spl)* family of helix-loop-helix proteins was discovered. Over 8×10^6 cDNA clones from various libraries were screened with the bHLH region and no positives were identified. Total mixed-stage RNA from *e1490* and the three *lin-22* alleles was isolated (Shackleford et al., 1993) and reverse transcribed (RT) using an oligo dT_{18}-*Bam*HI primer (Superscript 2, BRL). The putative 5′ end of this bHLH gene was isolated by RT-PCR using a primer to the SL1 splice leader (Krause and Hirsh, 1987) and two nested, internal helix 2 primers (Innes et al., 1990; H2A: 5′AGC-CAT-TTC-GAG-AAT-ATC-AGC3′; H2B: 5′TCC-CAT-TTG-GAA-TGT-TGG-AT3′). The fragment from *e1490* was cloned into pCRII (Invitrogen) and both strands sequenced. Double-strand PCR sequencing (dsDNA cycle sequencing kit, Gibco BRL) was used to obtain the sequence of this 5′ RT-PCR fragment, as well as the corresponding genomic region, from the *lin-22(n372)* allele. A PCR fragment was generated from the corresponding region of *n372* genomic DNA, and ds cycle sequencing was used to confirm the presence of the identified missense mutation. PCR was used to make genomic DNA fragments containing coding sequence from the remaining two alleles, which were gel-purified (Gene-Clean, Bio 101) and sequenced directly (see Fig. 4).

Poly(A)+ RNA was isolated from total *e1490* mixed stage RNA and L1-L3 stage-specific *e1490* RNA (Oligotex, Qiagen) and used in 3′ RACE reactions (Frohman, 1994). Two rounds of PCR were carried out using sets of nested primers homologous to *lin-22* coding sequence (H1A: 5′GAA-GAA-ACG-GAG-AGC-TCG-AAT3′ and H1B: 5′GAG-CTC-GAA-TAA-ACA-AGT-C3′; H2revA: 5′AAA-GCT-GAT-ATT-CTC-GAA-ATG3′ and H2revB: 5′GTC-GAA-TAC-CTC-CAA-CAA-CTC3′) and the RACE primers Q_o and Q_I. Potential cDNA clones containing a 3′UTR and a poly(A)+ tail were identified by sequence (see Fig. 4).

RESULTS

Mutations in *lin-22* alter cell fate specification within the V5 lineage, and also cause anterior V cells to generate V5-like lineages

In hermaphrodites, the *lin-22* phenotype can be considered to be a simple spatial homeotic transformation in which anterior V(1-4) cells adopt the fate of V5 (Horvitz et al., 1983; Fixsen, 1985; Fig. 2A). The hermaphrodite V5 lineage differs from the V(1-4) lineages in only one respect, the V5.pa cell becomes a postdeirid neuroblast instead of a seam cell (a seam cell is a stem cell, as described in the Introduction). Lineage transformations in *lin*-22 males were summarized in Fixsen (1985), but have not been analyzed in detail. To better understand the wild-type function of *lin*-22, we followed individual cell lineages in *lin-22(–)* males. Our findings confirmed and extended those of Fixsen (1985), and are shown in Fig. 2. *lin*-22 males differed from wild type in two respects. First, the V5 lineage generated two rays instead of one. This was because a cell fate transformation occurred within the V5 lineage. In the wild-type V5 lineage, the cell V5.pppp becomes a ray precursor cell, whereas its sister, V5.pppa, becomes a seam cell. However, in *lin*-22 mutants, both V5.pppp and V5.pppa became ray precursor cells and subsequently generated rays (Fig. 2B). The second *lin-22(–)* phenotype was more dramatic: this new V5-like lineage pattern was also generated by the anterior V1-V4 cells. Thus we conclude that in the wild type, *lin*-22 function is required to prevent the formation of an additional ray precursor

cell within the V5 lineage, and also to allow the V(1-4) lineages to generate their simple epidermal stem cell lineages rather than a V5-like lineage.

lin-22 encodes a helix-loop-helix protein homologous to the *hairy* and *E(spl)* genes of *Drosophila* and other organisms

To clone *lin-22*, we used positional cloning methods to identify eight Yeast Artificial Chromosomes (YACs) containing genomic DNA from the *lin-22* region (Costa et al., 1988; Ruvkun et al., 1989; Coulson et al., 1988). DNA from these YACs was gel purified and injected into the germline of *lin-22*(–) hermaphrodites along with the dominant co-injection marker Rol-6 (Mello et al., 1991). One of these YACs (Y68H6) rescued the Lin-22 phenotype and was subsequently used to probe an unamplified *C. elegans* genomic lambda library (see Materials and Methods). This led to the identification of a 15 kb lambda clone that also rescued the Lin-22 mutant phenotype in transformation experiments.

We sequenced subclones from the rescuing lambda clone and found a region containing a basic helix-loop-helix domain (bHLH) that was most similar to *hairy* and *hairy*-like genes (85% similar to the bHLH of the rat/mouse *HES-1* genes and 78% similar to the *Drosophila hairy* bHLH domain; see Fig. 3). The homology to these *hairy* family members appeared to be limited to the bHLH domain, which mediates DNA binding. Many other *hairy* family members contain two other conserved domains that appeared to be absent from LIN-22 (see Fig. 4, legend), an Orange domain, which has been shown to confer specificity among members of the Hairy/E(SPL) family (Dawson et al., 1996), and a conserved WRPW domain, which mediates protein-protein interactions between Hairy family members and Groucho (Paroush et al., 1995).

The structure and sequence of the putative *lin-22* cDNA (Fig. 4) was obtained by RT-PCR of wild-type total and poly(A)+ RNA, and confirmed by sequencing genomic DNA. To ask whether this gene was *lin-22* we sequenced PCR fragments of genomic DNA from the three *lin-22* mutant alleles. The weakest *lin-22* allele, *mu5* (Waring et al., 1992; Wrischnik, 1995), contained a stop codon 70 nucleotides downstream of the bHLH domain (Fig. 4). The stronger *mu2* mutation created a stop codon within helix 1, which would be predicted to produce a non-functional protein. The third allele, *n372*, contained a missense mutation in a glutamate residue that is completely conserved in *hairy*, the *E(spl)* genes, and their homologs (Figs 3, 4). A missense mutation of this specific glutamate residue is found in the *hairy*^{5H} allele of *Drosophila* (Wainwright and Ish-Horowicz, 1992) and also in the phenotypically strongest *lin-32* allele, *u282* (which is semi-dominant; Zhao and Emmons, 1995). *n372* has a phenotype virtually identical to that of *mu2*, except for a slight semi-dominant effect on postdeirid production (Waring et al., 1992; Wrischnik, 1995). From these data we conclude that *lin-22* encodes a bHLH protein homologous primarily to *hairy*.

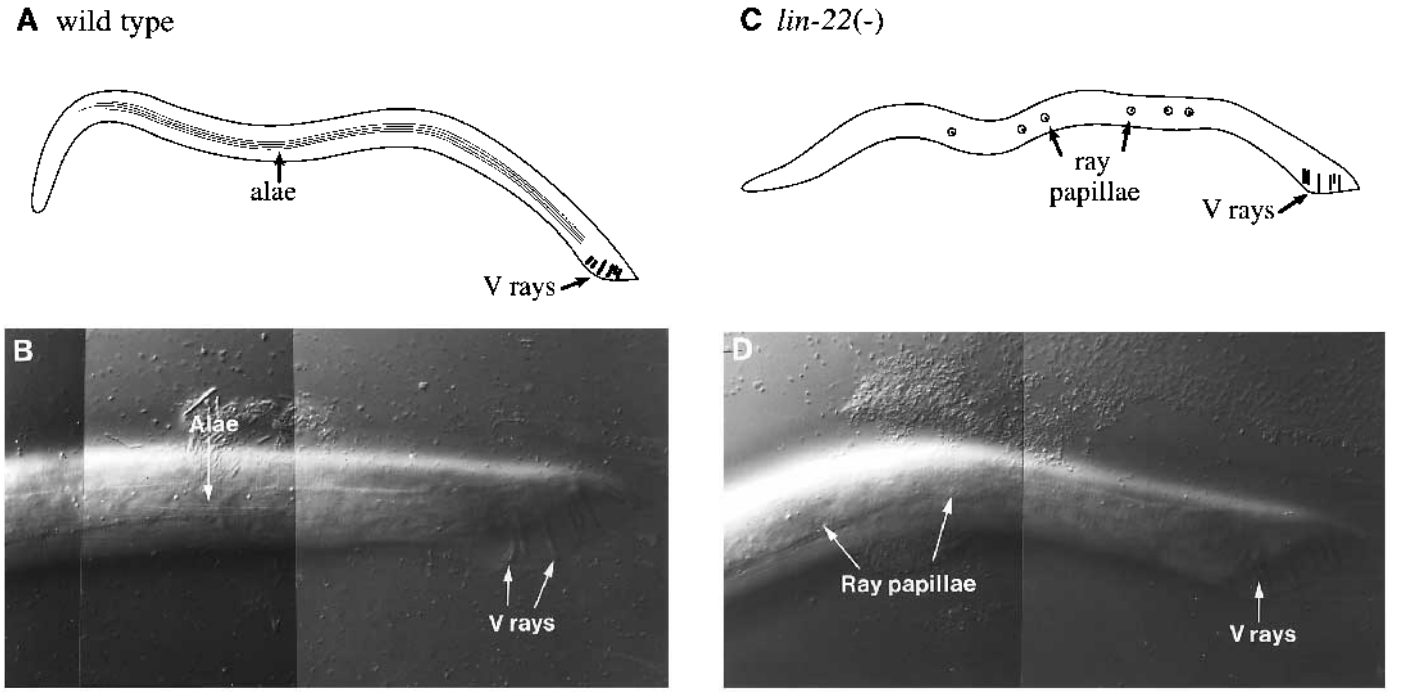


Fig. 1. Adult V cell phenotype of wild-type and *lin-22* mutant males. (A) Tracing of a wild-type adult male. In this and the following figures, anterior is to the left. Epidermal cells derived from the V cells generate ridges in the adult cuticle called alae. Six of the nine sensory rays found in the male tail also derive from the V cells. (B) Nomarski photomicrograph of the tail of the male shown in A. Alae and V-derived rays are indicated by arrows. (C) Tracing of an adult *lin-22*(-) male. The V cells generate neuroblasts in place of epidermal cells, so the alae is missing and is replaced by sensory rays. Although correct ray morphogenesis does not always occur along the body, ray papillae, the very tips of rays, can often be seen. (D) Nomarski photomicrograph of the tail of the *lin-22*(-) male shown in C. Two of the ray papillae are indicated. The length of the adult male is just shy of 1 mm.

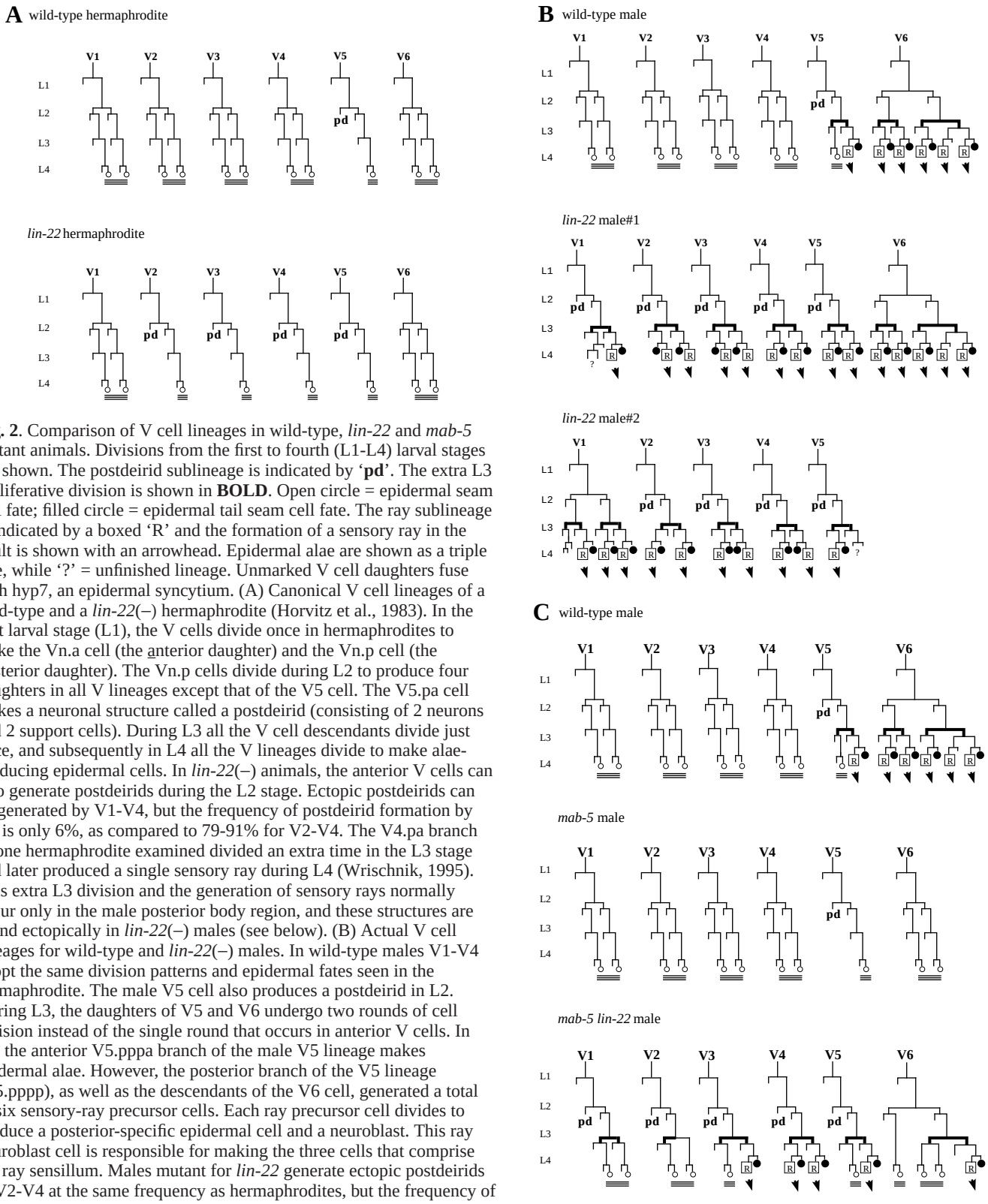


Fig. 2. Comparison of V cell lineages in wild-type, *lin-22* and *mab-5* mutant animals. Divisions from the first to fourth (L1-L4) larval stages are shown. The postdeirid sublineage is indicated by '**pd**'. The extra L3 proliferative division is shown in **BOLD**. Open circle = epidermal seam cell fate; filled circle = epidermal tail seam cell fate. The ray sublineage is indicated by a boxed 'R' and the formation of a sensory ray in the adult is shown with an arrowhead. Epidermal alae are shown as a triple line, while '?' = unfinished lineage. Unmarked V cell daughters fuse with hyp7, an epidermal syncytium. (A) Canonical V cell lineages of a wild-type and a *lin-22*(–) hermaphrodite (Horvitz et al., 1983). In the first larval stage (L1), the V cells divide once in hermaphrodites to make the Vn.a cell (the anterior daughter) and the Vn.p cell (the posterior daughter). The Vn.p cells divide during L2 to produce four daughters in all V lineages except that of the V5 cell. The V5.pa cell makes a neuronal structure called a postdeirid (consisting of 2 neurons and 2 support cells). During L3 all the V cell descendants divide just once, and subsequently in L4 all the V lineages divide to make alae-producing epidermal cells. In *lin-22*(–) animals, the anterior V cells can also generate postdeirids during the L2 stage. Ectopic postdeirids can be generated by V1-V4, but the frequency of postdeirid formation by V1 is only 6%, as compared to 79-91% for V2-V4. The V4.pa branch of one hermaphrodite examined divided an extra time in the L3 stage and later produced a single sensory ray during L4 (Wrischnik, 1995). This extra L3 division and the generation of sensory rays normally occur only in the male posterior body region, and these structures are found ectopically in *lin-22*(–) males (see below). (B) Actual V cell lineages for wild-type and *lin-22*(–) males. In wild-type males V1-V4 adopt the same division patterns and epidermal fates seen in the hermaphrodite. The male V5 cell also produces a postdeirid in L2. During L3, the daughters of V5 and V6 undergo two rounds of cell division instead of the single round that occurs in anterior V cells. In L4, the anterior V5.pppa branch of the male V5 lineage makes epidermal alae. However, the posterior branch of the V5 lineage (V5.pppp), as well as the descendants of the V6 cell, generated a total of six sensory-ray precursor cells. Each ray precursor cell divides to produce a posterior-specific epidermal cell and a neuroblast. This ray neuroblast cell is responsible for making the three cells that comprise the ray sensillum. Males mutant for *lin-22* generate ectopic postdeirids in V2-V4 at the same frequency as hermaphrodites, but the frequency of postdeirid production by V1 is significantly higher (43%). In addition, the anterior V cells undergo an extra round of cell division in L3, and can also generate ectopic sensory rays. Polarity reversals often occur during the L3 divisions and L4 ray precursor cell divisions, but only in anterior V1-V4 cells (polarity reversals were seen in 46% of these divisions in a sample of 6 animals lineaged). These reversals appear to occur in the ray neuroblast divisions as well. (C) V cell lineages in wild-type, *mab-5*(–), and *mab-5*(–); *lin-22*(–) males (data from Kenyon, 1986) Loss of *mab-5* function leads to the loss of the L3 proliferative divisions in V5 and V6 and blocks the formation of sensory rays. Loss of *mab-5* function in a *lin-22* mutant, however, does not lead to the complete loss of the L3 proliferative divisions in any of the V cell lineages (an additional 12 males scored).

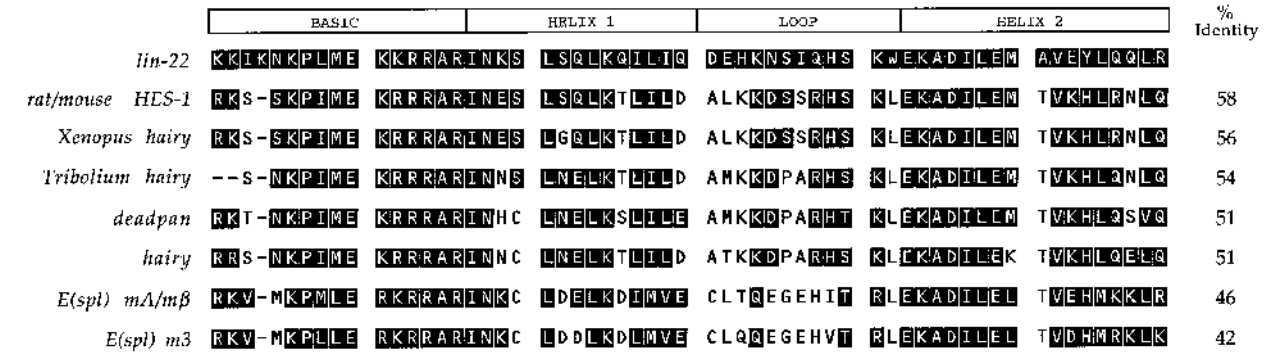


Fig. 3. Comparison of the predicted *lin-22* basic HLH domain with closely related sequences. The *lin-22* protein sequence is displayed on top followed by seven *hairy/Enhancer of split* homologs: *HES-1* protein from rat and mouse (Sasai et al., 1992; Takebayashi et al., 1994), *hairy*[1] from *Xenopus* (Dawson et al., 1995), Tribolium *hairy* (Sommer and Tautz, 1993), *deadpan* (Bier et al., 1992), *hairy* from *D. melanogaster* (Klambt et al., 1989), the *E(spl) mA/mβ* transcript (Knust et al., 1992; Delidakis and Artavanis-Tsakonas, 1992), and the *E(spl) m3* transcript (Delidakis and Artavanis-Tsakonas, 1992). The basic helix-loop-helix domains are indicated above the sequences and conserved amino acids (based on BLAST parameters; Altschul et al., 1990; Gish and States, 1993) are highlighted in black. The % amino acid identity between *lin-22* and a particular sequence is listed at the right.

Generation of ectopic postdeirids in *lin-22* mutants requires *lin-32*(+) activity

We next asked whether the cell fate transformations seen in *lin-22* mutants could be accounted for by changes in the activities of any known regulatory genes. First, we asked what gene activity (or activities) could be responsible for promoting the formation of the ectopic postdeirids produced by the anterior V(1-4) cells in *lin-22* mutants. A good candidate was the *lin-32* gene, since *lin-32* is required for the production of many different neuroblasts in *C. elegans*, including the postdeirid (Zhao and Emmons, 1995). To ask whether the ectopic postdeirids generated in the anterior of *lin-22* mutants required *lin-32*(+) activity, we constructed and analyzed *lin-22*; *lin-32* double mutants. We found that neither the normal V5 postdeirid nor the ectopic postdeirids were made in these double mutants (Fig. 5). Instead, cells at this position in the lineage became seam cells, as in the wild-type V(1-4) lineages. This indicates that formation of ectopic postdeirids in *lin-22* mutants requires *lin-32*(+) activity. Since the only difference between the anterior V cell lineages and the posterior V5 cell lineage in hermaphrodites is the formation of the postdeirid, ectopic *lin-32*(+) activity could account for the entire *lin-22*(–) hermaphrodite phenotype.

lin-22(+) inhibits expression of the posterior-specific Hox gene *mab-5* within the V5 lineage

In addition to producing ectopic postdeirids, males carrying mutations in *lin-22* exhibit two additional mutant features: the first is the generation of an additional ray precursor cell within the V5 lineage, and the second is the spatial homeotic transformation of anterior V1-V4 lineages into this new V5-like lineage. We first investigated the change that occurs within the V5 lineage, and asked what might cause the V5.ppp stem cell to generate a ray precursor cell instead of an epidermal cell. The *lin-32* gene described above is not required for the production of the ray precursor cell (Fig. 5; Zhao and Emmons, 1995). However, the activity of another gene, the Hox gene *mab-5*, is required for the production of this cell (Fig. 2C). During wild-type development, *mab-5* is expressed in the V5.pppp cell, which becomes a ray precursor cell, but not in its sister, V5.pppa. However, if *mab-5* is expressed ectopically in the V5.pppa cell using a heat-shock promoter fusion, the cell becomes a ray precursor cell and generates a ray (Salser and Kenyon, 1996). In addition, this ectopic ray is often missing in *mab-5; lin-22* mutants (Kenyon, 1986). We therefore used polyclonal anti-MAB-5 antisera (Salser et al., 1993) to ask whether *lin-22* mutations caused the V5.pppa cell to express *mab-5*. We found that MAB-5 protein was present in this cell in *lin-22* mutants (Fig. 6D,F). This suggests that ectopic *mab-5* expression is responsible for the additional ray. In addition, it indicates that in the wild-type male, *lin-22*(+) acts directly or indirectly to inhibit *mab-5*(+) expression within the anterior branch of the V5 lineage. Curiously, the ectopic *mab-5* expression seen in the anterior branch of V5 was consistently different from the staining in the posterior branch that would normally make a sensory ray: staining in V5.pppa appeared later and was weaker than the staining seen in its sister, V5.pppp (see Discussion).

lin-22(+) activity inhibits *mab-5* expression in anterior V cells

We next asked why the anterior V cells adopt posterior-specific, V5-like fates in *lin-22* mutants. As described above, ectopic postdeirid formation can be accounted for by ectopic acitivity of *lin-32*. What about the ectopic seam cell divisions and sensory rays? In the wild type, the Hox gene *mab-5* is required for all of the posterior-specific features of the V5 lineage except for the postdeirid. These features include the extra round of seam cell division in the L3 and the V5 ray (Kenyon, 1986). In addition, the ectopic expression of *mab-5*(+) using a heat-shock promoter is sufficient to elicit both L3 proliferative divisions and sensory ray production in the anterior V cells (Salser and Kenyon, 1996). Therefore it seemed possible that ectopic *mab-5* expression might be responsible for these lineage transformations. When we examined the distribution of MAB-5 protein in a *lin-22*(–) mutant, we found that MAB-5 expression, normally observed only in the daughters of V5 and V6, now occurred in the anterior V lineages (Fig. 6C,D,F). In the wild type, *mab-5* is expressed in a complex, dynamic pattern in the V5 lineage. We

found that this dynamic pattern of *mab-5* expression was reiterated by the anterior V1-V4 cells in *lin-22* mutants. MAB-5 expression first appeared during the second larval stage (L2) in only the posterior branch of the lineage; later in L2, MAB-5 expression disappeared, only to reappear during the L3 stage in both branches of the lineage that generates sensory rays (Fig. 6F). As in the *lin-22(–)* V5 lineage described above, the V(1-4).pppa cells, which produce the second ray during the L3 stage, often had weaker staining that appeared later than that seen in their posterior sisters (Fig. 6C,D).

lin-22 mutations affect the activities of the Hox genes *lin-39* and *egl-5*

The ectopic expression of *mab-5* seen in *lin-22* mutants suggested that ectopic *mab-5*(+) activity would be responsible for all of the extra rays generated in *lin-22*(–) males. Surprisingly, however, males mutant for both *mab-5* and *lin-22* can still make sensory rays, although they make fewer than are found in *lin-22* mutants alone (Kenyon, 1986). We wondered what gene activities were responsible for the residual rays seen in *mab-5*(–); *lin-22*(–) males. We suspected that the other Hox genes, *lin-39* and *egl-5*, could be involved because both are expressed in the lateral ectoderm and both can affect sensory ray production. Mutations in *egl-5*, an *Abdominal-B* homolog, result in the loss of one of the V6 sensory rays and affect the identities of the remaining V5 and V6 rays (Chisholm, 1991; Wang et al., 1993; Chow and Emmons, 1994). *lin-39*, a *Sex-combs-reduced*/*Deformed* homolog, is needed for the proper development of cells in the central body region, and does not normally affect the rays (Ellis, 1985; Wang et al., 1993; Clark et al., 1993).

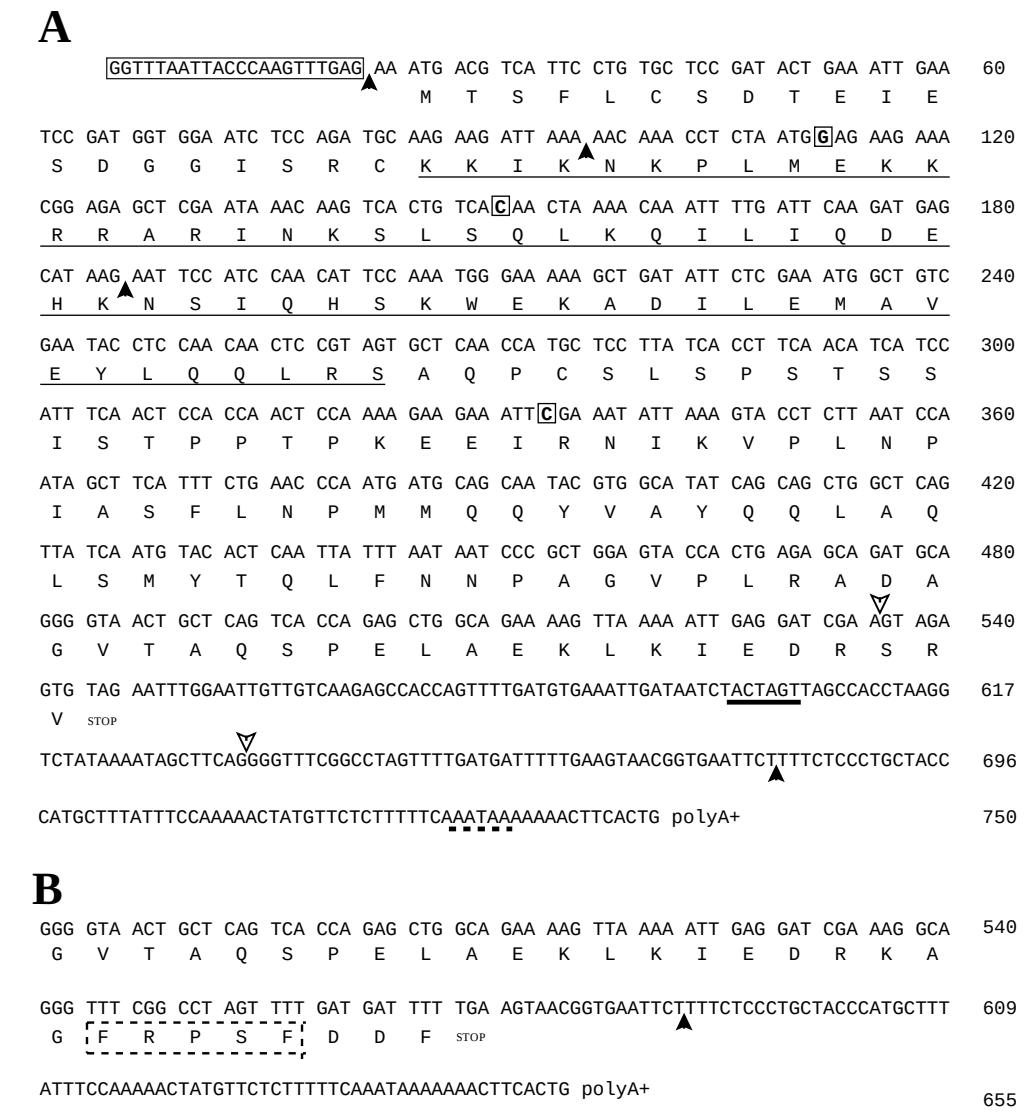


Fig. 4. Predicted nucleotide and amino acid sequences of the *lin-22* gene. The cDNA sequence is listed above the predicted protein sequence. Boxed DNA contains the SL1 splice leader sequence (Krause and Hirsh, 1987). Filled arrowheads denote splice junctions, based on comparisons of cDNA with genomic sequence. The conserved basic helix-loop-helix (bHLH) domain is underlined. A putative polyadenylation site is underlined with dashes. That this gene encodes LIN-22 has been demonstrated by identifying the DNA lesions in the three known *lin-22* alleles. In the *lin-22(n372)* allele, a G→A transition at position 112 changes a conserved glutamate residue to a lysine residue in the basic region of the protein (boxed G nucleotide). This glutamate is 100% conserved among members of this family. The *lin-22(mu2)* allele contains a C→T transition at position 183 (boxed C) that results in the formation of a stop codon within helix 1 of the bHLH domain. The *lin-22(mu5)* allele contains a C→T transition at position 234 (boxed C) which leads to the generation of a stop codon after the bHLH domain. The actual C terminus of the cDNA has not been identified unambiguously. The primary cDNA product isolated (A) contains no obvious Orange domain (a domain that confers specificity among members of the *Hairy/E(spl)* family; Dawson et. al., 1995) 3′ to the bHLH region, and does not contain the canonical WRPW tetrapeptide found at the C terminus in virtually all other *hairy* and *E(spl)* homologs (and implicated in *hairy*/*Groucho* interactions; Paroush et al., 1995). It is possible that LIN-22 protein does not require this domain, or that another amino acid sequence serves its function. No obviously related amino acid sequences are present in the protein inferred from this cDNA sequence. This cDNA does contain a potential intron from postition 536-635 (open arrowheads) which, if removed, would generate a C terminus containing an FRPSF sequence (dashed box), which might be a candidate for a domain with a function normally provided by the canonical WRPW motif (B). Transformation rescue experiments (see Materials and Methods) show that loss of genomic information 3′ to the *Spe*I site (heavy underline) is not necessary for rescue of the *lin-22* loss-of-alae phenotype.

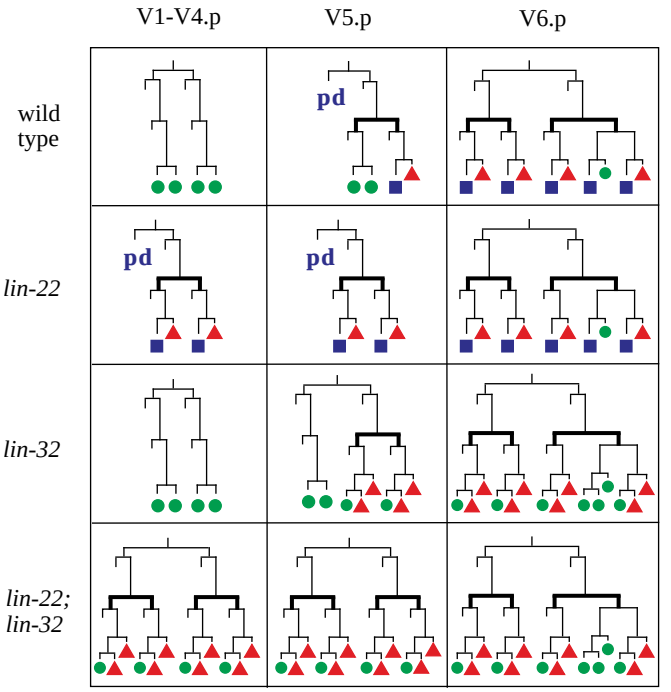


Fig. 5. *lin-32* mutations specifically block neuroblast production in wild-type and *lin-22*(–) males. Lineages start at the second larval stage (L2). Green circles indicate an epidermal cell fate, while red circles represent a specific epidermal fate, that of the tail seam cell. The postdeirid sublineage is indicated by a blue '**pd**', and a blue square represents the ray neuroblast sublineage. Mutations in *lin-32* prevent the formation of larval neuroblasts, and as a consequence neither the V5 postdeirid nor the V5 and V6 sensory rays are made, and epidermal cells are made instead (Zhao and Emmons, 1995; for additional males examined see Materials and Methods). This is shown schematically by the loss of the blue neuronal lineages. However, we found that the extra L3 proliferative divisions seen in V5.ppp and the V6 descendants still occurred in a *lin-32* mutant (n=15). In addition, although the ray neuroblast adopts an epidermal fate in *lin-32* mutants, its parent, the ray precursor cell, still divides to generate what would have been the ray neuroblast and its sister, the tail seam cell (Zhao and Emmons, 1995). Males with mutations in both *lin-22* and *lin-32* failed to generate either postdeirid or ray neuroblasts, but the extra L3 proliferative divisions (n=2) and the generation of ectopic ray precursors (indicated by the formation of ectopic tail seam cells) still occurred in the anterior V cells. The presence of anterior tail seam cells in *lin-22; lin-32* mutants was scored directly (n=12) or by their ability to disrupt alae formation when present in large numbers (n=38). When *mab-5*(+) activity is removed from *lin-22*(–); *lin-32*(–) males (n=76), these tail seam cells are often no longer made because the formation of the ectopic ray precursor cells are dependent on *mab-5* activity (data not shown).

However, ectopic expression of *lin-39* can lead to the occasional formation of a sensory ray in *mab-5*(–) males (Hunter and Kenyon, 1995).

To assess whether these other Hox genes play a role in ray production in *lin-22* mutants, we removed either *lin-39* or *egl-5* activity from *mab-5; lin-22* double mutants by generating animals of the genotypes *lin-39 mab-5*; *lin-22* and *mab-5 egl-5*; *lin-22*. We found that removing either *lin-39*(+) or *egl-5*(+) gene activity led to a reduction in the number of sensory rays produced, indicating that the formation of many of these ectopic rays requires *lin-39*(+) or *egl-5*(+) activity (Fig. 7). The loss of either *lin-39*(+) or *egl-5*(+) activity reduced ray production only within the normal domain of function of that gene.

lin-22 may regulate an unknown activity that promotes cell proliferation

In otherwise wild-type males, *mab-5*(+) activity is necessary and sufficient for the L3 proliferative divisions. Therefore, we expected that the production of the ectopic L3 proliferative divisions seen in *lin-22* mutants would require *mab-5*(+) activity. However, we found that these divisions were generally unaffected in *lin-22*(–) males containing additional mutations in *mab-5*, both *mab-5* and *lin-39*, or both *mab-5* and *egl-5* (data not shown; Wrischnik, 1995). This suggests that *lin-22* may act on an as yet unidentified target gene to prevent cell proliferation in the anterior lateral ectoderm.

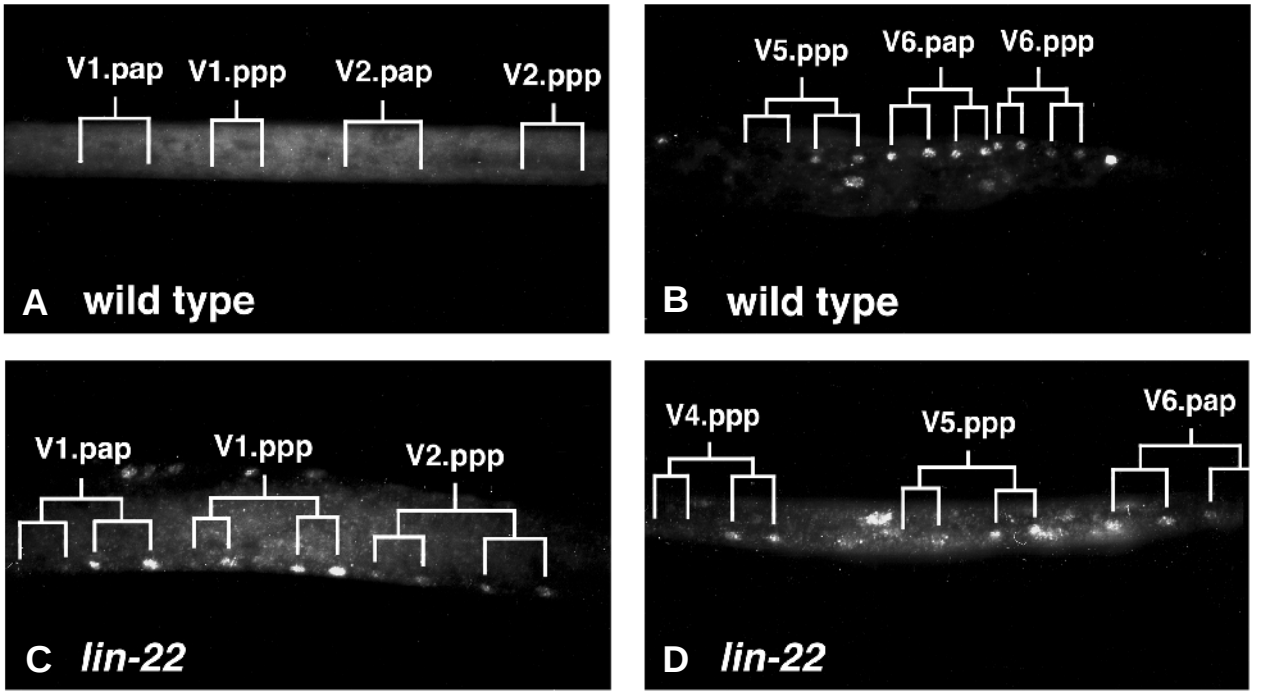
lin-22 affects Hox gene function in the hermaphrodite

Curiously, in *lin-22* mutants, not all V cells produced postdeirids at the same frequency. In *lin-22*(–) hermaphrodites, the V2, V3 and V4 cells generated postdeirids at a frequency of 79%, 82% and 91%, respectively. However, the V1 cell generated ectopic postdeirids at a frequency of only 6% (see Fig. 8). Why does the V1 cell behave differently from the other V cells? The Hox gene *lin-39* patterns the central body region and is expressed in V2-V5, but not V1 (Wang et al., 1993; J. Maloof and C. Kenyon, unpublished results), so we wondered whether *lin-39*(+) activity caused V2-V4 to act differently from V1 in a *lin-22* mutant. To test this we examined postdeirid production in *lin-39*; *lin-22* double mutants and found that the V2, V3 and V4 cells still made ectopic postdeirids, but at a frequency of only 9-15%, much closer to that of V1 (6%). This suggests that in *lin-22* mutants, LIN-39 protein may differentiate V(2-4) from V1.

DISCUSSION

The findings from this and previous work (Fixsen, 1985; Waring et al., 1992) demonstrate that wild-type *lin-22* activity controls two different types of cell fate decisions in *C. elegans*. First, it inhibits the production of a second ray neuroblast within the V5 lineage. Second, it creates large-scale regional differences in A/P pattern by preventing anterior V(1-4) cells from adopting a V5-like fate. Since the lineage generated by V5 contains both postdeirid and ray neuroblasts whereas the anterior V cell lineages consist only of epidermal cells, wild-type *lin-22* activity has the effect of markedly restricting the amount of neurogenesis that takes place in the lateral ectoderm.

We have found that *lin-22* encodes a *hairy/E(spl)* homolog, and that it exerts its effects by changing the activities of a number of other regulatory genes. First, it inhibits the expression and function of the Hox gene *mab*-5 within one specific branch of the V5 lineage. Second, it inhibits expression of this same Hox gene in anterior body regions. Third, it inhibits the activities of the Hox genes *lin-39* and *egl-5* within their normal domains of function, the central body region and tail regions, respectively. Fourth, it inhibits the activity of the *C. elegans achaete-scute* homolog, *lin-32*, in the anterior body region. Finally, it must regulate an unidentified gene function



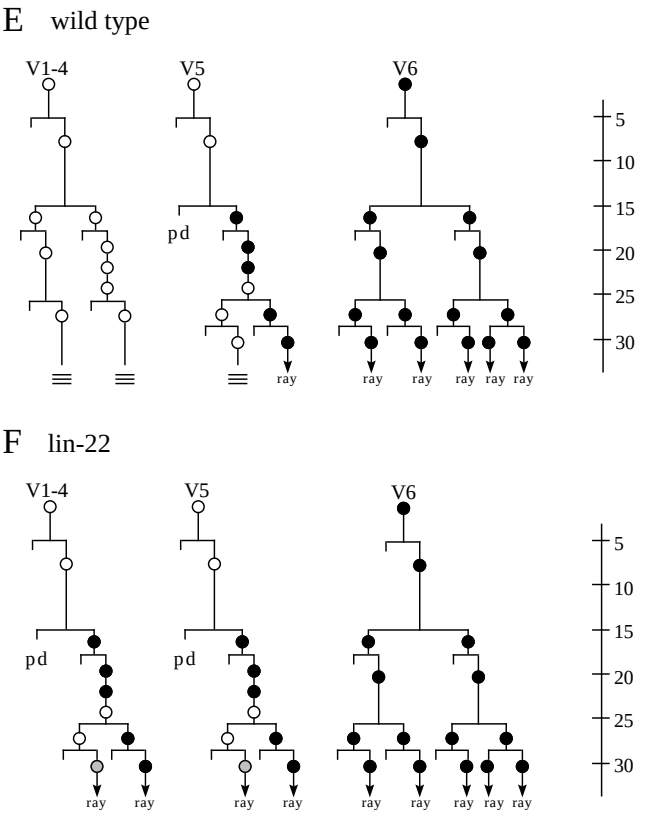


Fig. 6. MAB-5 protein expression in wild-type and *lin-22*(−) males. (A,B) Distribution of MAB-5 protein in the anterior (A) and posterior (B) of a wild-type male during the beginning of the L3 stage (approx. 25 hours after hatching at 20°C). No anterior staining was detected. In contrast, all the V6 descendants expressed *mab-5*. Only the two posterior daughters of V5 that will make a sensory ray (V5.ppppa and V5. ppppp) express *mab-5* in the V5 cell. The location of the Vn.pap and Vn.ppp daughters are indicated by the lineage drawings. Polyclonal rabbit α-MAB-5 antiserum was used (see Materials and Methods). (C,D) MAB-5 protein expression in a *lin-22*(−) male. All the daughters of V6 stain as in wild type, but the daughter cells from both branches of the V5 lineage express *mab-5* (D). Expression in the anterior branch was consistently weaker than that seen in the posterior branch. In addition, MAB-5 protein was also seen in the anterior V cell descendants of V1 and V2 (C), and V4 (D). Expression in the anterior cells mimics the pattern seen in V5, with anterior daughters often staining more weakly than their posterior sisters. (E,F) Schematic of MAB-5 protein expression seen in the V1-V6 lineages through the L3 stage in wild type (E) and *lin-22* (F) males. Filled circles indicate cells staining with α-MAB-5 antibody. (Staining in pre-syncytial cells is not shown). Ray formation is indicated by 'ray' and an arrow. Time after hatching is given at the right. The wild-type pattern is that seen by Salser and Kenyon (1996). In *lin-22*(−), the V6 pattern of expression was identical to that seen in wild type. Early V5 staining was also identical: initially *mab-5* was not expressed during the first larval stage (0-16 hours post hatching), but staining subsequently appeared during the second larval stage (L2; 16-25 hours after hatching). This staining persisted throughout L2 until just before the proliferative L3 division (at 27-28 hours). Staining returned after the first L3 division only in the posterior daughter, but then came on in both branches after the next division. This identical pattern of expression was displayed by the anterior V cells (including the H2 cell of the head region) in a *lin-22* mutant. The numbers of *lin-22* animals examined at each stage was 8-12 for the following time points: 4-6 hours, 8-10 hours, 15-17 hours, 19-21 hours, 21-23 hours, and 25+ hours. The pattern of *mab-5* expression appears to be identical for *lin-22*(−) hermaphrodites and males through the L2 stage. Wild-type controls showed no staining in any anterior V cell descendants at any timepoint (15-17 hours, n=19; 25+ hours, n=22; all others n>10). After the L3 divisions, the number of hermaphrodites expressing ectopic *mab-5* dropped to only 11/25 as opposed to 25/27 in males. In addition, if a hermaphrodite had anterior staining, it was generally weaker and occurred in fewer cells than in the males.

that controls stem cell proliferation. Some of these activities of *lin*-22 in *C. elegans* may be analogous to those of *hairy* in *Drosophila*.

lin-22 regulates the expression of the Hox gene *mab-5* within the V5 lineage

One of the major findings of this study is that *lin-22* activity plays a central role in the spatial regulation of expression of the Hox gene *mab-5*. *mab-5* has an interesting pattern of expression in the wild type. In the V(1-4) lineages, *mab-5* is not expressed. In the V5 lineage, *mab-5* is expressed in a highly complex and dynamic fashion, where it acts multiple times to regulate many different types of cell fate decisions independently of one another. In the V6 cell, *mab*-5 expression is activated in the embryo and persists throughout the V6 lineage. In this lineage, *mab-5* appears to act only once, very early in postembryonic development, to specify the entire V6 lineage (Salser and Kenyon, 1996). How such a complex pattern of expression and function is achieved during development is a fascinating question.

We have found that *lin-22* regulates one specific aspect of the complex *mab-5* expression pattern within the V5 lineage. In the wild type, V5.pppa does not express *mab-5* and becomes a seam cell, whereas its sister switches on *mab-5* and becomes a ray precursor cell. *mab-5* expression in these cells is known to be both necessary and sufficient to distinguish between the ray precursor and seam cell fates (Salser and Kenyon, 1996). In this study we have found that *lin-22* activity is required for these two cells to express *mab-5* differently: in *lin-22* mutants, both cells express *mab-5*, and both become ray precursor cells.

It is interesting that *mab-5* expression is not identical in these two sister cells in *lin-22* mutants; instead it appears later, and fainter, in the V5.pppa cells than in their sisters. It is not clear whether this is because these *lin*-22 mutations may not remove all *lin-22* activity, or whether it reflects the existence of an underlying difference in the potential of these cells to express *mab-5*. A similar phenomenon was observed in *lin-22; mab-5* double mutants: in these animals, the V cells generated fewer rays than in *lin*-22 single mutants, and the Vn.pppp cells were much more likely to become ray precursor cells than their sisters were (Kenyon, 1986; unpublished observations). Thus we favor the hypothesis that there is an underlying bias that potentates ray formation within the posterior sisters.

lin-22 activity defines the spatial domain of *mab-5* expression by inhibiting its expression in anterior V cell lineages

In addition to affecting the pattern of cell fates generated within the V5 lineage, *lin-22* also acts as a more global spatial

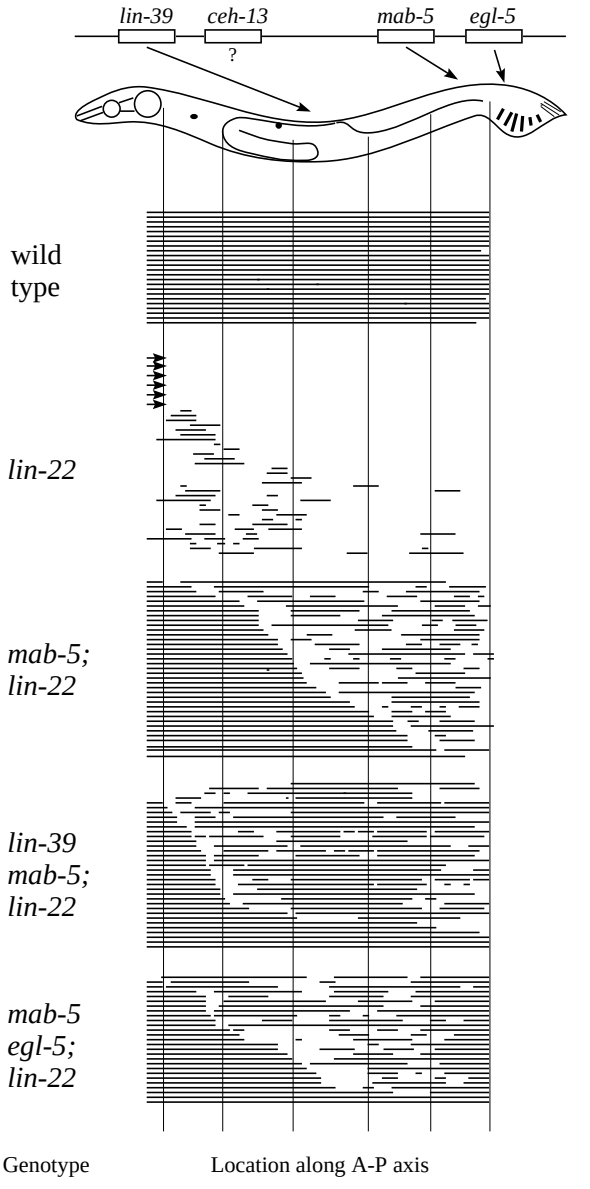


Fig. 7. Effects of Hox mutations on alae production in *lin-22* mutant males. Each line represents the location of epidermal alae along the A-P axis from an individual male worm. The locations of patches of alae are shown relative to cellular and anatomical markers positioned along the A-P axis (see Materials and Methods). The *C. elegans* Hox cluster is shown as a reference, and the body region influenced by each gene is indicated with an arrow. Arrowheads represent males with no alae. Alae extends along the length of the male from the head to the tail in wild-type and *mab-5*(−) males. Mutations in *lin-22* led to the generation of ectopic sensory rays in the male and the concomitant loss of alae-producing epidermal cells. The loss of *mab-5*(+) activity in a *lin-22*(−) mutant greatly decreased the formation of ectopic sensory rays, especially in the anterior of the animal, and led to an increase in alae production. This was first observed by Kenyon (1986). *lin-39*(+) and *egl-5*(+) activities play a role in generating the ectopic sensory rays seen in *mab-5; lin-22* mutants. When either *lin-39*(+) or *egl-5*(+) activity was removed from the *mab-5; lin-22* double mutant, the formation of ectopic sensory rays was reduced, primarily in the domain-of-function of that Hox gene (the center three zones for *lin-39*; the final tail zone for *egl-5*). In an effort to discern whether we could abolish all sensory ray production by removing the activities of the three known Hox genes, we made a strain mutant for *lin-22*, *lin-39*, *mab-5*, and *egl-5*. The strain had poor viability, but males that could be scored were still capable of making some sensory rays. However, these rays were produced randomly along the A-P axis (data not shown). Perhaps these residual rays result from the activity of the final *C. elegans* Hox gene, a *labial* homolog for which there is no known mutation (Schaller et al., 1990). Alternatively, these rays could be the products of elevated *lin-32* activity, since extra ray neuroblasts may sometimes be generated when *lin-32* is expressed ectopically using a heat-shock promoter (see Zhao and Emmons, 1995).

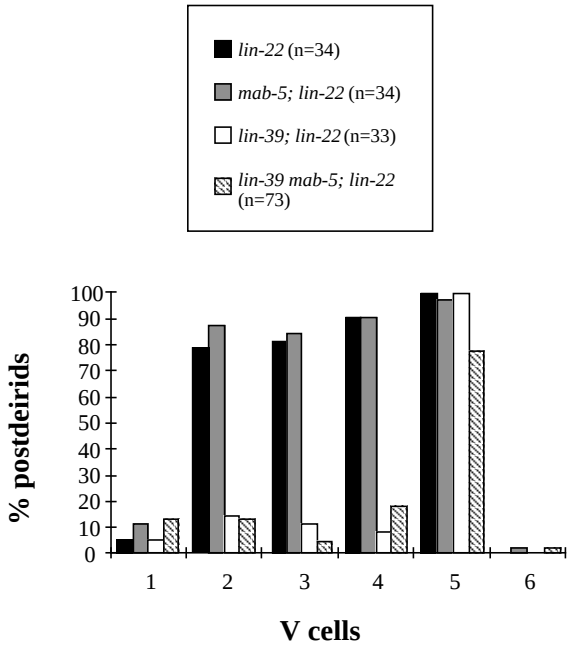


Fig. 8. *lin-39*(+) activity promotes postdeirid formation in *lin-22*(–) hermaphrodites. The graph shows the percentage postdeirid production by each V cell in hermaphrodites of the four indicated genotypes. Mutations in *mab-5* had no effect on ectopic postdeirid formation in *lin-22*(–) animals. However, the addition of the *lin-39* mutation to either *lin-22*(–) alone or to *mab-5*(–); *lin-22*(–) resulted in lowering the levels of postdeirid production in V2-V4 compared to that of V1. This phenomenon also occurs in males (Wrischnik, 1995). Although mutations in *lin-39* have no obvious effect on the V cells, these data suggest a role for *lin-39*(+) in patterning the V cells. Supression of V1 postdeirid formation in *lin-22*(–) was also seen for the *lin-39(mu26)* allele (*n*=50).

homeotic gene, causing anterior V cells to generate their normal lineages instead of copies of a V5-like lineage. In *lin-22* mutants, the anterior V(1-4) cells each undergo the same V5-like lineage that V5 does, and expresses *mab-5* in the same complex and dynamic fashion. This indicates that one of the roles of *lin-22(+)* activity in the wild-type is to prevent the anterior V1-V4 cells from activating the complex, dynamic pattern of *mab*-5 expression normally seen in V5.

Mutations in *lin-22* alter the activities of the Hox genes *lin-39* and *egl-5*

Since *mab-5* activity is required for all the rays produced by V5 and V6 in wild-type animals, it was surprising to find that in *mab-5; lin*-22 double mutants, some sensory rays are still generated (Kenyon, 1986). These rays are preferentially generated by cells in the central and posterior body regions. In a *lin-22* mutant background, if the *mab-5* gene dosage is incrementally decreased from two, to one, to zero gene copies, the boundary between alae and rays is progressively shifted towards the posterior. Conversely, if *mab-5* activity is increased above the wild-type level in a *lin-22* mutant background, then the boundary shifts further toward the anterior (Kenyon, 1986). The correlation between A/P position and *mab-5* gene dosage suggested the model that some component of the system that regulates the potential of V cells to produce alae is present in a graded distribution along the body axis (Kenyon, 1986). However, in this study we have found that the reason cells in the central and posterior body regions still generate rays in a *lin-22* mutant lacking *mab-5* activity is because two other Hox genes, *lin-39* and *egl-5*, have acquired the capacity to generate rays. Both of these Hox genes promote ray production in *lin-22* mutants, but only within their normal domains of function. This implies that wild-type *lin-22* activity does not define the domains of *lin-39* or *egl-5* function, but that it instead sets a functional threshold for these Hox genes within their primary domains of function. The interpretation that *lin-22* activity sets a rigid threshold for ray production is consistent with the observation that changing the level of *mab-5* activity shifts the alae/ray boundary along the A/P axis in a *lin-22* mutant background but not in the wild type.

lin-22 mutants lacking both *mab-5* and *lin-39* activity still produce some rays in the anterior body region (see Fig. 7). The gene activity responsible for these rays has not been identified; one candidate is the *C. elegans* Hox gene *ceh-13*, a *labial* homolog, whose function has not been determined, but which would be predicted to generate anterior-specific body pattern (Wang et al., 1993).

lin-22 activity may down-regulate a target gene that promotes the L3 proliferative divisions

In the wild-type V5 and V6 lineages, the seam cells undergo a proliferative cell division during the third larval stage that is not observed in the anterior V(1-4) lineages. In the wild type, this cell division depends on the activity of *mab-5*, and moreover, ectopic *mab-5* activity can cause the homologous cells in the anterior V(1-4) lineages to undergo a similar proliferative division. Thus it was surprising to find that mutations in *mab-5* did not prevent these proliferative cell divisions in *lin-22* mutants. It seems likely that these ectopic divisions are regulated by an as-yet unknown gene subject to inhibition by *lin-22*(+) activity. Mutations in *lin-22* also affect ventral ectodermal cells, specifically the P(3-8).p cells (Fixsen, 1985). In wild-type males these cells normally fuse with the epidermal syncytium during the L1 stage, but in *lin-22* mutant males P(3-8).p fail to fuse in L1 and undergo a division two stages later. Perhaps *lin-22* regulates a similar activity in both processes.

lin-22(+) prevents production of the postdeirid sensillum by inhibiting the activity of *lin-32*

One pattern element generated by V5 forms independently of all Hox genes currently known: the postdeirid neuroblast, which arises in the V5.pa lineage position. We have found that production of both the normal and ectopic postdeirid neuroblasts requires a different gene, the *achaete-scute* homolog *lin-32*. This suggests that in the wild type, *lin-22* activity directly or indirectly inhibits *lin-32* activity, and thereby prevents *lin-32* activity from generating postdeirid neuroblasts in the V1-V4 lineages.

Postdeirid production in the anterior body region of *lin-22* hermaphrodites also requires the activity of the Hox gene *lin-39. lin-39* activity is not required for V5 to produce a postdeirid in either wild type or *lin-22* mutants (Clark et al., 1993; Wang et al., 1993), so this finding was unexpected. *lin-39* is expressed in the V(2-4) lineages more strongly than in the V1 lineage (Wang et al. 1993; J. Maloof and C. Kenyon, unpublished observations), which probably explains why V(2-4) produce

postdeirids in *lin-22* hermaphrodites but V1 does so less frequently.

What restricts *lin-22* activity to the anterior V cell lineages?

The V-cell transformations seen in *lin*-22 mutants suggest that *lin*-22 is an anterior-specific patterning gene. It is not clear what allows the V5 lineage (with the exception of V5.pppa) to escape from the effects of *lin-22* activity in the wild type. However, it appears that the *caudal* homolog *pal-1*, described above, allows V6 to ignore *lin-22* activity. *pal-1* is required for V6 to develop differently from V(1-4) (Waring and Kenyon, 1990, 1991). In *pal-1(e2091)* mutants, which are defective in a late function of *pal-1*, *mab-5* expression in the V6 lineage is decreased, and V6 generates a V(1-4)-like lineage (Salser and Kenyon, 1996; Hunter and Kenyon, unpublished). Furthermore, in this mutant, V6 responds to *lin-22* activity the way that V1-V4 do: if a *lin-22* mutation is introduced, then V6 generates the same V5-like lineage that V(1-4) generate in *lin-22* mutants (Waring and Kenyon, 1990). Thus, *pal-1* plays a role in restricting *lin-22* activity to anterior cells during wild-type development.

Have the *lin-22* and *hairy/E(spl)* functions been conserved during evolution?

The LIN-22 protein contains a basic helix-loop-helix domain that bears strong homology to the transcriptional regulators *hairy* and *Enhancer of split* complex [*E(spl)*-C] genes of *Drosophila*. These genes play important roles in neural and anteroposterior pattern formation in *Drosophila*, so it is interesting compare their functions at the cellular level with those of *lin-22*.

Regulation of neurogenesis

In *Drosophila*, the *hairy* protein acts during postembryonic development to limit the boundaries of proneural clusters, groups of cells from which single sensory organ neuroblasts are derived, by inhibiting expression of proneural genes within the *achaete-scute* complex (Skeath and Carroll, 1991; Orenic et al., 1993). Loss of *hairy* function results in ectopic sensory bristles in the wing, thorax and head of *Drosophila* (Mocoso del Prado and Garcia-Bellido, 1984; Ingham et al., 1985). *hairy* inhibits neurogenesis by binding directly to the upstream regulatory region of the *achaete* gene and repressing *achaete*'s proneural activity (Ohsako et al., 1994; Van Doren et al., 1994). Homologs of *hairy*/*E(spl)* have now been identified in a number of organisms (*Tribolium*, Sommer and Tautz, 1993; rats and mice, Sasai et al., 1992; Feder et al., 1993; Takebayashi et al., 1994; and *Xenopus*, Dawson et al., 1995), where they may also function in the regulation of neurogenesis. For example, disruption of the murine *HES-1* gene leads to physical defects in the developing brain, and also results in increased expression of *Mash-1*, an *achaete-scute* homolog (Ishibashi et al., 1995).

The peripheral nervous system phenotypes of *hairy* and *lin-22* mutants have much in common. First, in both mutants many extra neuroblasts are produced. Worms carrying *lin*-22 mutations make five instead of one postdeirid neuroblast and up to nine additional rays; and there is a corresponding decrease in the number of epidermal cells produced. Furthermore, genetic experiments indicate that the additional postdeirid neuroblasts are generated because the *C. elegans achaete-scute* homolog functions ectopically, suggesting the possibility that an ancient *hairy/achaete-scute* regulatory relationship has been conserved during evolution. The additional rays also require *lin-32* activity; however, *lin-32* is required to produce the ray neuroblast itself, which is nested in the so-called ray-precursor sublineage initiated by expression of the Hox gene *mab-5*. For this reason, to prevent ray production it would be sufficient for *lin-22* activity to block *mab-5* expression rather than *lin-32* expression.

The *E(spl)*-C genes are part of the signaling pathway that turns off proneural gene expression in all but a single neuroblast precursor cell within the proneural cluster itself (for review see Ghysen et al., 1993). Like *hairy*, the *E(spl)*-C genes block neurogenesis by inhibiting the proneural genes of the *achaete-scute* complex. However, the *E(spl)* genes act in response to *Notch*/*Delta*-mediated cell signaling events within proneural clusters, whereas *hairy* helps to determine which potential proneural clusters are allowed to form in the first place. In *C. elegans*, intercellular signals between the daughters of the V cells are required for postdeirid formation (Sulston and White, 1980; Austin and Kenyon, 1994), which suggests the model that intercellular signals promote postdeirid formation by inhibiting *lin-22* activity. However, this model is ruled out by the finding that intercellular signaling is still required for postdeirids to form in *lin-22* mutants (Waring et al., 1992). In addition, the known *C. elegans Notch*/*Delta* homologs such as *lin-12* and *glp-1* are not involved in these signaling events (Austin and Kenyon, 1994).

Signals between V cells also inhibit the production of ray precursor cells (Sulston and White, 1980). Since *lin-22* activity also inhibits the production of ray precursor cells, these intercellular signals could, in principle, exert their effects by activating *lin-22*. However, this hypothesis is not completely supported by the data, since *lin-22* mutations trigger the production of extra rays in anterior V cells, which do not appear to be sensitive to intercellular signals (Sulston and White; 1980; Waring et al., 1992). Thus, at this point there is no compelling reason to think that *lin-22* functions similarly to the *E(spl)* genes to regulate neural development.

Regulation of cell division

In addition to regulating neuroblast formation, *lin-22* activity prevents seam cells from undergoing a proliferative division in the third larval stage. Likewise, *hairy* inhibits cell cycle progression and differentiation in the region just anterior to the morphogenetic furrow in the eye imaginal disc (Brown et al., 1991, 1995). *hairy*, in conjunction with *extramacrochaete*, may act to block photoreceptor differentiation by preventing progression of the cell cycle, thus inhibiting a cell proliferation event required for later neuronal differentiation. The occurrence of the L3 proliferative divisions greatly potentates subsequent ray formation in the V cells (Salser and Kenyon, 1996). Thus, the role of *lin-22* could parallel that of *hairy* in acting to block neurogenesis by inhibiting cell proliferation.

Regulation of A/P patterning

hairy also plays an important role in patterning the A/P axis of the *Drosophila* embryo. As a pair-rule gene, *hairy* is expressed in seven stripes of cells in the embryo, where it transmits A/P positional information to other pair-rule genes, such as *even-*

skipped, *runt* and *fushi tarazu* (Ingham et al., 1985; Carroll and Scott, 1986; Howard and Ingham, 1986; Ish-Horowicz and Pinchin, 1987; Carroll et al., 1988, Pankratz et al., 1990; Howard and Struhl, 1990; Riddihough and Ish-Horowicz, 1991). This ultimately affects Hox gene expression (Jack and McGinnis, 1990; Macias et al., 1994). Because it is expressed in seven stripes along the A/P axis, *Drosophila hairy* provides patterning information for many different body regions. In vertebrates, the *hairy* homologs initially identified were not expressed in patterns that suggested roles for them in axial patterning (Ishibashi et al., 1993; Feder et al., 1993; Takebayashi et al., 1994). However, more recently, one *hairy/E(spl)* homolog called *her-1* has been found that is expressed in a pair rule pattern in the developing zebrafish embryo. This interesting finding has suggested that a common precursor of insects and zebrafish may have been a segmented animal (Muller et al., 1996; Kimmel, 1997).

The *C. elegans lin-22* gene also plays an important role in anteroposterior patterning; however, nothing in the *lin-22* phenotype suggests that it functions in a periodic, or pair-rule, fashion. Instead, by influencing the functions of a number of downstream regulatory genes, including Hox genes, *lin-22* activity causes a large anterior body region (spanning V1 -V4) to develop differently from the posterior V5 body region. Thus, whereas the hypothesis that a regulatory relationship between *hairy* and *achaete-scute* has been conserved during evolution seems plausible, it is less clear whether *lin-22*'s role in regional A/P patterning arose through convergent or divergent evolution.

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