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The bromodomain protein LIN-49 and trithorax-related protein LIN-59 affect development and gene expression in *Caenorhabditis elegans*

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SUMMARY

We have molecularly characterized the *lin-49* and *lin-59* genes in *C. elegans*, and found their products are related to *Drosophila* trithorax group (trx-G) proteins and other proteins implicated in chromatin remodelling. LIN-49 is structurally most similar to the human bromodomain protein BR140, and LIN-59 is most similar to the *Drosophila* trx-G protein ASH1. In *C. elegans*, *lin-49* and *lin-59* are required for the normal development of the mating structures of the adult male tail, for the normal morphology and function of hindgut (rectum) cells in both males and hermaphrodites and for the maintenance of structural integrity in the hindgut and egg-laying system in adults. Expression of the Hox genes *egl-5* and *mab-5* is reduced in *lin-49* and *lin-59* mutants, suggesting *lin-49* and *lin-59* regulate HOM-C gene expression in *C. elegans* as the trx-G genes do in *Drosophila*. *lin-49* and *lin-59* transgenes are expressed widely throughout *C. elegans* animals. Thus, in contrast to the *C. elegans* Polycomb group (Pc-G)-related genes *mes-2* and *mes-6* that function primarily in the germline, we propose *lin-49* and *lin-59* function in somatic development similar to the *Drosophila* trx-G genes.

Key words: HOM-C gene expression, Trithorax group (trx-G) genes, Hindgut development, *Caenorhabditis elegans*

INTRODUCTION

Correct regulation of gene expression during development requires a variety of different proteins, ranging from cell-type specific transcription factors to the basal transcription machinery. Since DNA is tightly packaged in vivo, one important class of proteins involved in transcription is proteins that affect the accessibility of DNA. Such proteins are proposed to regulate chromatin structure and thereby either allow for or interfere with the expression of genes. Recent work has focused on a collection of multiprotein complexes that can affect gene expression by directly modifying nucleosomes and altering chromatin structure (reviewed by Kingston et al., 1996; Pollard and Peterson, 1998). Although a large number of these complexes have been described, how their function correlates with gene regulation during development in vivo largely remains to be characterized.

Of the proteins that participate in the maintenance of transcriptional states in animal development, the best characterized have been those encoded by the Polycomb group (Pc-G) and trithorax group (trx-G) genes in *Drosophila*. These genes were originally identified for their role in affecting expression of genes in the Bithorax and Antennapedia complexes that specify regional identity along the anterior/posterior axis (HOM-C genes; reviewed by Kennison, 1995). Genetically, Pc-G and trx-G genes represent two distinct classes, but the genes in each class encode a variety of different types of proteins. Nevertheless, many Pc-G and trx-G proteins include sequence motifs that suggest they associate with chromatin and participate in transcriptional regulation. In vivo, Pc-G genes act as transcriptional repressors, and trx-G genes act as transcriptional activators. Both molecular and genetic results point to a model in which Pc-G and trx-G proteins influence gene expression by affecting chromatin structure and thus contribute to establishing and maintaining specific transcriptional states (discussed by Papoulas et al., 1998).

Molecular homologues of Pc-G and trx-G genes are found in all animals. Recent work in *C. elegans*, however, suggests these genes may function differently in the development of nematodes compared to flies. Two genes (called *mes-2* and *mes-6*) are the orthologues of the Pc-G genes *E(z)* and *esc* (Holdeman et al., 1998; Korf et al., 1998). In *C. elegans*, mutations in these genes confer a maternal-effect sterile phenotype (Capowski et al., 1991). *mes-2* and *mes-6* are required for transcriptional repression (Kelly and Fire, 1998), but their expression and function is mostly limited to the germline. Thus, in *C. elegans* these Pc-G homologues act differently from Pc-G genes in *Drosophila*, as they don't appear to play a critical role in the developmental regulation of HOM-C gene expression, or in other aspects of somatic development. In addition, the sequenced *C. elegans* genome includes homologues to only these two of the nine molecularly characterized Pc-G genes (*C. elegans* sequencing consortium, 1998). Because of these differences between *C. elegans* and

Drosophila Pc-G genes, an important question is whether the developmental functions of the trx-G genes is likewise different between the two species. Homologues of some trx-G genes are present in the *C. elegans* genome (Table 1). However, the function of none of these *C. elegans* trx-G-related genes has previously been reported.

Because the mating structures of the *C. elegans* adult male tail derive from precursor cells in the posterior body region, alleles of the posterior HOM-C genes *egl-5* and *mab-5* have been recovered in genetic screens for mutations that disrupt male tail development (Hodgkin, 1983; Kenyon, 1986; Chow and Emmons, 1994; Chamberlin et al., 1999). In such a screen, we identified two new genes (*lin-49* and *lin-59*) with mutant phenotypes similar to each other. We have molecularly characterized these two genes, and found that they both encode products with similarity to trx-G gene products and other proteins that affect gene expression. We provide phenotypic and gene expression data suggesting that these genes affect the expression of the *C. elegans* HOM-C genes *egl-5* and *mab-5* like trx-G genes do in *Drosophila*. Thus *lin-49* and *lin-59* are important in somatic development and share some developmental functions with the *Drosophila* trx-G genes.

Table 1. Trx-G homologues in *C. elegans*

	Drosophila protein	*C. elegans* protein	Blast score
Orthologues	BRM	F01G4.1	1124
	TRR	T12D8.1	358
	ASH2	Y17G7B.2a	309
Similar*	OSA/ELD	C01G8.1	160
	TRX	T12D8.1‡	145
	FSH	F57C7.1	145
	ASH1	LIN-59	127
Homeodomain§	HTH	CEH-25	111
	BA/HDL	C28A5.4	95

No *C. elegans* homologues were found for Trx-G proteins TRL/GAGA, KIS and MOR.

*Although these proteins have modest blast scores they share similarity within several domains across the protein.

‡A second protein, C26E6.9/10, has a higher blast score (167), but T12D8.1 has more similarity over the full extent of the protein.

§These proteins share similarity only within the homeodomain, but the Trx-G protein is the closest *Drosophila* homologue for the *C. elegans* protein.

MATERIALS AND METHODS

Strains

Nematodes were cultured according to standard techniques (Sulston and Hodgkin, 1988). Mutations and strains used are described by Hodgkin (1997) and as indicated.

Linkage group (LG) I: *lin-59(sa489)*

LG III: *unc-119(e2498)*

LG IV: *lin-22(n372), lin-49(s1198), lin-49(sy238), lin-49(sa470), unc-22(s7), unc-31(e169). sDf18. nT1*

LG V: *him-5(e1490). muIs3* (*mab-5::lacZ*; Cowing and Kenyon, 1992), *muIs13* (*egl-5::lacZ*; Wang et al., 1993)

Extrachromosomal arrays: *saEx422, saEx423* (*R107.1::gfp*). *saEx491,* (*lin-49::gfp*). *saEx425, saEx426* (*lin-59::gfp*)

Germline transformation and rescue of *lin-49* and *lin-59* mutant phenotypes

lin-49 and *lin-59* were mapped to genetic intervals on LG IV and I, respectively (Chamberlin et al., 1999). We tested cosmids that map within each interval for transgenic rescue of mutations. DNA was microinjected into the mitotic germline of hermaphrodites according to the method of Mello et al. (1991). 100 ng/μl of plasmid containing the semi-dominant *rol-6(su1006)* allele (pRF4) was coinjected as a marker into animals with 1-10 ng/μl of test DNA. For *lin-49* rescue, animals of genotype *lin-49(s1198) unc-22(s7) unc-31(e169)/nT1* were injected. Heritable lines were tested for rescue by assaying for the presence of viable Unc-22 Unc-31 progeny (*lin-49(s1198)* animals normally die at hatching or as young larvae with characteristic hindgut defects). For *lin-59* rescue, animals of genotype *lin-59(sa489); him-5(e1490)* were injected. Heritable lines were tested by assaying Rol males for rescue to wild-type tail morphology.

Isolation and characterization of *lin-49* and *lin-59* genomic and cDNA clones

Cosmids and DNA sequence from the genomic regions of *lin-49* and *lin-59* were provided by Alan Coulson and the *C. elegans* sequencing consortium. Deletion derivatives and subclones of the cosmids were prepared using standard methods (Sambrook et al., 1989). pTJ1033 is a 9.2 kb *Sac*I/*Pst*I subclone from F42A9 into pBluescript (Stratagene) that rescues *lin-49(s1198).* pTJ1041 is a *Nar*I deletion derivative of T12F5 that rescues *lin-59(sa489).*

Several cDNA clones for both *lin-49* and *lin-59* were identified by Yuji Kohara and the *C. elegans* cDNA sequencing project. They provided three clones for *lin-49* (yk323c10, yk42a12, yk33c1) and three for *lin-59* (yk40b5, yk32b5, yk18a4). Using restriction enzyme digests, we verified that the clones in each set were similar. We then sequenced the longest for each gene to identify splice junctions and 3′ and 5′ ends (see DNA sequence analysis, below). For *lin-49*, the clone yk33c1 included a 3687 bp cDNA of the entire predicted coding region and the 3′ untranslated region, but lacked the full 5′ untranslated region. We have submitted the sequence of this cDNA to the GenBank database (accession no. AF163018). For *lin-59*, the clone yk32b5 included a cDNA of the 3′ untranslated region and the coding regions of exons 5-8. To complete the 5′ end, we used random primed first strand cDNA as template for the polymerase chain reaction (RT-PCR) using *lin-59*-specific primers and a primer to the SL1 splice leader as in Chamberlin et al. (1997). The resultant composite cDNA sequence of 4696 bp was submitted to the GenBank database (accession no. AF163019).

Construction of *lin-49::gfp*, *lin-59::gfp* and *R107.1::gfp* transgenes

We made a green fluorescent protein (GFP) reporter construct for *lin-49* expression (pTJ1241) by cloning a 3.7 kb *Pst*I/*Bgl*II promoter fragment from pTJ1033 into pPD95.67 (all pPD vectors were received from Andy Fire). We made a GFP reporter construct for *lin-59* expression (pTJ1068) by cloning a 4.7 kb *Nhe*I/*Nae*I promoter fragment from T12F5 into pPD95.70. We made a GFP reporter construct for the sodium dicarboxylate cotransporter-related gene R107.1 (pTJ996) by cutting the 7.6 kb *Sal*I/*Xba*I insert from pUL#AL32 (Lynch et al., 1995) and cloning it into pPD95.70. Transgenes were produced by microinjection of plasmid DNA into animals, as described above. For *lin-49::gfp*, 45 ng/μl pTJ1241 was coinjected with 30 ng/μl pDP#MM016 (*unc-119(+)* plasmid; Maduro and Pilgrim, 1995) and 100 ng/μl pBluescript KS+ into *unc-119(e2498); him-5(e1490)* hermaphrodites. For *lin-59::gfp*, 100 ng/μl pTJ1068 was coinjected with 30 ng/μl pDP#MM016. For *R107.1::gfp*, 200 ng/μl pTJ996 was coinjected with 15 ng/μl pDP#MM016.

DNA sequence analysis

PCR fragments and cDNA clones were sequenced by the University of Washington Biochemistry sequencing facility using an ABI PRISM sequencer (Perkin-Elmer). We amplified genomic DNA from mutant animals according to the single worm PCR method of Barstead et al. (1991). PCR fragments covering all exons of each gene were

sequenced. The DNA from at least two independent reactions was used as template to sequence both strands in regions containing the mutations. We used BLAST 2.0 (Altschul et al., 1997; http://www.ncbi.nlm.nih.gov/BLAST/) to identify and evaluate the molecular homologues of trx-G proteins, LIN-49 and LIN-59, and ClustalW 1.7 (Thompson et al., 1994b; http://dot.imgen.bcm.tmc.edu:9331/) and Boxshade 3.21 (http://www.isrec.isb-sib.ch:8080/software/BOX_form.html) to align and display the sequence motifs presented in Figs 1 and 2.

Phenotypic analysis of mutants

Young adult males were observed using Nomarski optics at 1000× magnification. The morphology of their tail structures was compared to wild type, and the extent of lateral alae differentiation was scored according to the method of Kenyon (1986), using the landmarks of Wrischnik and Kenyon (1997). Differentiation of ectopic spicule socket cells in adult *lin-48* mutant males was scored after killing the B or B.a cell in L1 or L2 larval stage males, as previously described (Chamberlin et al., 1999). The male animals were also scored for distended intestinal lumen (Con). Early L4 hermaphrodites were observed using Nomarski optics at 1000× to evaluate the extent of vulval development according to the criteria of Sternberg and Horvitz (1986). Viability of adult hermaphrodites was scored by placing one to five L4 hermaphrodites on fresh plates, and then scoring their morphology and viability each day. Animals that did not move when prodded were scored as dead. For all experiments, both the scored animals and their mothers were raised at the indicated temperature (15°C or 20°C).

Analysis of transgene expression in mutants

lin-49(sy238), *lin-49(sa470)* and *lin-59(sa489)* are all temperature sensitive, with mutant phenotypes enhanced at 25°C (Chamberlin et al., 1999). However, homozygous mutant strains cannot be maintained at high temperatures. To test transgene expression at 25°C, parents were shifted from 20°C and their progeny were analyzed for expression 2 days later. The animals raised at 25°C for 2 days are still viable (they still move, eat, etc.), but they are enhanced for mutant defects. Animals of both sexes and all larval stages were counted.

The strain JT9876 *unc-119(e2498); him-5(e1490); saEx423* was used as the parent strain and as the wild-type control for *R107.1::gfp* expression. *R107.1::gfp* expression initiates in late embryogenesis and persists through all larval stages to adulthood. *saEx423* was crossed into strains and maintained on the basis of its ability to rescue *unc-119* mutations. To score expression, non-Unc larvae were anesthetized on pads of 5% agar in water containing 5 mM sodium azide, and scored for sex, larval stage and GFP expression at 1000× magnification. Transgenic animals were verified by confirming expression of *R107.1::gfp* in non-hindgut cells prior to scoring. This method may over-represent the level of expression in mutant animals, as transgenic animals without any detectable expression would not be counted. Cells were scored positive for expression if any GFP fluorescence was detected above background.

Larvae of all stages exhibit expression of *egl-5::lacZ* and *mab-5::lacZ* transgenes, although the expression patterns change with development. For our tests, animals were scored for presence and level of expression but not for which cells express. To detect β-galactosidase activity, mixed stage animals were stained according to the method of Fire (1986). Staining was stopped by rinsing animals three times with excess PBS. Mutant and wild-type strains were always stained in parallel. Because mutations in *lin-49* and *lin-59* reduce, but don't eliminate, transgene expression, extended exposure of animals to the X-gal solution results in dark staining of both mutant and wild-type animals. To detect the effects of *lin-49* and *lin-59* mutations, animals were exposed long enough for >50% of wild-type animals to develop staining, but not to saturation. *muIs13* (*egl-5::lacZ*)-bearing animals in Table 6 were stained for 15 minutes at 37°C.

RESULTS

We recovered alleles of *lin-49* and *lin-59* in a genetic screen for mutations that disrupt *C. elegans* male tail development (Chamberlin et al., 1999). Mutations in these two genes confer similar developmental defects and are pleiotropic, and genetic analysis indicated that *lin-49* and *lin-59* are essential genes. We initiated a molecular analysis of these genes to better understand their developmental functions.

lin-49 encodes a bromodomain protein similar to human BR140

We genetically mapped *lin-49* to linkage group IV between *mes-6* and *fem-3*, which provided a left-most and right-most boundary for *lin-49* on the physical map. We identified one cosmid in this region (F42A9) that rescued *lin-49(s1198)* in 4 of 4 heritable lines. A 9.2 kb subclone of F42A9 that includes only one predicted gene, F42A9.2, also rescued *lin-49(s1198)* in 2 of 2 heritable lines. This transgenic rescue, in combination with the sequence of *lin-49* mutations (below), identified F42A9.2 as *lin-49*.

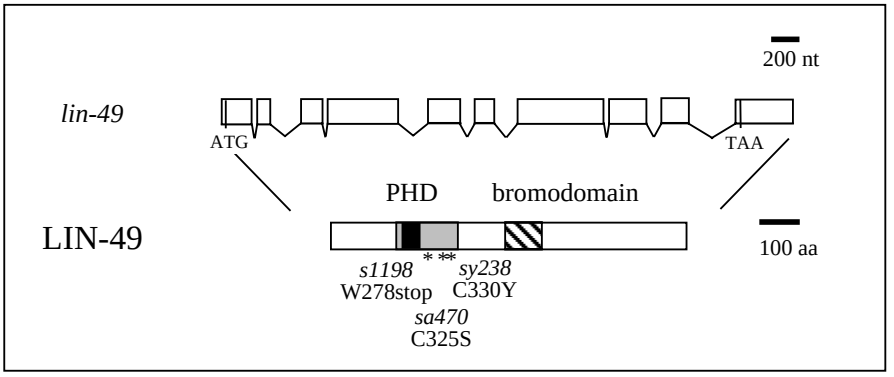
To determine the structure of *lin-49* mRNAs, we obtained and completed the sequence of a corresponding cDNA clone identified by Y. Kohara (Fig. 1). The sequence can be translated to produce a 1042 amino acid protein which contains a bromodomain and a region with regularly spaced cysteines and histidines (the cysteine rich region). Each of these domains is found in several proteins, including two *Drosophila* trx-G proteins (Fig. 1B,C). However, besides LIN-49, the only protein reported to contain both domains is human BR140. Since a function for BR140 has not been reported, LIN-49 provides the first functional characterization for this class of bromodomain proteins.

The bromodomain is found in many chromatin-associated proteins, including most known histone acetyltransferase (HAT)-associated transcriptional co-activators (Jeanmougin et al., 1997). In vitro studies indicate the domain participates in protein-protein interactions (e.g., Barlev et al., 1998). In particular, the bromodomain of the HAT co-activator P/CAF can interact specifically with acetylated lysine, suggesting the bromodomain may confer on proteins the capacity of acetylation-dependent protein-protein interactions (Dhalluin et al., 1999). However, where it has been tested, deletion of the domain does not disrupt protein function in vivo, indicating other regions of bromodomain-containing proteins can compensate in its absence (Candau et al., 1997; Elfring et al., 1998).

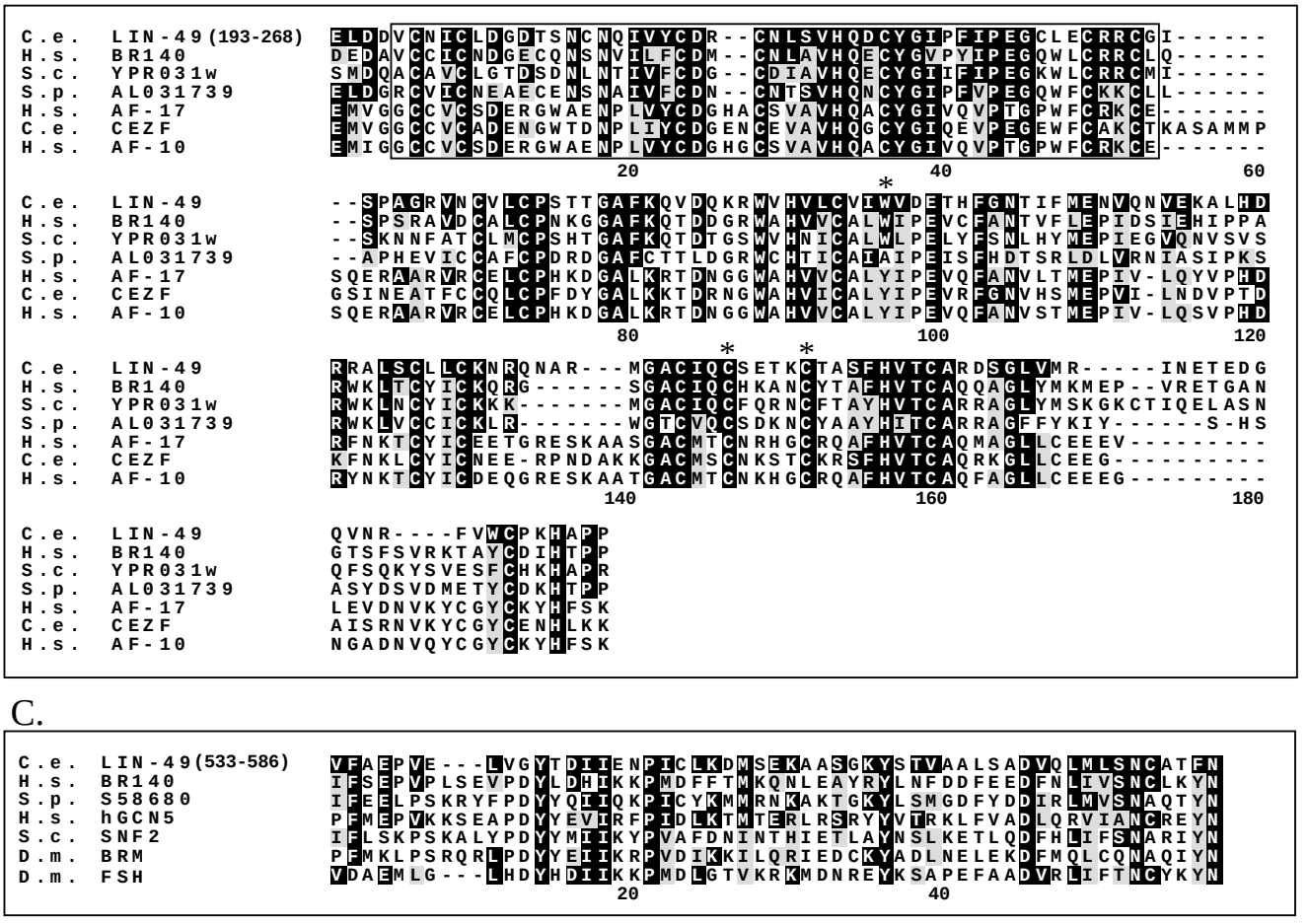
The cysteine rich region of LIN-49 includes seventeen cysteines and four histidines that are regularly spaced and invariant between LIN-49 and other proteins that contain this sequence motif (Fig. 1B). The cysteine rich region includes one embedded PHD finger (C4-H-C3). The PHD finger is a recognized sequence motif found in some chromatin-associated proteins, but its function is not known (Aasland et al., 1995). The extended similarity throughout the cysteine rich region identifies LIN-49 and other proteins that contain this motif as a distinct subgroup of PHD-containing proteins. In addition, the functional importance of the cysteine rich region is suggested by the observation that two *lin-49* mutations are missense mutations within this motif (below).

We sequenced DNA from *lin-49* mutant animals (Fig. 1A,B). The lethal allele, *s1198*, is a tgG → tgA transition that

A.



B.



C.

Fig. 1. The *lin-49* gene and its product. The complete sequence of a *lin-49* cDNA was submitted to GenBank (accession no. AF163018). A. *lin-49* consists of ten exons and encodes a protein with a bromodomain (striped) and a cysteine rich region (gray) that includes a PHD finger (black). One nonsense and two missense mutations localize to the cysteine rich region. B. Alignment of the cysteine rich region from LIN-49 and other proteins. The PHD finger is boxed. The amino acids affected in mutants are indicated with *. Six representative proteins with the cysteine rich region are shown in addition to LIN-49. At least ten additional protein sequences with this domain are in GenBank. C. Alignment of the bromodomain from LIN-49 and other proteins. C.e., *Caenorhabditis elegans*; H.s., *Homo sapiens*; S.c., *Saccharomyces cerevisiae*; S.p., *Schizosaccharomyces pombe*.

results in a Trp278 → Stop. This mutation should result in a truncated protein lacking half the cysteine rich region and the bromodomain. This mutation is consistent with genetic results, which indicate *s1198* is a strong reduction of function or potential null allele of *lin-49*. The viable alleles, *sa470* and *sy238*, each result in an amino acid substitution at an invariant cysteine in the cysteine rich region. *sa470* is a Tgc → Agc transversion that results in a Cys325 → Ser substitution. *sy238* is a tGt → tAt transition that results in a Cys330 → Tyr substitution. The phenotype of animals bearing *sa470* or *sy238*

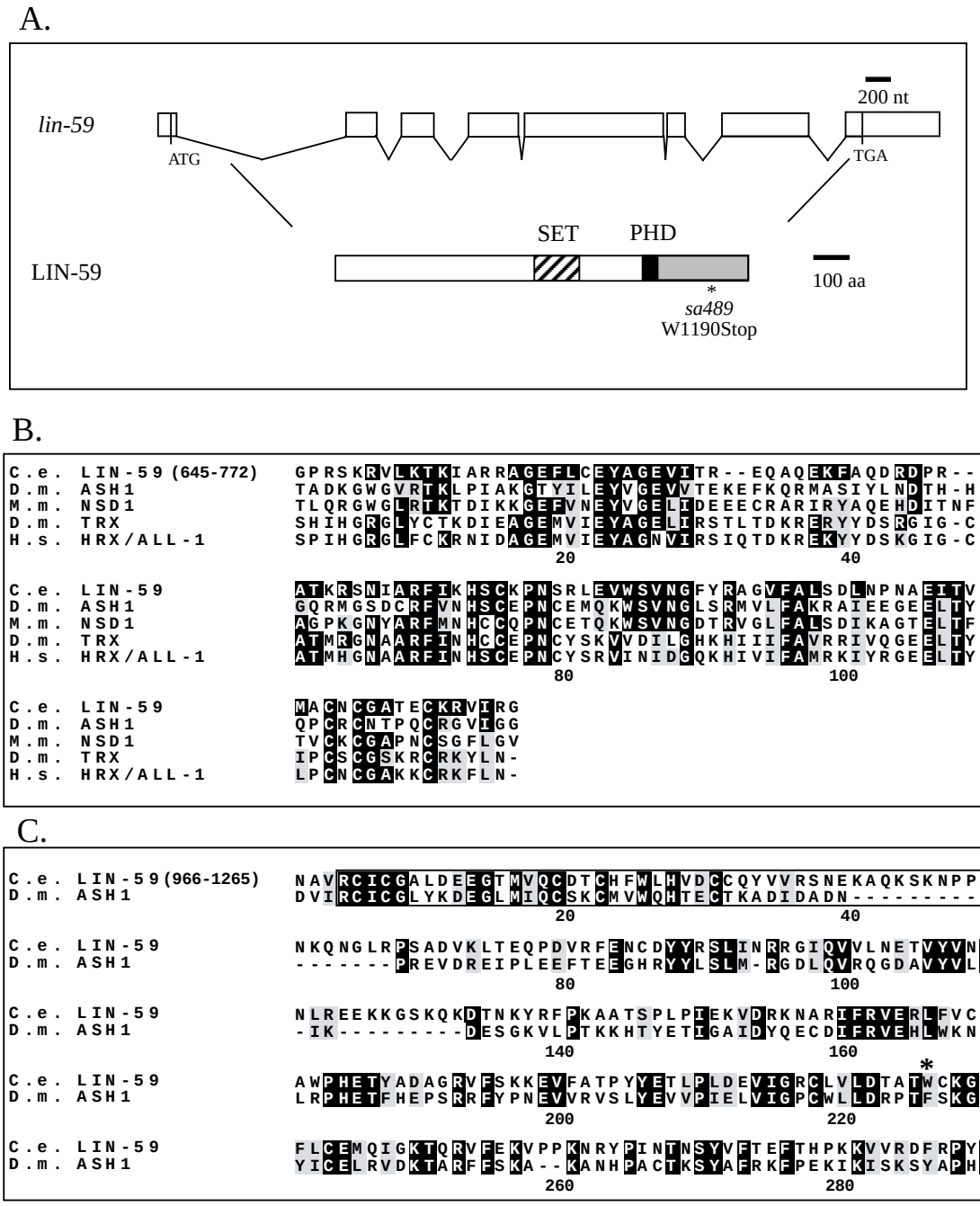


Fig. 2. The *lin-59* gene and its product. The complete sequence of a *lin-59* cDNA was submitted to GenBank (accession no. AF163019). A. *lin-59* consists of eight exons and encodes a protein with a SET domain (striped) and a carboxyl terminal region similar to the *Drosophila* ASH1 (gray) which includes a PHD finger (black). The *sa489* nonsense mutation is indicated. B. Alignment of the SET domain from LIN-59 and other proteins. C. Alignment of the carboxyl terminal region of LIN-59 and ASH1. The PHD finger is boxed. The tryptophan affected in *sa489* is indicated with *. C. e., *Caenorhabditis elegans*; D.m., *Drosophila melanogaster*; M.m., *Mus musculus*; H.s., *Homo sapiens*.

in *trans* to deletions that remove *lin-49*, or in *trans* to the lethal allele *s1198*, is enhanced compared to homozygous mutants (Chamberlin et al., 1999). Thus we believe these mutations reduce, but do not eliminate *lin-49* activity.

lin-59 encodes a SET domain protein similar to the product from the *Drosophila* trx-G gene *ash1*

We genetically mapped *lin-59* to linkage group I between *fog-1* and *unc-11*, which provided a left-most and right-most boundary for *lin-59* on the physical map. We identified two cosmids in the region (F21A9 and T12F5) that each rescued *lin-59(sa489)* (4 of 4 and 1 of 1 heritable lines, respectively). The approx. 20 kb overlap region between the two cosmids included three predicted genes. We created a deletion derivative of T12F5 that included only one of these predicted genes (T12F5.4) and showed that this cosmid rescued *lin-*

59(sa489) (4 of 4 heritable lines). Based on this transgenic rescue and the sequence of the *lin-59(sa489)* mutation, we conclude T12F5.4 is *lin-59*.

To determine the structure of *lin-59* mRNAs, we completed the sequence of a cDNA clone identified by Y. Kohara. This cDNA corresponds to the 3′ portion of *lin-59*, and includes the 3′ untranslated region and exons 5-8. To complete the cDNA, we used RT-PCR to amplify and sequence the 5′ end. This produced a full length cDNA sequence of 4696 bp (Fig. 2). The sequence can be translated to produce a 1312 amino acid protein. LIN-59 is most similar to the product of the *Drosophila* trx-G gene, *ash1* (Tripoulas et al., 1996). LIN-59 and ASH1 both contain a central SET domain, and share similarity in the carboxyl terminus which includes one PHD finger. The SET domain is found in several classes of proteins, including some products of Pc-G and trx-G genes, and it has been implicated in mediating protein-protein interactions (Cui et al., 1998; Rozenblatt-Rosen et al., 1998). Although both LIN-49 and LIN-59 contain a PHD finger, the carboxyl terminal region shared by LIN-59 and ASH1 is not otherwise similar to the cysteine rich region found in LIN-49-related proteins (discussed above).

We sequenced DNA from *lin-59(sa489)* mutant animals. *sa489* is a tGg to tAg transition that results in a premature amber stop codon. This mutation would result in a truncated protein missing the 122 carboxyl terminal amino acids, including part of the region conserved between LIN-59 and ASH1. The phenotype of animals bearing *sa489* in *trans* to a deletion that removes *lin-59* is enhanced compared to homozygous mutants (Chamberlin et al., 1999). Thus, we believe *sa489* is not a null allele.

lin-49::gfp and *lin-59::gfp* transgenes are expressed broadly in somatic tissues

Human BR140 and *Drosophila ash1* are both expressed broadly throughout the organism (Thompson et al., 1994a; Tripoulas et al., 1994). To investigate the expression of *lin-49* and *lin-59* we constructed reporters consisting of each promoter driving expression of GFP, and expressed them in transgenic animals. We found that embryonic expression of *lin-59::gfp* is first detected in mid-blastula stage. Subsequently it is expressed in most cells of the animal, throughout development and into adulthood (Fig. 3). *lin-49::gfp* transgenes are expressed at lower levels, but with a similar pattern. This transgene expression is consistent with *lin-49* and *lin-59* being expressed throughout the animal.

Mutations in *lin-49* and *lin-59* confer some phenotypes found in HOM-C mutants

lin-49 and *lin-59* mutants share a range of phenotypes, and the viable alleles of *lin-49* and *lin-59* are temperature sensitive for embryonic and larval lethality (Chamberlin et al., 1999). To further characterize the mutant phenotypes and the temperature sensitivity, we scored both male and hermaphrodite animals for morphological defects. In particular, we focused on male alae production and fan morphogenesis and hermaphrodite vulval development since these tissues are known to be affected by mutations in HOM-C genes.

Mutations in *C. elegans* HOM-C genes interact with mutations in *lin-22*, a *C. elegans hairy* homologue (Kenyon, 1986; Wrischnik and Kenyon, 1997). *lin-22* affects the anterior/posterior identity of a set of lateral epidermal cells called V cells. Normally, the more anterior V cells (V1-V4) produce seam cell progeny that generate alae (lateral cuticular ridges). In male animals, the more posterior V cells (V5-V6) produce progeny that differentiate as structures in the adult male tail called rays. In males mutant for the HOM-C gene *mab-5*, V5 and V6 behave like their more anterior neighbors, and produce alae rather than rays. In contrast, in *lin-22* males V1-V4 behave like their posterior neighbor V5, and produce rays at the expense of alae. The *lin-22* mutant phenotype results, at least in part, from ectopic MAB-5 expression. Consequently, *mab-5; lin-22* double mutants produce more alae than *lin-22* single mutants. Mutations in the HOM-C genes *egl-5* and *lin-39* can also modify the *lin-22* mutant phenotype (Wrischnik and Kenyon, 1997).

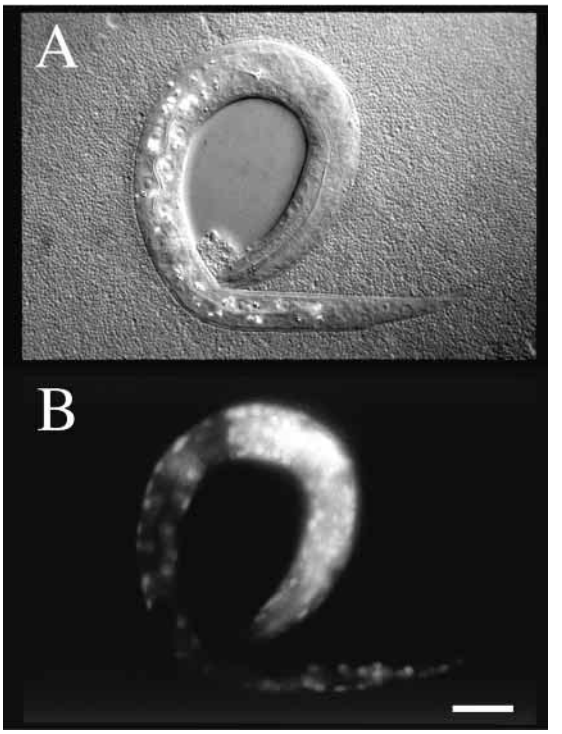


Fig. 3. *lin-59::gfp* expression. (A) Nomarski photomicrograph of an L1 transgene-containing animal. (B) Fluorescence image, showing widespread expression of *lin-59::gfp*. Scale bar, 20 μm.

To investigate the role of *lin-49* and *lin-59* in anterior/posterior patterning, we asked whether mutations in these genes affect alae production and interact with *lin-22* (Fig. 4). We found that *lin-49* and *lin-59* mutants have defects in anterior/posterior patterning of V cells. At both 15°C and 20°C, mutant males produce occasional ectopic rays, and alae that fail to extend to the normal posterior position and that include gaps. In addition, the alae can extend further anterior than normal. Thus, *lin-49* and *lin-59* affect anterior/posterior patterning, but they are distinct from *mab-5* and *lin-22*. To test for genetic interactions, we constructed double mutants with *lin-22*. At 15°C *lin-22 lin-49* double mutant males generally produced more alae than *lin-22* single mutants (the *lin-22 lin-49* double mutants are sick and difficult to grow at 20°C). This indicates *lin-49* genetically interacts with *lin-22* in a manner similar to *mab-5*. *lin-59; lin-22* doubles do not produce

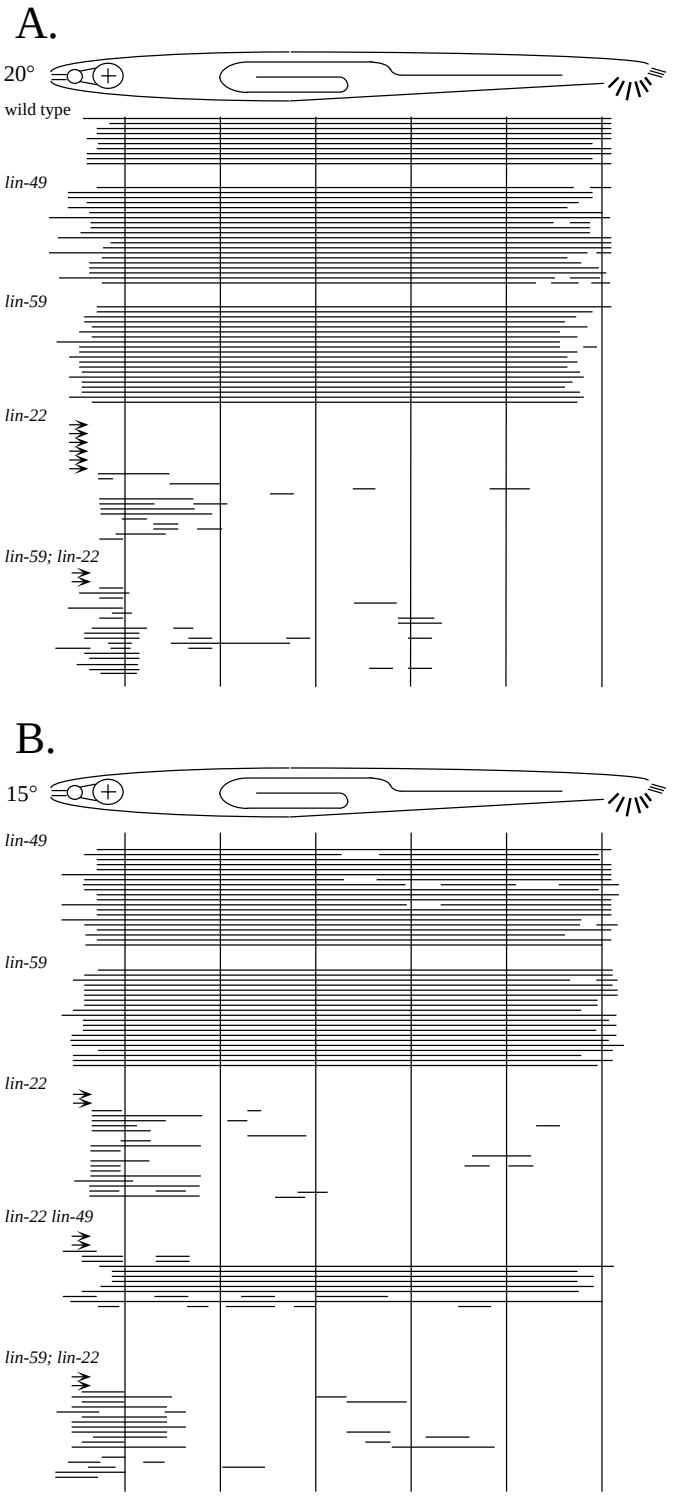


Fig. 4. Alae production in *lin-49* and *lin-59* mutant males at 20°C (A) and 15°C (B). Each line represents the location of lateral alae along the anterior/posterior axis for one side of an individual male animal. The locations of alae patches and gaps are shown relative to cellular and anatomical markers (Wrischnik and Kenyon, 1997). Arrows represent males with no alae. In *lin-49* and *lin-59* single mutants, alae can extend further anterior than normal, can fail to extend to the normal posterior position and can include gaps. Like mutations in the HOM-C gene *mab-5*, a mutation in *lin-49* can partially suppress the defect in alae production of *lin-22* mutants. *lin-59; lin-22* doubles exhibit phenotypes associated with both mutants, as alae extend more anterior than normal, but generally fail to differentiate in midbody and posterior regions. Alleles used: *lin-22(n372)*, *lin-49(sa470)*, *lin-59(sa489)*. All strains also include *him-5(e1490)*.

appreciably more alae than *lin-22* single mutants, although they still produce alae that extend further anterior than normal. Since the *lin-59* allele is not null, we can not distinguish between the possibility that *lin-59* is different from *lin-49* in its interaction with *lin-22* and the possibility that the *lin-59* mutation is not strong enough to show an effect.

In addition to the occasional ectopic rays that result from defects in patterning V cells, *lin-49* and *lin-59* mutant males have defects affecting the fan and bursa (Table 2). These defects range from animals with simple ray fusions to more general defects in morphogenesis (Table 3; Fig. 5E,F). Chow and Emmons (1994) have shown that reduction of *egl-5* and *mab-5* activity can result in a transformation of the identity of ray 4 to that of ray 3. Consequently, these rays fuse together to form a single ray. We have observed similar ray fusions in *lin-49* and *lin-59* mutants. For example, of *lin-49(sa470)* males raised at 15°C with a fused or missing ray phenotype, 10 of 22 (45%) had ray 3 and ray 4 fused together. *lin-49(sy238)* and *lin-59(sa489)* mutants showed similar types of ray fusion defects. In addition to ray fusions, ray 5 and ray 6 were sometimes misplaced or even transposed (Fig. 5E and data not shown), suggesting failure of the normal migration or morphogenesis of these cells.

To further investigate the role of *lin-49* and *lin-59* in the development of structures that require normal HOM-C gene activity, we characterized the development of the hermaphrodite-specific vulval structures in the midbody region. Normally, the hermaphrodite vulva forms from the progeny of three ventrally located epidermal cells (P5.p, P6.p, and P7.p). In hermaphrodites mutant for the HOM-C gene *lin-39*, these cells behave like their anterior and posterior counterparts and fuse with the syncytial hypodermis (hyp 7) rather than divide to form the vulva (Clark et al., 1993). The

Table 2. Adult male phenotypes in *lin-49* and *lin-59* mutants

		Mab (%)				
Genotype	Temp. (°C)	Fan	Spicules	Hook	Con (%)	*n*
Wild type	15	2	2	0	0	51
	20	0	0	2	0	50
lin-49(sa470)	15	51	25	47	12	51
	20	96	90	78	32	50
lin-49(sy238)	15	84	57	52	18	49
	20	100	98	84	58	50
lin-59(sa489)	15	51	37	25	14	51
	20	84	94	30	60	50

Numbers indicate percentage of animals with abnormal morphology.

n in this and all other tables is total number of animals. Hook phenotype could not be scored in all mutant animals. *n* for this phenotype is: *lin-49(sa470)* 15°C: 49; 20°C: 37; *lin-49(sy238)* 15°C: 42; 20°C: 25; *lin-59(sa489)* 15°C: 40; 20°C: 23.

All genotypes include *him-5(e1490)*.

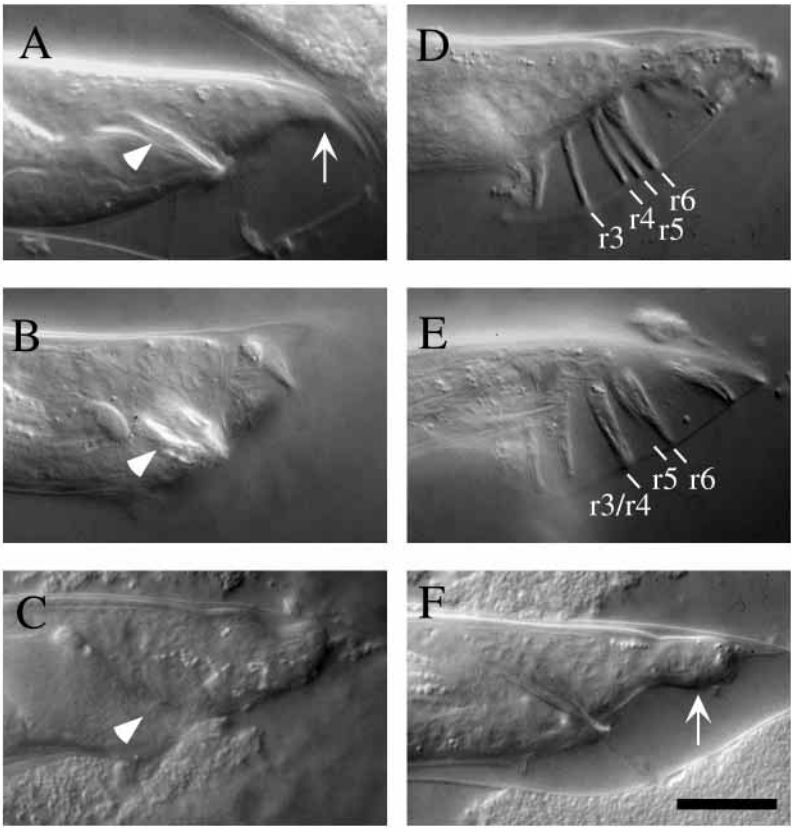


Fig. 5. Male tail defects in *lin-49* and *lin-59* mutants. Nomarski photomicrographs illustrate defects in spicule development and fan formation. In wild type, the spicules are long and straight (A, arrowhead). In mutants the spicules can be crumpled and short (B), or even absent (C). The fan includes 18 bilaterally symmetric rays. Rays 3-6 (r3 – r6) on the left side of a wild type animal are indicated (D). In mutants (E), ray 3 and ray 4 are frequently fused. Ray 5 is also misplaced and slightly out of the plane of focus. More severe defects are seen in other animals (F). In these animals anterior migration of the tail cells during fan morphogenesis is incomplete (compare the tail tip area (arrow) in wild type (A) to mutant (F)). After molting to the adult stage, these cells generally collapse back, filling in the space normally occupied only by the ray processes. Males in A and F are late L4 larval stage. Males in B-E are young adults. Genotypes and growth conditions: A, D, *him-5(e1490)*, 20°C. B, C, *lin-59(sa489); him-5(e1490)*, 20°C. E, *lin-49(sa470); him-5(e1490)*, 15°C. F, *lin-49(sy238); him-5(e1490)*, 20°C. Scale bar, 20 μm.

resulting animals are vulvaless, and incapable of laying eggs. In general, hermaphrodites of *lin-49* and *lin-59* genotypes are capable of laying eggs (Table 4 legend), indicating they are not vulvaless. We scored the extent of vulval differentiation in *lin-49(sy238)* mutants, and found that P7.p, or sometimes P5.p, failed to divide in 12 of 43 (28%) *lin-49(sy238)* mutant hermaphrodites raised at 20°C, suggesting some similarity to *lin-39* mutants. However, five of five *lin-49(sy238)/sDf18* animals that survived to L4 stage and 16 of 16 *lin-49(sa470)* homozygotes had normal vulval development. We have not ruled out the possibility that the observed vulval defect results from another mutation in the *lin-49(sy238)* background. Thus, *lin-49* and *lin-59* mutant hermaphrodites have only minor, if any, defects in vulval development.

Table 3. Severity of fan defects is temperature sensitive in *lin-49* and *lin-59* mutants

		Fan defects (%)		
Genotype	Temp. (°C)	Fused/ missing rays	Swollen/def. morphogenesis	*n*
lin-49(sa470)	15	85	15	26
	20	46	54	48
lin-49(sy238)	15	83	17	41
	20	8	92	50
lin-59(sa489)	15	77	23	26
	20	40	60	42

All males with fan defects were classified as having either minor (fused/missing rays) or severe (swollen/def. morphogenesis) fan defects. Numbers represent percentage of total males with fan defects. Animals are the same scored in Table 2.

Mutations in *lin-49* and *lin-59* affect development of male spicule and hook structures

In *lin-49* and *lin-59* males the spicules can be misformed, and in some animals they are greatly reduced in size or apparently missing, suggesting a defect in spicule development or differentiation (Table 2; Fig. 5B,C). Normally, all of the cells of the spicules derive from the precursor cell B (Sulston et al., 1980). Males with mutations in a gene called *lin-48* make ectopic spicule cells that derive from another precursor, U

Table 4. Adult *lin-49* and *lin-59* mutant hermaphrodites have reduced viability

Genotype	Temp. (°C)	inviable*	*n*
Wild type	15	2	55
	20	0	50
lin-49(sa470)	15	9	55
	20	23	43
lin-49(sy238)	15	40	58
	20	41	61
lin-59(sa489)	15	85	46
	20	59	51

All genotypes include *him-5(e1490)*.

Six animals (of the 419 total tested) died because they bagged (the progeny hatched inside the mother and they killed her). These include one wild-type animal at 15°C, two *lin-49(sy238)* animals at 20°C, two *lin-49(sa470)* animals at 20°C and one at 15°C. Three of these lin-49 mutants were initially egg-laying compentent, but bagged after they developed hernia at the vulva.

*Numbers indicate percentage of adult hermaphrodites who died within one generation (3 days at 20°C and 5 days at 15°C). Since metabolism is slowed at lower temperatures, we believe these time points are an appropriate comparison. Percentage inviable after 3 days at 15° are *lin-49(sa470)*: 2%; *lin-49(sy238)*: 19%; *lin-59*: 61%.

Table 5. Reduced production of ectopic spicule cells in *lin-59* mutants

Genotype	Cell killed	% with spicule cuticle	*n*
Wild type*	B‡	0	10
*lin-48**	B	67	21
lin-59; lin-48§	B	9	11

*Data from Chamberlin et al. (1999).
‡B includes animals in which B or B.a was killed (see Materials and Methods).
§Full genotype: *lin-59(sa489)*; *lin-48(sy234)*; *him-5(e1490)*.

(Chamberlin et al., 1999). To test the genetic relationship between *lin-49*, *lin-59* and *lin-48* we constructed double mutants. We found 9% (one of eleven animals) of *lin-59; lin-48* double mutants produced ectopic spicule cells, compared to 67% of *lin-48* single mutants (Table 5). Since the U cell can develop abnormally in *lin-59* mutants (Chamberlin et al., 1999), the *lin-59* mutation may block ectopic spicule cell production either by affecting the precursor cell or by affecting differentiation. We found that *lin-48; lin-49* double mutants were inviable, so these animals could not be tested for spicule differentiation.

Mutant males also have deformities of the hook (Table 2). The hook can be abnormal or small, or completely absent. In some animals it is also displaced anterior to its normal position. Adult males with hook and spicule defects were more likely to have a constipated (Con) phenotype, suggesting these defects may contribute to blocking the intestine or disruption of defecation behavior. However, these abnormalities in male mating structures are not the sole cause of the Con phenotype, since it is seen in mutants of both sexes at all stages (Chamberlin et al., 1999).

Mutations in *lin-49* and *lin-59* affect the maintenance of organ integrity

Both male and hermaphrodite *lin-49* and *lin-59* mutant larvae have cellular and morphological defects within the hindgut (rectum). These defects are associated with defects in structural integrity, and can result in lethality when animals herniate at the anal pore (Chamberlin et al., 1999). In males these defects affect subsequent larval development of the mating structures of the tail (discussed above). In addition to larval defects, we have found that hermaphrodite animals that survive to adulthood are more sickly than wild type, and tend to die young. In particular, adults are prone to defects in structural integrity, and develop hernia at the anal and vulval openings over time. The herniated animals soon die, such that at 20°C after 3 days from 23 to 59% of mutant adult hermaphrodites (depending on genotype) are dead, compared to 0% for wild type (Table 4). This suggests either that *lin-49* and *lin-59* play a role in adult viability and the maintenance of organ integrity or that earlier developmental defects somehow compromise the durability of the adult structures.

Table 6. Expression of *egl-5::lacZ* is moderately reduced in *lin-49* and *lin-59* mutants

Expt.	Genotype	Expression (%) Strong	Weak	None	*n*
1	Wild type	60	20	21	136
	lin-49	10	32	58	107
2	Wild type	60	23	17	87
	lin-49	12	23	65	125
1	Wild type	31	26	43	111
	lin-59	12	25	63	124
2	Wild type	47	14	39	137
	lin-59	19	35	47	133

Animals were tested at 25°C, as described in Materials and Methods. Animals with detectable β-gal activity were subdivided into 'weak' (showing very faint levels) and 'strong' (all others).
lin-49(sa470) and *lin-59(sa489)* were used. All genotypes include *him-5(e1490)*.
In some cases the totals do not add up to 100 due to rounding.

Table 7. Expression of a Rep cell marker (*R107.1::gfp*) is moderately reduced in *lin-49* and *lin-59* mutants

Genotype	Temp. (°C)	Rep expression 3	2	1	0	n
Wild type	20	47	40	11	2	110
	25	76	16	7	1	113
lin-49	20	34	32	25	9	118
	25	6	20	23	51	114
lin-59	20	28	29	26	17	149
	25	37	29	25	9	139

Animals were tested at 25°C, as described in Materials and Methods.
Numbers indicate percent of animals with expression in 3, 2, 1 or 0 of the three Rep cells.
lin-49(sa470) and *lin-59(sa489)* were used. All genotypes include *him-5(e1490)*

Mutations in *lin-49* and *lin-59* affect gene expression

We had previously found that expression of a hindgut cell marker (*cdh-3::gfp*) is reduced in *lin-49* and *lin-59* mutants (Chamberlin et al., 1999). Since *lin-49* and *lin-59* encode products with similarity to *Drosophila* HOM-C gene regulators, we tested whether expression of two *C. elegans* HOM-C genes (*egl-5* and *mab-5*) is affected in mutants. We found that, like *cdh-3::gfp*, expression of *egl-5* and *mab-5* reporter transgenes is moderately reduced in *lin-49* and *lin-59* mutants (Table 6 and data not shown). Finally, to ask whether *lin-49* and *lin-59* also affect gene expression in anterior hindgut cells, we tested the marker *R107.1::gfp*. We found that this transgene is expressed in the three Rep (rectal epithelial cells: RepD, RepVL, RepVR), and that its expression in these cells is reduced in mutants (Table 7).

DISCUSSION

LIN-49 and LIN-59 share similarity with proteins implicated in gene expression and chromatin modulation

We have molecularly characterized the *C. elegans* genes *lin-49* and *lin-59*. These genes represent the first trx-G-related genes functionally characterized in *C. elegans*. *lin-59* encodes a product similar to that of the *Drosophila* trx-G gene *ash1*. LIN-59 and ASH1 share a centrally positioned SET domain and sequence similarity in their carboxyl termini. *lin-49* encodes a product that includes a bromodomain, a domain also found in the trx-G proteins FSH and BRM. LIN-49 is most similar to the mammalian BR140 protein. We have shown that *lin-49* is an

essential gene that plays a role in *C. elegans* development, and that it is functionally related to *lin-59*, thus providing the first evidence for function of this class of bromodomain proteins.

The function of *lin-49* and *lin-59* in *C. elegans* development

We recovered mutations in *lin-49* and *lin-59* in a genetic screen for mutations that disrupt male tail development. These mutations result in abnormal development of male-specific tail structures. They also disrupt the morphology, function and gene expression of hindgut cells in both male and hermaphrodite animals. In addition, these mutations interfere with organ integrity in the hindgut and in the egg-laying system. Consistent with a broad range of functions, *lin-49* and *lin-59* transgenes are expressed throughout the animal. Nevertheless, in *lin-49* and *lin-59* mutants we observe the most severe developmental defects in the posterior body region of the animals. Since the viable alleles of *lin-49* and *lin-59* used in this study are not null, further work will be required to distinguish between the interpretation that these mutations preferentially affect development in the tail region because the tail has a greater requirement for the activity of *lin-49* and *lin-59* and the interpretation that *lin-49* and *lin-59* function more broadly, but these specific mutations preferentially disrupt function in the posterior body region.

The molecular identity of LIN-49 and LIN-59 indicates they may function like *Drosophila* trx-G proteins in activating or maintaining HOM-C gene expression, and our observation that expression of *egl-5* and *mab-5* transgenes is affected in mutants is consistent with this notion. Genetic analysis also indicates *lin-49* and *lin-59* affect HOM-C gene activity, as some mutant phenotypes are similar to those seen in *egl-5* and *mab-5* mutants. Mutants have ray fusions similar to weak *mab-5* and *egl-5* mutants, and *lin-49* acts like *mab-5* as a partial suppressor of *lin-22*. In addition, *lin-49* and *lin-59* affect the expression or activity of more than HOM-C genes. Mutants have defects in organ integrity that have not been observed in HOM-C mutants. *lin-49* and *lin-59* single mutant males produce lateral alae with gaps, a phenotype more similar to *mab-5; lin-22* double mutants than *mab-5* or other HOM-C single mutants (Kenyon, 1986; Wrischnik and Kenyon, 1997). The alae also extend further anterior than normal, a phenotype associated with *vab-3* (*C. elegans Pax-6*) mutants (Chisholm and Horvitz, 1995), but not reported for HOM-C mutants. Expression of additional transgenes (*cdh-3* and *R107.1*) is affected in *lin-49* and *lin-59* mutants, whereas, where tested, these transgenes are expressed normally in HOM-C mutants (Chamberlin et al., 1999; H. M. C., unpublished). Taken together, these data suggest *lin-49* and *lin-59* affect the expression of genes in addition to HOM-C genes, as do some of the trx-G genes in *Drosophila* (Brizuela et al., 1994; Brizuela and Kennison, 1997). Despite the range of affected tissues and genes, the modest and variable effects we observe on individual genes and processes suggest *lin-49* and *lin-59* play a facilitative rather than essential role in gene expression.

trx-G and Pc-G genes in *C. elegans*

Our results are consistent with the idea that trx-G and Pc-G-related genes in *C. elegans* function mechanistically like their *Drosophila* counterparts. The roles of *C. elegans* trx-G and Pc-G orthologues in development of the animal, however, are distinct from the roles of the *Drosophila* genes. In *C. elegans*, the Pc-G-related genes *mes-2* and *mes-6* are similar to *Drosophila* Pc-G genes in acting as transcriptional repressors, but differ in having little role in somatic development (Holdeman et al., 1998; Korf et al., 1998; Kelly and Fire, 1998). *Drosophila* trx-G genes positively regulate HOM-C gene expression. Our results suggest *lin-49* and *lin-59* similarly promote HOM-C gene expression, and are important for somatic development. Thus the developmental functions of the *C. elegans* Pc-G-related genes is altered without a similar change in the function of at least some trx-G-related genes.

The *C. elegans* genome contains seven Hox genes that are likely orthologues of HOM-C genes. However, they are not as tightly clustered as those in *Drosophila* and vertebrates are. Ruvkun and Hobert (1998) have proposed that the phenomena of the absence of most Pc-G genes and the partial dispersion of the HOM-C genes are linked, resulting from a loss of the long-range Pc-G regulation of HOM-C genes in an ancestral nematode. Consistent with this, the remaining Pc-G homologues no longer have a significant role in HOM-C gene regulation in *C. elegans*. In contrast, our results suggest that, like *Drosophila* trx-G genes, *lin-49* and *lin-59* do participate in HOM-C gene regulation. Because we have studied these genes in *C. elegans*, our results support the idea that trx-G genes can function in animal development to initiate and maintain transcriptional states independent of any function they have to counterbalance Pc-G gene repression.

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