Dartmouth College
Dartmouth Digital Commons

Open Dartmouth: Faculty Open Access Articles

4-8-2004

The C. Elegans Heterochronic Gene lin-46 Affects Developmental Timing at Two Larval Stages and Encodes a Relative of the Scaffolding Protein Gephyrin

A. S.-R. Pepper
Fox Chase Cancer Center

Jill E. McCane
Fox Chase Cancer Center

Kevin Kemper
Fox Chase Cancer Center

Dennis Au Yeung
Dartmouth College

Rosalind C. Lee
Dartmouth College

See next page for additional authors

Follow this and additional works at: https://digitalcommons.dartmouth.edu/facoa

Part of the Genetics Commons, and the Molecular Biology Commons

Recommended Citation

Authors

A. S.-R. Pepper, Jill E. McCane, Kevin Kemper, Dennis Au Yeung, Rosalind C. Lee, Victor Ambros, and Eric G. Moss

The *C. elegans* heterochronic gene *lin-46* affects developmental timing at two larval stages and encodes a relative of the scaffolding protein gephyrin

Anita S.-R. Pepper[1], Jill E. McCane[1], Kevin Kemper[1,3], Denise Au Yeung[2], Rosalind C. Lee[2], Victor Ambros[2] and Eric G. Moss[1,3,*]

[1]Fox Chase Cancer Center, Philadelphia, PA 19111, USA
[2]Department of Genetics, Dartmouth Medical School, Hanover, NH 03755, USA
[3]Department of Molecular Biology, UMDNJ, Stratford, NJ 08084, USA
*Author for correspondence (e-mail: mosseg@umdnj.edu)

Summary

The succession of developmental events in the *C. elegans* larva is governed by the heterochronic genes. When mutated, these genes cause either precocious or retarded developmental phenotypes, in which stage-specific patterns of cell division and differentiation are either skipped or reiterated, respectively. We identified a new heterochronic gene, *lin-46*, from mutations that suppress the precocious phenotypes caused by mutations in the heterochronic genes *lin-14* and *lin-28*. *lin-46* mutants on their own display retarded phenotypes in which cell division patterns are reiterated and differentiation is prevented in certain cell lineages. Our analysis indicates that *lin-46* acts at a step immediately downstream of *lin-28*, affecting both the regulation of the heterochronic gene pathway and execution of stage-specific developmental events at two stages: the third larval stage and adult. We also show that *lin-46* is required prior to the third stage for normal adult cell fates, suggesting that it acts once to control fates at both stages, and that it affects adult fates through the *let-7* branch of the heterochronic pathway. Interestingly, *lin-46* encodes a protein homologous to MoeA of bacteria and the C-terminal domain of mammalian gephyrin, a multifunctional scaffolding protein. Our findings suggest that the LIN-46 protein acts as a scaffold for a multiprotein assembly that controls developmental timing, and expand the known roles of gephyrin-related proteins to development.

Keywords: *C. elegans*, Larval development, Hypodermis, Heterochronic genes, *lin-14*, *lin-28*, *lin-42*, *lin-46*, *lin-57*, *let-7*, Gephyrin, MoeA, *moc-1*, *moc-2*

Introduction

The succession and synchrony of diverse developmental events in the larva of *Caenorhabditis elegans* are controlled by the heterochronic genes (Ambros, 2000). In many cell lineages, cell division patterns and differentiation events differ during each of the four larval stages (L1-L4). A mutation in a heterochronic gene results in either precocious or retarded development in which stage-specific patterns of cell division and differentiation are either skipped or reiterated, respectively. For example, *lin-14* and *lin-28* are two heterochronic genes that act early in larval development: *lin-14* loss-of-function (lf) mutant animals precociously execute events specific to the second larval stage (L2) and later, and *lin-28(lf)* animals precociously execute events specific to the third larval stage (L3) and later (Fig. 1) (Ambros and Horvitz, 1984). The heterochronic genes are like other developmental regulators in that they define a component of the fate of a cell, but unlike spatial or cell-type regulators, they govern the temporal component of that fate.

In general, each heterochronic gene governs cell fates at a particular larval stage, and a gene that acts early in larval development may affect later stages as a consequence. For example, *lin-28* is involved immediately in the L2 fate decision, but indirectly affects adult differentiation programs (Ambros, 1989). In particular, *lin-28(lf)* causes precocious execution of the larva-to-adult (L/A) switch, during which lateral hypodermal seam cells cease dividing, differentiate, and produce adult alae, a cuticle structure. However, molecular analysis has shown that *lin-28* expression is repressed three stages before the L/A switch (Moss et al., 1997; Seggerson et al., 2002). *lin-28* affects the L/A switch via the expression of other heterochronic genes, *let-7*, *lin-41*, *lin-57* and *lin-29* (see Fig. 5; Ambros; 1989; Rougvie and Ambros, 1995; Slack et al., 2000; Abrahante et al., 2003; Lin et al., 2003).

lin-14 has two roles during larval development: first to determine L1 fates only, and then later to affect the L2 and subsequent events (Ambros and Horvitz, 1987). These two activities differ not only by when they occur, but in the nature of their interactions with other genes. Specifically, *lin-14* apparently acts alone to govern L1 cell fates, then it interacts with *lin-28* to choose cell fates that will occur in the L2 (Ambros, 2000). *lin-14* and *lin-28* are engaged in a complex genetic circuitry that involves both positive and negative regulatory interactions (see Fig. 5A) (Seggerson et al., 2002).

This circuitry involves the microRNA lin-4 as well as other as yet unidentified components (Lee et al., 1993; Arasu et al., 1991; Moss et al., 1997; Seggerson et al., 2002). In later larval stages, both *lin-14* and *lin-28* are repressed, allowing late stage events to be executed. The changes in activity and interactions of the heterochronic genes through the larval stages lead to the normal succession of larval developmental events.

The products of the heterochronic genes include at least two microRNAs (lin-4 and let-7), an evolutionarily conserved protein with a unique pairing of RNA-binding motifs (LIN-28), a protein related to the circadian rhythm regulator *period* of *Drosophila* (LIN-42), and a nuclear protein of unknown function (LIN-14) (Ruvkun et al., 1989; Ruvkun and Giusto, 1989; Lee et al., 1993; Moss et al., 1997; Jeon et al., 1999; Reinhart et al., 2000). Although insight has been gained into how the expression of certain heterochronic genes is regulated, it is not yet known whether the identified heterochronic genes constitute all the components of the pathway. Nor is it known how the products of the heterochronic genes interact among themselves and with other cellular components to effect the precise timing of developmental events in worm larvae.

We have identified a new heterochronic gene, *lin-46*, which occupies a unique position in the larval developmental timing mechanism of *C. elegans*. Our analysis indicates that *lin-46* functions at a step immediately downstream of *lin-28*, and acts at a branch-point in the heterochronic pathway. *lin-46* encodes a protein that is homologous to bacterial and mammalian proteins, but as a developmental regulator, is novel.

Materials and methods

Culturing, mutants, and strain construction

C. elegans strains were cultured as described (Wood et al., 1988) and grown at 20°C, unless otherwise noted. Genetic screens were performed after EMS (*ma164*) and UV (*ma174*) mutagenesis, and new alleles were outcrossed prior to further analysis. All mutant alleles, except those of *lin-46*, have been described previously. A *lin-46* mutant allele can be followed in crosses by its retarded phenotype, although in most strain constructions we relied on a linked marker, *unc-76(e911)*, which has no developmental timing effects (A.S.-R.P. and E.G.M., unpublished) (Bloom and Horvitz, 1997). The presence of the *lin-46* mutant allele in strains was confirmed upon outcrossing.

Strains

The *lin-28(lf)* alleles used are null based on molecular analysis (Moss et al., 1997; Seggerson et al., 2002). The nature of the different *lin-14* alleles used are indicated in the text and tables. The *lin-46* allele *ma164* was used in all experiments, except where indicated. N2 (wild type), ME1 *lin-28(ga54) I; lin-46(ma164) V*, ME11 *lin-42(n1089) II; lin-46(ma164) unc-76(e911) V*, ME12 *lin-46(ma164) unc-76(e911) V; lin-14(n360) X*, ME13 *aeEx1* (pJJ.1[*lin-46:GFP*] pRF4[*rol-6(su1006)*]), ME20 *lin-46(ma164) V*, ME21 *lin-46(ma174) V*, ME42 *lin-46(ma164) unc-76(e911) V; lin-14(ma135)/szT1 X*, ME58 *lin-42(n1089) II; lin-46(ma164) unc-76(e911) V; lin-14(n179) X*, ME63 *lin-42(n1089) I; lin-14(n179ts) X*, ME89 *lin-28(n719) I; wIs78*, ME90 *lin-28(n719) I; wIs78; lin-14(n536n540)/+ X*, ME91 *wIs78; lin-46(ma164) unc-76(e911) V*, ME92 *lin-28(n719) I; wIs78; lin-46(ma164) unc76(e911) V*, ME94 *aeEx33* (pLT.18 [*lin-46Δ:GFP*] pRF4[*rol-6(su1006)*]), ME97 *lin-28(n719) I; wIs78; lin-46(ma164) unc-76(e911) V; lin-14(n536n540)/+ X*, ME98 *wIs78; lin-14(ma135)/szT1 X*, ME99 *wIs78; lin-46(ma164) unc-76(e911) V; lin-14(ma135)/szT1 X*, ME101 *lin-41(ma104) I, lin-46(ma164) V*, ME105 *wIs78; lin-14(n179ts) X*, ME107 *wIs78; lin-46(ma164) unc-76(e911) V; lin-14(n179ts) X*, CT8 *lin-41(ma104) I*, MT2257 *lin-42(n1089) II*,

MT1848 *lin-14(n360) X*, PV5 *lin-28(ga54) I, RG733 ced-1(e1735) I; wIs78* and *VT284 lin-14(ma135)/szT1 X. wIs78*: [pDP#MM016B (*unc-119*) + pJS191 (*ajm-1::gfp* + pMF1(*scm::gfp*) + F58E10] (Abrahante et al., 2003).

Microscopy and phenotype analysis

Egg-laying ability was scored using a dissecting microscope. Gonad development, seam cell number, and adult lateral alae formation were scored using DIC and fluorescence microscopy at high magnification. Lateral hypodermal cell lineages were deduced from counting seam cells of animals of multiple ages from the L1 to the L4. These cells were identified by GFP expression from *wIs78*, which contains *scm-1:GFP* and *ajm-1:GFP* that mark seam cell nuclei and seam cell junctions, respectively (Koh and Rothman, 2001). The stage of development was determined by extent of gonad and germline development. Data were collected from animals both early and late in each larval stage. Because cell division was ongoing near the beginning of each stage, we present only seam cell numbers when most or all cell divisions appeared to be complete.

Cold-sensitive period

lin-46(ma174) animals were raised at either the restrictive (15°C) or permissive (25°C) temperature until specific stages in development, when they were shifted to the other temperature for the remainder of larval development. The animals were shifted when they were in the lethargus period that precedes each molt. At adulthood, animals were then scored for the presence of gaps in alae.

Genetic and physical mapping

Genetic mapping and physical mapping were performed using standard methods (Fig. 3A; data available at wormbase.org).

Plasmids and transformation

Cosmid genomic clones were obtained from *C. elegans* genome sequencing consortium. Subclones were made in pBluescript. In rescue experiments, clones were co-injected with pRF4(*rol-6(su1006)*) into a strain of the genotype *lin-28; lin-46*. Rolling animals were scored for egg-laying ability and the presence of adult alae in the L4 stage. Clones that caused the transgenic F1 animals to display a precocious phenotype were scored as positive for the ability to rescue the *lin-46* suppression phenotype.

Poly(A)+ RNA from a mixed stage population was subjected to RT-PCR using oligo(dT), SL1- and *lin-46* specific primers. PCR products were cloned and sequenced to determine the structure of the gene.

A translational fusion reporter construct (pJJ.1) was made by introducing GFP in frame into the *Eag*I site, nine codons from the stop codon, in the *Bgl*I-*Ngo*MI subclone shown in Fig. 3A. A transcriptional fusion construct (pLT.18) was derived from the translational fusion by creating a 1.5 kb deletion within the gene, from *Bgl*II site in the second exon, 20 codons from the AUG, to the *Eco*RV site in the last exon, 15 codons from start of GFP. Both constructs were co-injected with pRF4(*rol-6(su1006)*). Four translational fusion lines and two transcriptional fusion lines showed consistent expression patterns as described in Results.

RNA interference

RNA-interference of *lin-57* was achieved culturing worms on bacterial strain HT115(DE3) containing a plasmid expressing *lin-57* dsRNA (gift of A. Rougvie) in the presence of IPTG, ampicillin and tetracycline (Timmons et al., 2001; Abrahante et al., 2003; Lin et al., 2003).

Results

lin-46 mutations suppress precocious development

To identify additional early-acting components of the heterochronic pathway, we conducted genetic screens for

**Table 1. *lin-46(lf)* suppresses *lin-28(lf)* and *lin-14(ts)*
precocious phenotypes**

		Number of seam cells[†] (*n*)[‡]				
	Strain*	L1	L2	L3	L4	% Egl[§]
1	Wild type	10 (27)	17 (10)	16 (12)	16 (8)	0 (180)
2	*lin-28(lf)*	10 (19)	11 (20)	11 (19)	N/A	100 (264)
3	*lin-28(lf); lin-46(lf)*	10 (23)	17 (19)	16 (11)	16 (12)	0 (138)
4	*lin-28(lf); lin-46(lf)*, 15°C	10 (13)	17 (26)	16 (14)	16 (24)	0 (30)
5	*lin-14(ts)*, 25°C	16 (12)	15 (11)	14 (16)	N/A	46 (26)
6	*lin-46(lf); lin-14(ts)*, 25°C	16 (33)	17 (16)	19 (15)	17 (7)	3 (39)
7	*lin-46(lf)*	10 (27)	17 (15)	16 (8)	16 (19)	0 (119)
8	*lin-46(lf)*, 15°C	10 (12)	17 (19)	22 (24)	20 (26)	0 (24)

*Strains used: RG733, ME89, ME92, ME105, ME107, ME91. Grown at
20°C, except where noted.

[†]Number of seam cells per animal side presented as the mode of the data;
L2 cell counts were often high by one cell because of the comparatively late
division of cells of the T lineage, causing both daughters to be scored.

[‡]Number of animals examined, one side per animal

[§]Egl, egg-laying defective. The ability to lay any eggs was considered
Egl+.

Fig. 1. Lineages of hypodermal cells in heterochronic mutants.
Schematics of post-embryonic cell lineages for lateral (seam;
Vn=V1-V4 and V6) and ventral (Pn=P3-P8) cells are shown for
animals of each genotype indicated. Vulva precursor cells (VPC) are
the posterior daughters of P5-P7. A horizontal line depicts cell
division. To the left in each set of lineages are indicated the cell-type
specific lineage pattern interpreted from the data: 1, L1-specific cell
lineage pattern (etc.); A, adult. 2/3 and 4/A indicate that cells at the
indicated stage may execute a mix of alternative fates (see text).
Broken lines indicate cells not depicted. Three horizontal
lines indicate fusion of seam cells and secretion of adult lateral alae. The
anterior daughters in Vn (seam) lineages, indicated by short lines,
appear to fuse with the main hypodermal syncytium. Wild-type and
lin-28(lf) lineages are taken from previous studies (Sulston and
Horvitz, 1977; Ambros and Horvitz, 1984).

mutations that suppress the precocious developmental
phenotypes of *lin-14(lf)* and *lin-28(lf)* mutations. These
mutations cause multiple cell lineage defects that result in a
protruding vulva and an inability to lay eggs as adults (Ambros
and Horvitz, 1984). Suppressor mutations were identified in
animals with a normal vulva that could lay eggs. Two such
mutations were characterized: *ma164* and *ma174*. Each is
recessive to wild type and they define a complementation group
on linkage group V (see Fig. 3). Because these mutations cause
changes to the normal cell lineage patterns (as described
below), the gene they define was given the name *lin-46*, for cell
lineage abnormal, according to standard nomenclature
(Horvitz et al., 1979).

Both mutant alleles of *lin-46* completely suppressed the
protruding vulva (Pvul) and egg-laying defective (Egl)
phenotype of three different *lin-28* null alleles (Table 1, lines
2 and 3, and data not shown) (Moss et al., 1997). The Pvul and
Egl phenotypes of *lin-28(lf)* are due in part to precocious vulva
precursor cell (VPC) divisions and vulva development (Euling
and Ambros, 1996). We observed that *lin-28(lf); lin-46(lf)*
animals had wild-type timing of vulva development, with VPC
divisions occurring in the L3 stage (Fig. 1; data not shown).
lin-28 also governs the choice between L2- and L3-specific
fates in the lateral hypodermal seam cells (Ambros and
Horvitz, 1984). Normally during the L1, L3 and L4 stages,
most seam cells undergo a single asymmetric cell division,
after which the anterior daughter joins the hypodermal
syncytium and the posterior daughter remains a seam cell (Fig.
1, wild type, Vn). However, in the L2 of wild-type animals, the
asymmetric division of certain seam cells (V1-4, V6) is
preceded by a symmetric division, which increases the number
of seam cells on each side of an animal by five; additional seam
cells are also added at this stage by descendants of other
hypodermal cells (H1 and T; Table 1, line 1; Fig. 1) (Sulston
and Horvitz, 1977). *lin-28(lf)* animals have fewer seam cells
than wild type, because they skip L2 lineage patterns that
generate these additional seam cells; *lin-28(lf)* animals also
cease the molting after only three cycles because of precocious
execution of the L/A switch (Table 1, line 2; Fig. 1) (Ambros
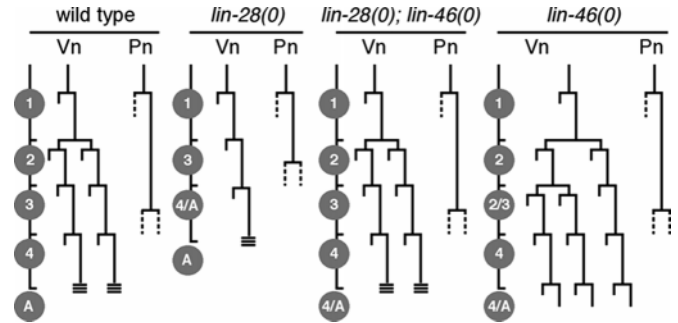and Horvitz, 1984). We found that the *lin-28(lf); lin-46(lf)*

animals had the normal number of seam cells at every stage,
as well as the normal four molts, indicating suppression of the
L2 lineage defects *of lin-28(lf)* by the *lin-46* mutation (Table
1, line 3; Fig. 1). Furthermore, *lin-46(lf)* completely suppressed
the precocious L/A switch, as indicated by synthesis of adult
alae, caused by *lin-28(lf)* (Table 2, lines 3 and 4; Fig. 1). These
data indicate that *lin-46* affects all fate decisions governed by
lin-28 and that the absence of *lin-46* activity completely
bypasses the requirement of the animal for *lin-28*.

By contrast, we observed no suppression of any of the
precocious heterochronic defects of a *lin-14* null allele. For
example, like *lin-14(0)* animals, *lin-46(lf); lin-14(0)* animals
are completely egg-laying defective and execute the L/A
switch precociously (Table 2, lines 5 and 6; data not shown).
However, surprisingly, one of the *lin-46* mutant alleles (*ma174*)
was isolated as a suppressor of *lin-14(n179ts)* animals. *lin-14(n179ts)* is a loss-of-function and temperature-sensitive
allele (Ambros and Horvitz, 1984; Ambros and Horvitz,
1987). As mentioned above, *lin-14* acts twice during larval
development: once to affect L1 cell fates (*lin-14A* activity), and
again to affect L2 and later fates (*lin-14B* activity) (Ambros
and Horvitz, 1987). When *lin-14(n179ts)* animals are raised at
25°C they display both a loss of *lin-14A* activity, causing a
precocious increase in seam cell number and a loss of *lin-14B*
activity, which leads to precocious execution of the L/A switch
(Table 1, line 5; Table 2, line7). We found that a mutation in
lin-46(lf) suppressed *lin-14B* defects, but not the *lin-14A*
defects, of the *lin-14(n179ts)* allele. Specifically, *lin-46(lf); lin-14(n179ts)* animals had an abnormal number of seam cells in
the L1, but had the normal number of molts (Table 1, line 6).
Furthermore, compared with *lin-14(n179ts)* animals, the *lin-46(lf); lin-14(n179ts)* animals were significantly less egg-
laying defective and showed reduced amounts of precocious

Table 2. Genetic interactions between *lin-46* and heterochronic mutants

	Strain*	% with full-length/ partial precocious alae[†] (n)[‡]
1	Wild type	0/0 (27)
2	lin-46(lf)	0/0 (26)
3	lin-28(lf)	100/0 (20)
4	lin-28(lf); lin-46((lf)	0/0 (42)
5	lin-14(0)	100/0 (15)
6	lin-46(lf); lin-14(0)	100/0 (7)
7	lin-14(ts), 25°C	74/26 (23)
8	lin-46(lf); lin-14(ts), 25°C	17/22 (23)
9	lin-14(A+B–)[§]	77/23 (22)
10	lin-46(lf); lin-14(A+B–)	5/90 (20)
11	lin-41(lf)	14/32 (22)
12	lin-46(lf); lin-41(lf)	13/30 (23)
13	lin-42(lf)	100/0 (28)
14	lin-46(lf); lin-42(lf)	0/63 (24)
15	lin-57(RNAi)[¶]	75/25 (20)
16	lin-46(lf); lin-57(RNAi)[¶]	25/75 (20)

*Strains used: N2, ME20, PV5, ME1, MT1848, ME12, VT284, ME42, CT8, ME101, MT2257, ME11, ME63 and ME58. Grown at 20°C, except where noted.

[†]Precocious adult alae observed during the L4 stage; partial alae ranged from small patches to nearly full-length; one side examined per animal.

[‡]Number of animals examined.

[§]A+B–, an allele of *lin-14* that is wild type for *lin-14A* activity, but mutant for *lin-14B* activity (Ambros and Horvitz, 1987).

[¶]RNAi was performed on wild-type or *lin-46(ma164)* strains as described in the Material and methods.

Table 3. Suppression of *lin-28(0)* by *lin-46(0)* does not require *lin-14*

	Strain*	Number of seam cells[†] (n)[‡]	
		at hatching	after L1
1	lin-14(0)	10 (2)	15 (16)
2	lin-46; lin-14(0)	10 (2)	15 (12)
3	lin-28; lin-14(0)	10 (2)	10 (10)
4	lin-28; lin-46; lin-14(0)	10 (3)	15 (20)

*Strains used: ME98, ME99, ME90, ME97; *lin-14(0)*, *lin-14* null allele.

[†]Counts made on one side of each animal and presented as the mode of the data.

[‡]Number of animals examined.

for suppression, and, furthermore, that *lin-46(lf)* suppresses the phenotype of *lin-28(lf)* by raising *lin-14* activity to a compensatory level. Importantly, a requirement for *lin-14* in the suppression would suggest that *lin-14* can act independently of *lin-28* to control late stage fates. Because of its implications for how the heterochronic pathway functions, we further investigated this possibility.

Suppression of *lin-28* does not require *lin-14*

To determine whether *lin-14* is indeed required for the suppression of *lin-28(lf)* phenotype by *lin-46(lf)*, we assessed hypodermal development in strains bearing a null allele of *lin-14*. *lin-14(0)* animals skip L1-specific lineage patterns, and precociously execute the L2-specific division patterns that increase the seam cell number (Ambros and Horvitz, 1987). It has been previously shown that a *lin-28(lf); lin-14(0)* strain executes the L/A switch one stage earlier than a *lin-14(0)* strain; however, the effect of this mutant combination on seam cell number was not assessed (Ambros, 1989). We observed that the increase of 10 to 15 seam cells that occurs abnormally early during the L1 in *lin-14(0)* animals did not occur in the absence of *lin-28*, consistent with an L3 lineage pattern occurring two stages early (Table 3, lines 1 and 3). This observation indicates the hypodermal lineage patterns executed in the L1 in *lin-14(0)* animals are controlled by *lin-28*, and is evidence that *lin-28* acts independently of *lin-14* to govern these cell fate choices.

Importantly, although *lin-46(lf)* had no effect on the number of seam cells of *lin-14(0)* animals with wild-type *lin-28*, it increased the number of seam cells of a *lin-28(lf); lin-14(0)* strain to that of a *lin-14(0)* strain (Table 3). Thus, *lin-46(lf)* suppressed the *lin-28(lf)* defect, despite the absence of *lin-14*. In addition, the L/A switch, as indicated by seam cell fusion and adult alae formation, occurred one stage later in triple mutant animals relative to *lin-28(lf); lin-14(0)* animals (data not shown). Therefore, the elevation of *lin-14* activity is not the mechanism by which *lin-46(lf)* compensates for the loss of *lin-28*.

Interactions of *lin-46* with other heterochronic mutants

To further establish the position of *lin-46* in the developmental timing pathway, we examined its interactions with other precocious heterochronic mutants. In addition to *lin-14* and *lin-28*, several other genes have been identified that result in precocious expression of the L/A switch when mutated: *lin-41*, *lin-42* and *lin-57* (Z. Liu, PhD thesis, Harvard University,

adult alae (Table 1, line 6; Table 2, line 8). Similarly, the precocious adult alae formation of *lin-14(n360)*, a *lin-14(lf)* allele that displays only *lin-14B* defects, was suppressed by *lin-46(lf)* (Table 2, lines 9 and 10) (Ambros and Horvitz, 1987). In addition, we observed that intestinal nuclei of *lin-46(lf); lin-14(n179ts)* animals fail to divide in the L1 as they normally do, which is a *lin-14A* defect, but did not display precocious vulva development, a *lin-14B* defect (data not shown) (Ambros and Horvitz, 1987; Euling and Ambros, 1996). Thus, *lin-46(lf)* suppressed the defects that *lin-14* hypomorphic alleles have in common with *lin-28(lf)*, but not those unique to *lin-14*, although it suppressed none of the defects of a *lin-14* null allele.

Interestingly, the suppression of the *lin-28(lf)* phenotype was quite complete: whereas 100% of *lin-28(lf)* animals have precocious adult alae, the *lin-28(lf); lin-46(lf)* double mutant animals were devoid of adult alae at the L4 stage (Table 2, lines 3 and 4). By contrast, the suppression of *lin-14* hypomorphic alleles was substantial, albeit incomplete. For example, for *lin-14(n360)*, the percentage of animals with full-length precocious adult alae was reduced from 77% to 5%; however, 95% of double mutant animals had some amount of precocious alae (Table 2, lines 9 and 10).

Because *lin-14* and *lin-28* act together to govern the cell fates of the L2 and later, our findings suggest that *lin-46* has different relationships with these two genes. The ability of *lin-46(lf)* to suppress *lin-14* hypomorphic alleles but not a null allele may reflect that at least some *lin-14* activity is required

Fig. 2. Retarded development of the lateral hypodermis in a *lin-46* mutant. A DIC micrograph of an adult animal of the genotype *lin-46(ma164)* grown at 20°C showing the same field in two planes of focus. Left, a pair of recently divided lateral hypodermal seam cells. Right, the gap in adult lateral alae overlying these cells. These gaps initially overly an even number of seam nuclei, but later, the anterior cell fuses with the main hypodermal syncytium.

1990) (Abrahante et al., 1998; Abrahante et al., 2003; Lin et al., 2003). For this analysis, we again examined the ability of a *lin-46(lf)* to suppress the precocious execution of the L/A switch that is characteristic of these mutants.

lin-46(lf) partially suppressed the precocious synthesis of adult alae of a *lin-42(lf)*, whereas it had little or no effect on the precocious alae caused by loss of the activities of *lin-41(lf)* and *lin-57(lf)* (Table 2, lines 11-16). Thus, *lin-46* appears to function upstream of *lin-41* and *lin-57* and downstream or in parallel with *lin-42* in controlling the L/A switch. These results are consistent with *lin-46* acting early in the heterochronic pathway, because both *lin-41* and *lin-57* are thought to act late in development, downstream of *lin-28* in controlling the L/A switch (see Fig. 5) (Slack et al., 2000; Lin et al., 2003). The earliest time of action of *lin-42* has not been determined, but previous genetic analysis, as well as these data, are consistent with it acting early in the heterochronic pathway (Z. Liu, PhD thesis, Harvard University, 1990).

lin-46 mutations cause retarded development at two stages

In examining the effects of *lin-46(lf)* in the absence of other heterochronic mutations, we observed an increase in the number of seam cells after the L2 stage when the animals were grown at 15°C (Table 1, line 8). This increase probably reflects a reiteration of the L2-specific lineage pattern at the L3 stage, in which the five seam cells that divide twice in the L2, do so again in the L3 (Fig. 1). This retarded lineage defect was not observed when animals were raised at 20°C, indicating a strict cold-sensitivity for this defect (Table 1, line 7). Because the mutant alleles of *lin-46* are likely to result in the complete loss of function of the gene (see below), a reiteration of L2 lineages in *lin-46(0)* would indicate that the normal role of *lin-46* is to promote the expression of L3-specific cell fates at the appropriate time.

Moreover, we observed that two mutant alleles of *lin-46* each caused a similar failure of the L/A switch at the end of larval development, resulting in gaps in adult alae (Table 4, lines 4 and 7; Figs 1, 2). The penetrance and expressivity of this phenotype are significantly enhanced by growth at low temperature. For example, the retarded L/A switch defect of *lin-46(ma164)* showed 6% penetrance at 25°C, 32% at 20°C, and 86% at 15°C (Table 4, lines 4-6). Because this cold sensitivity was true of mutant alleles of *lin-46*, which, as described below, are likely to be null by molecular criteria, it appears to be a property of a strain lacking *lin-46* activity, rather than a property of the molecular lesions themselves.

Remarkably, the early and late retarded lineage defects of *lin-46(lf)* are not equivalently penetrant. Specifically, 32% of

lin-46(ma164) animals raised at 20°C show L/A switch defects, whereas none grown at the same temperature showed reiteration of L2 lineage patterns (Table 1, line 7, and Table 4, line 5). Thus, the late stage defect is not dependent on an earlier lineage defect (Fig. 1). As discussed below, this is consistent with *lin-46* either acting twice, early and late, or acting at one time and separately affecting two downstream processes that are differently sensitive to its level of activity.

Interestingly, the retarded phenotype of *lin-46(lf)* is nearly completely suppressed by *lin-28(lf)*. For example, the penetrance of the L/A switch defect in *lin-28(lf); lin-46(ma164)* was 8% at 15°C, which is significantly reduced relative to 86% for *lin-46(ma164)* alone (Table 4, lines 4 and

Table 4. Cold-sensitivity and suppression of *lin-46* retarded phenotype

	Strain* and temperature	% with gaps in adult alae[†] (*n*)[‡]
	Wild type	
1	15°C	0 (21)
2	20°C	0 (44)
3	25°C	0 (23)
	lin-46(ma164)	
4	15°C	86 (22)
5	20°C	32 (22)
6	25°C	6 (16)
	lin-46(ma174)	
7	15°C	96 (25)
8	20°C	10 (21)
9	25°C	0 (20)
	lin-28	
10	15°C	0 (20)
11	20°C	0 (22)
12	25°C	0 (24)
	lin-28; lin-46(ma164)	
13	15°C	8 (39)
14	20°C	0 (31)
15	25°C	0 (21)
	lin-14(0)	
16	15°C	0 (13)
	lin-46; lin-14(0)	
17	15°C	67 (21)

*Strains used: N2, ME20, ME21, PV5, ME1, MT1848 and ME12.
[†]Gaps observed in young adult animals; gaps covered one to several seam cells; strains with more frequent gaps generally had longer gaps; one side examined per animal.
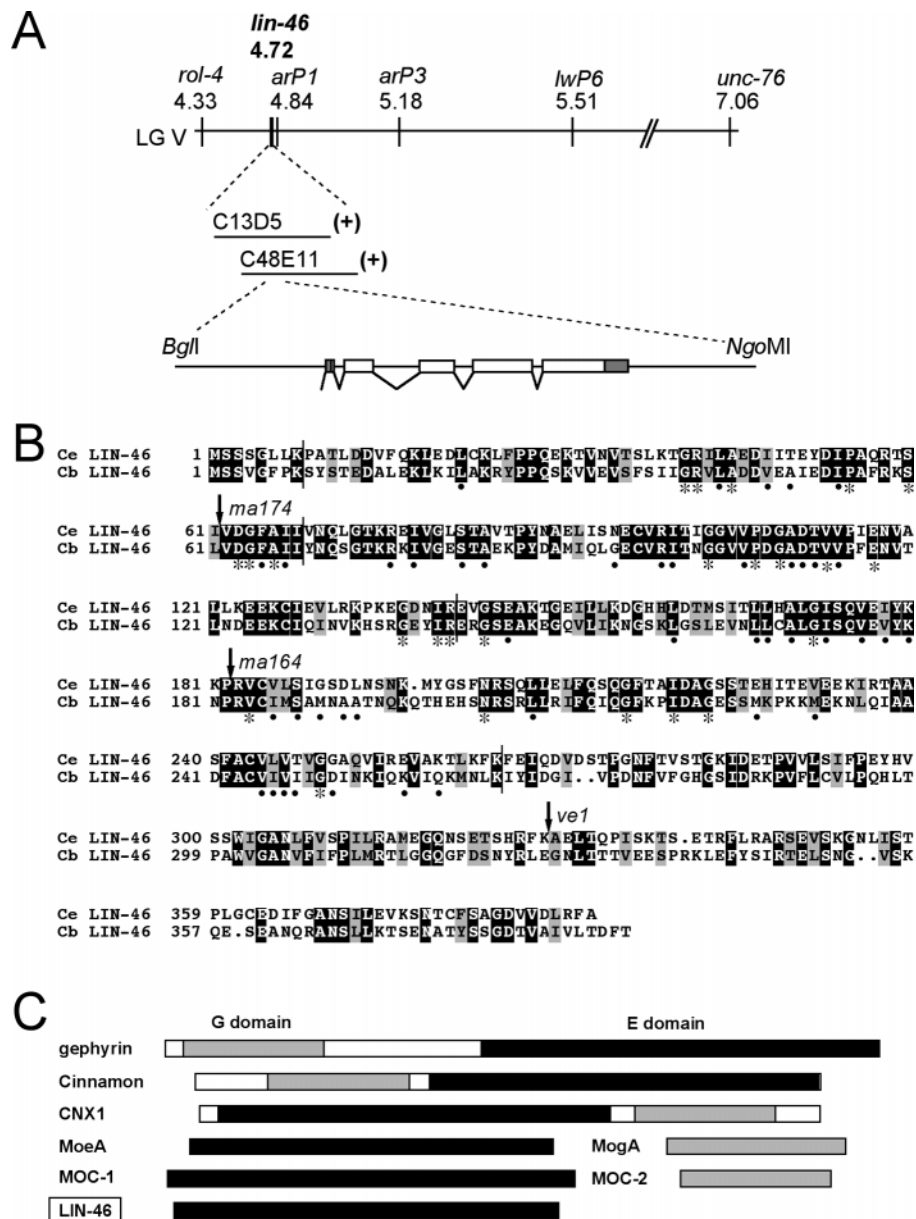[‡]Number of animals examined.

A

lin-46
4.72

rol-4   arP1   arP3   lwP6   unc-76
4.33   4.84   5.18   5.51   7.06

LG V

C13D5 (+)

C48E11 (+)

BglI   NgoMI

B

```
          1
Ce LIN-46   1  MSSSGLLKPATLDDVFQKIEDLCKLFPPCEKTVNVTSLKTGRILAEDIITEYDIPAQRTS
Cb LIN-46   1  MSSVGFPKSYSTEDALEKIKILAKRYPPQSKVVEVSFSIIGRVLADDVEAIEDIPAFRKS
                                                        *  *   *   *     *  *  *

          ↓ ma174
Ce LIN-46  61  IVDGFAIIVNCLGTKRBIVGLSTAVTPYNAELISNECVRITIGGVVPDGADTVVPIENVA
Cb LIN-46  61  LVDGFAIIYNQSGTKRKIVGESTAEKPYDAMIQLGECVRITNGGVVPDGADTVVPFENVT
               *  *   *    *    *  *       *         *     *   *  *  *

Ce LIN-46 121  PLKEEKCIEVLRKPKEGDNIREVGSEAKTGEILLKDGHHLDTMSITLLHALGISQVEIYK
Cb LIN-46 121  INDEEKCIQINVKHSRGEYIRERGSEAKEGQVLIKNGSKLGSLEVNLLCALGISQVEVYK
                  *     *         *  *   *                        *    *  *

          ↓ ma164
Ce LIN-46 181  KPRVCVLSIGSDLNSNK.MYGSFNRSQLIELFCSQGFTAIDAGSSTEHITEVEEKIRTAA
Cb LIN-46 181  NPRVCINSAMNAATNQKQTHEHSNRSRLLRIFQIQGFKPIDAGESSMKPKKMEKNLQIAA
                                  *        *    *      *         *

Ce LIN-46 240  SFACVLVTVGGAQVIREVAKTLKFKFEIQDVDSTPGNFTVSTGKIDETPVVLSIFPEYHV
Cb LIN-46 241  DFACVIVIIGDINKIQKVIQKMNLKIYIDGI..VPDNFVFGHCSIDRKPVFLCVLPQHLT
                *   *       *   *                     *            *  *

Ce LIN-46 300  SSWIGANLFVSPILRAMEGQNSETSHRFKAELTQPISKTS.ETRFLRARSEVSKGNLIST
Cb LIN-46 299  PAWVGANVFIFPLMRTLGGQGFDSNYRLEGNLTTTVEESPRKLEFYSIRTELSNG..VSK

Ce LIN-46 359  PLGCEDIFGANSILEVKSNTCFSAGDVVDLRFA
Cb LIN-46 357  QE.SEANQRANSILKTSEKATYSSGDTVAIVLTDFT
```

C

```
              G domain                    E domain
gephyrin
Cinnamon
CNX1
MoeA                              MogA
MOC-1                             MOC-2
LIN-46
```

Fig. 3. Cloning of *lin-46* and its homology to MoeA and gephyrin. (A) Regions of the genetic and physical maps of linkage group V of *C. elegans* depicting the location of *lin-46*. Two cosmids shown rescued a *lin-46* mutant. One of these was subcloned to yield the minimal rescuing *Bgl*I-*Ngo*MI restriction fragment. The gene structure was determined by RT-PCR and sequence analysis. (B) An alignment of the predicted amino acid sequences of LIN-46 of *C. elegans* and *C. briggsae.* Black, identical residues; gray, similar residues. Vertical lines indicate exon boundaries. Arrows indicate positions of lesions in the mutant alleles: *ma164*, *ma174* and *ve1*. Dots and asterisks indicate residues partially and highly conserved among gephyrin/MoeA homologues, respectively. (C) Schematics of LIN-46 related proteins: gephyrin, mammals; Cinnamon, *Drosophila*; CNX1, *Arabidopsis*; MoeA and MogA, *E. coli*; MOC-1, MOC-2 and LIN-46, *C. elegans*. MoeA-like (E) and MogA-like (G) regions are indicated.

13). Likewise, we observed no early cell lineage defects in the double mutant grown at 15°C (Table 1, line 4; Fig. 1). This mutual suppression of *lin-46(lf)* and *lin-28(lf)* suggests that *lin-46* is not in a simple linear pathway downstream of *lin-28*, but rather that their activities converge, possibly on common targets.

By contrast, the retarded phenotype of *lin-46(lf)* was not suppressed by a *lin-14* null mutation. Rather, *lin-46(lf)* causes a severe retarded defect in a *lin-14* null mutant background (67%), as it does in an otherwise wild-type background (86%; Table 4, lines 4 and 17). This result further indicates that *lin-14* is not required for the effect of *lin-46* on developmental timing.

lin-46 encodes a homologue of bacterial MoeA and vertebrate gephyrin.

lin-46 was cloned by genetic mapping and transformation rescue (Fig. 3A; see Materials and methods). The smallest rescuing fragment encodes one complete gene, annotated as R186.4 in the genome sequence. Northern analysis and cDNA library screening failed to detect a transcript from this gene (data not shown). RT-PCR analysis revealed that the gene is expressed, has four introns, and its mRNA is trans-spliced, polyadenylated, and 1.4 kb long (Fig. 3A). The sequence of amplified genomic DNA from three *lin-46* mutant strains revealed mutations in all three that would affect the protein encoded by this gene (Fig. 3B). Two of these lesions, from alleles *ma164* and *ma174*, probably result in severe truncations of the protein, strongly suggesting they are molecular nulls. Furthermore, frameshift mutations introduced into the rescuing clone rendered it unable to complement the *lin-46* mutant (data not shown). A clone of the orthologous gene from the related species *C. briggsae* rescued the *C. elegans lin-46* mutant phenotype, indicating that its function is conserved between these species (data not shown). We therefore concluded that *lin-46* corresponds to R186.4.

lin-46 has the potential to encode a 391 amino acid protein with homology along its entire length to MoeA of bacteria and the C-terminal domains (referred to as the E-domain) of the mammalian protein gephyrin; other related proteins are Cinnamon of *Drosophila* and CNX1 of *Arabidopsis* (Fig. 3C) (Kamdar et al., 1994; Stallmeyer et al., 1995). Gephyrin is a submembraneous scaffolding protein that aids in clustering glycine and GABA receptors at postsynaptic neurons (Kneussel and Betz, 2000). MoeA is involved in the last step of the biosynthesis of molybdenum co-factor, a metal coordinating molecule in many molybdo-enzymes, although the exact function of MoeA in this process is not known. We
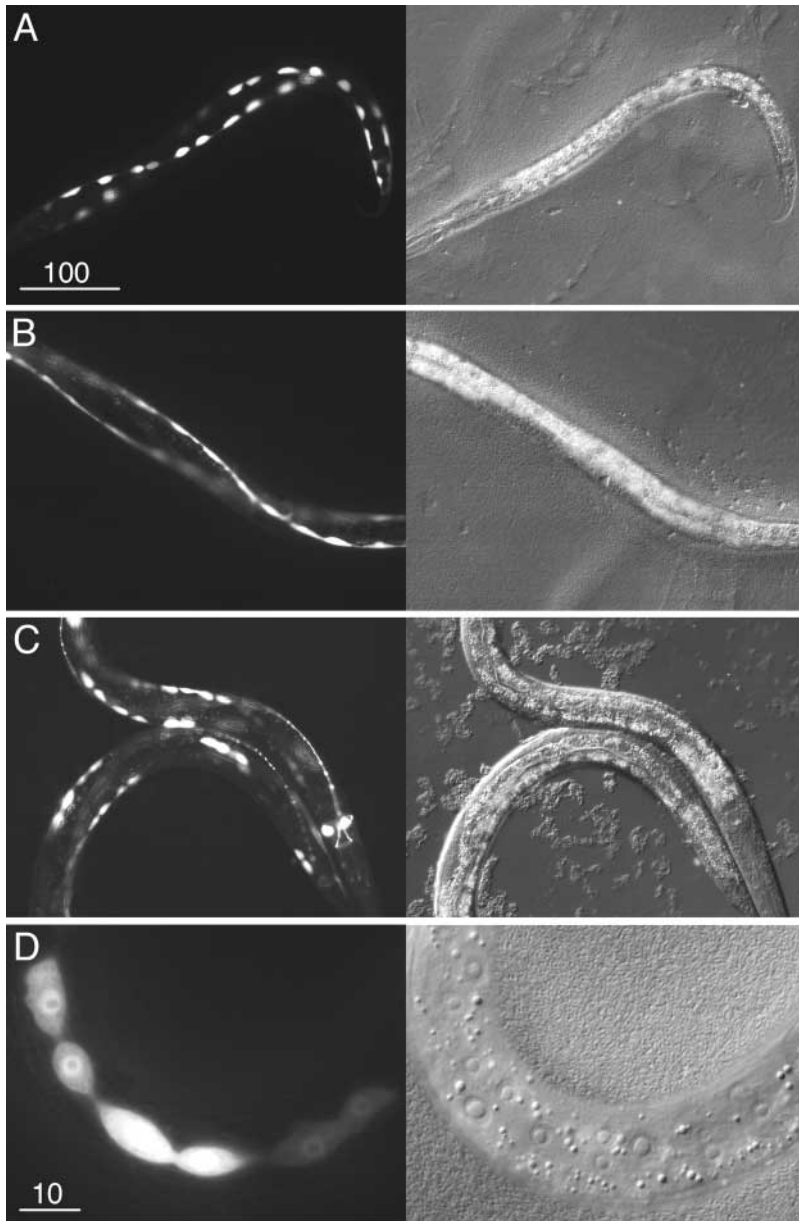
Fig. 4. Expression of *lin-46:GFP* in hypodermal cells during larval development. Fluorescence and DIC micrographs of animals bearing *lin-46:GFP* transgenes. Transgene arrays include a *rol-6* marker that causes twists in the body. (A) L2 animal expressing *lin-46:GFP* transcriptional fusion showing fluorescent lateral hypodermal seam cells. (B) An L4-stage animal bearing the transcriptional fusion showing fluorescent seam cells apparently undergoing fusion. (C) L2 animals expressing a *lin-46:GFP* translational fusion. Fluorescence is seen in seam cells as well as two neurons (see text). The exposure time is three to eight times longer than for animals expressing transcriptional fusion. (D) Higher magnification showing nuclear and cytoplasmic localization of LIN-46:GFP in an L2 animal expressing the translational fusion. Pairs of seam cells often show similar fluorescence intensity. Scale bars: in A, 100 μm for A-C; in D, 10 μm.

lin-46 is expressed in hypodermal cells during larval development

Green fluorescent protein (GFP) reporter constructs indicated that *lin-46* is expressed in hypodermal cells, particularly the lateral and ventral hypodermis, in brief periods during larval development (Fig. 4 and data not shown). Animals bearing a transcriptional fusion (see Materials and methods) showed intense fluorescence in lateral hypodermal cells around the time of each molt, especially during the L2- and L4-lethargus (Fig. 4A,B). In L2 animals, not all seam cells fluoresced simultaneously, and often only a cluster of an even number of cells fluoresced in a given animal, suggesting the expression of the fusion is related to seam cell division, which occurs somewhat asynchronously during larval development (Sulston and Horvitz, 1977). A translational fusion reporter showed a similar expression pattern, although it was less intense (Fig. 4C). The translational fusion also showed strong constitutive expression in the cell bodies and axons of the bilateral motor interneurons AVB, but we do not know the significance of this expression. The LIN-46:GFP fusion protein localized to both cytoplasm and nucleus, but mostly in the non-nucleolar part of the nucleus (Fig. 4D). Thus, LIN-46 is present in cells whose fates it governs near the time they execute stage-specific fates. Its appearance at multiple times suggested the possibility that *lin-46* functions more than once during larval development.

The cold-sensitive period for the late-stage retarded phenotype is early in larval development

To assess whether *lin-46* might function twice during larval development, we used the pronounced cold sensitivity of the *lin-46* mutant phenotype to define the cold-sensitive period for its late stage retarded defect. We observed that it was sufficient that animals be at the restrictive temperature only until the L2 lethargus for nearly 90% of the population to display gaps in adult alae (Table 5, 15°C to 25°C). Futhermore, we found it was necessary that the animals be at 15°C at least until L2 lethargus for a high penetrance of the alae defect; if animals

discuss possible molecular functions of the LIN-46 protein below.

Gephyrin, as well as Cinnamon and CNX1, are essentially fusions of homologues of the bacterial proteins MoeA and MogA. Indeed, all three of these proteins from higher eukaryotes are believed to participate in molybdenum cofactor biosynthesis, as the bacterial proteins do (Kamdar et al., 1994; Stallmeyer et al., 1995; Feng et al., 1998). We noted that *C. elegans* is unusual among multicellular eukaryotes in having its MoeA and MogA homologues encoded separately, and, furthermore, having two MoeA paralogues, one of which is encoded by *lin-46* (Fig. 3C). We have named the genes encoding the other MoeA homologue and the MogA homologue of *C. elegans moc-1* and *moc-2*, respectively (for molybdenum cofactor biosynthesis related; Fig. 3C). The biological roles of these genes in *C. elegans* are not yet known.

Table 5. Cold-sensitive period of late-stage developmental defect of *lin-46(0)*

	% with gaps in adult alae[†] (*n*)[‡]	
Stage*	15°C to 25°C	25°C to 15°C
Late embryo	2 (64)	97 (31)
L1 lethargus	31 (29)	92 (38)
L2 lethargus	88 (40)	7 (41)
L3 lethargus	90 (61)	2 (66)
L4 lethargus	97 (34)	0 (34)

*Stage at the time of shift from one temperature to the other.
[†]*lin-46(ma174)* animals were scored on both sides.
[‡]Number of animals examined.

were shifted from the permissive to the restrictive temperature at the L2 lethargus then 7% or less showed the defect (Table 5, 25°C to 15°C). These data indicate that the period that *lin-46* mutant animals are cold sensitive for the adult alae defect is prior to the L3 stage. Because the early fate defect of *lin-46(lf)* is executed early in the L3 stage, these observations suggest that *lin-46* is required during the L2 stage to affect cell fates executed at both early and late stages.

Discussion

We have identified a new heterochronic gene, *lin-46*, and determined that it acts early in the *C. elegans* larval developmental timing pathway to promote both L3-specific and adult-specific cell fates. Our genetic results support a model in which *lin-46* supports the activity of a downstream complex of factors that promotes specific fates. The nature of these factors, whether involved in signaling or gene regulation, is not known, however, some may be direct regulatory targets of *lin-28*. *lin-46* encodes a novel developmental regulator, a protein that resembles the multifunctional vertebrate protein gephyrin, and therefore may act, like gephyrin, as a scaffold for a multiprotein complex.

Interactions governing the L2/L3 fate choice

The heterochronic pathway is characterized by dynamic interactions among regulators that make binary choices in stage-specific cell fates (Ambros, 1989). We previously presented a model for the interactions governing the choice of L2 versus L3 cell fates (Seggerson et al., 2002). In this model, *lin-14* and *lin-28* are repressed by the microRNA lin-4 at the same time they support each other's expression via a lin-4-independent feedback circuit. This positive regulatory circuit is proposed to occur by the repression of two negative regulators, *x* and *y*, whose identities are not known.

Here we extend this model by proposing that *lin-46* acts specifically to promote the activity of *y*, and furthermore, that *y* acts both to regulate *lin-14* expression and to control developmental timing at two larval stages (Fig. 5A). Therefore, *y* represents both the point of convergence of the *lin-28* and *lin-46* activities, as well as a branch-point in the pathway that affects *lin-14* expression and L3 cell fates. We have no reason yet to believe that *lin-14* expression and L3 cell fates are regulated by distinct factors. Therefore, we continue to indicate this as a single activity, the hypothetical factor *y*, which may consist of multiple factors.
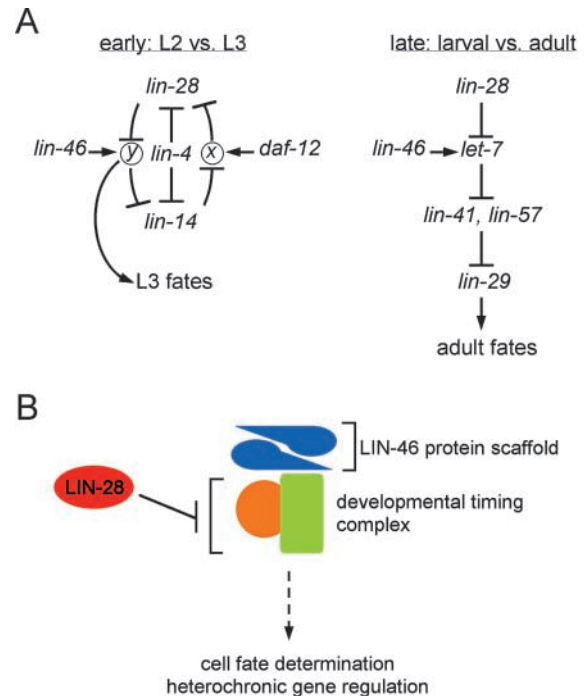

Fig. 5. Genetic and molecular models for LIN-46 activity in developmental timing. (A) Genetic models for the heterochronic pathway governing fate choices at two stages. (Left) Interactions governing the L2/L3 fate choice. The circled regulators *x* and *y* have not been identified and may in fact represent multiple factors. *lin-14* is depicted in its role in the L2/L3 fate decision only (*lin-14B* activity). The position of *daf-12* is based on previous studies (Antebi et al., 1998; Seggerson et al., 2002); its role does not appear to be analogous to that of *lin-46*. (Right) Interactions governing the L/A switch. The effect of *lin-28* or *lin-46* on *let-7* expression or activity has not been determined, and may be indirect. The linear pathway from *let-7* to adult fates is based on previous studies (Abrahante et al., 2003; Lin et al., 2003). (B) A molecular model for LIN-46 protein function. LIN-46 may scaffold a multiprotein assembly constituting a developmental timing complex that regulates the expression of downstream heterochronic genes and promotes L3- and adult-specific cell fates. Broken arrow indicates that the effects of the complex may be either positive or negative, and either direct or indirect. LIN-28 may act as a negative regulator of one or more components of that complex, probably at the level of gene expression.

In our model, we have placed *lin-46* outside of the feedback circuit based on the relative penetrance of the *lin-28(lf)* and *lin-46(lf)* phenotypes and their mutual suppression (Tables 1 and 4). Based on molecular analysis, the alleles of *lin-28* and *lin-46* used in these experiments are null (Fig. 3) (Moss et al., 1997). Thus, their mutual suppression implies that *lin-28* and *lin-46* affect a common process, but do not act in a simple linear pathway. If it were a simple pathway, then the *lin-28(lf); lin-46(lf)* animals would display the same degree of retarded development as *lin-46(lf)* animals. Furthermore, the difference in penetrance of the *lin-28(lf)* and *lin-46(lf)* phenotypes suggests they have different degrees of influence on cell fates. Specifically, whereas a *lin-28(lf)* is always fully precocious, the *lin-46(lf)* retarded phenotype is incomplete and conditional. For these reasons, we propose that *lin-46* is not directly part of

the *lin-14/lin-28* positive feedback circuit, and therefore is not itself *y*, but rather acts to promote the activity of *y* (Fig. 5A).

The difference in the penetrance between the suppression by *lin-46(lf)* of *lin-28(lf)* and the retarded L2 phenotype of *lin-46(lf)* also suggests that changes occur in how the L2/L3 fate decision is made at different times. In other words, cell fates at the L2 stage in a *lin-28(lf)* are more sensitive to the loss of *lin-46* than are fates at the L3 in wild type (Table 1; Fig. 1). Perhaps the decision to execute L3 fates is more robust at the third larval stage in wild type because of additional activities that are not present at the second larval stage in a *lin-28* mutant. Furthermore, in no situation does the absence of *lin-46* cause L2-specific cell fates to be reiterated indefinitely, further suggesting that additional factors come to influence the L2/L3 fate decision, ultimately in favor of L3 fates.

The role of *lin-14* in late stage cell fates

Our observations help to further illuminate the relationship between *lin-14* and *lin-28* in controlling the execution of cell fates after the first larval stage. Two models have been proposed to explain the activities of *lin-14*, *lin-14A* and *lin-14B* that independently affect L1 and later developmental events (Ambros and Horvitz, 1987). One is that different isoforms of the LIN-14 protein possess these distinct activities (Wightman et al., 1991; Reinhart and Ruvkun, 2001). However, this model is not supported by the finding that a molecular clone of a single isoform of LIN-14 can fully rescue *lin-14(lf)* (Hong et al., 2000). A second model is that the LIN-14 protein forms a temporal gradient through larval development, analogous to morphogen gradients in the *Drosophila* embryo (Ruvkun and Giusto, 1989; Reinhart and Ruvkun, 2001). However, our findings indicate that *lin-14* affects L2 developmental events only through *lin-28*, which requires a genetic circuit between these genes that engages late in the first larval stage (Table 3) (Seggerson et al., 2002). In other words, the *lin-14B* activity essentially reflects the participation of *lin-14* in a positive feedback circuit with *lin-28*.

This model explains why *lin-46(lf)*, which bypasses the requirement for *lin-28*, suppresses the L2 defects of *lin-14(lf)* but not its L1 defects. Hypomorphic alleles of *lin-14*, such as *lin-14(n360)* and *lin-14(n179ts)* used in these studies, reduce the activity of *lin-14*, but do not eliminate it. At the end of the first larval stage, when the positive feedback circuit between *lin-14* and *lin-28* is engaged, a mutation in *lin-14* would lead to a reduction in *lin-28* expression, and then a further reduction in *lin-14*. We have previously proposed that *y* in our model mediates the reduction of *lin-14* in this circuit (Fig. 5A, left) (Seggerson et al., 2002). However, if *lin-46* is absent, the activity of *y* would be attenuated and the reduction of *lin-14* would not be further amplified. Therefore, *lin-46(lf)* suppresses a *lin-14* hypomorph by essentially upregulating the existing *lin-14* activity. As a corollary, we propose that part of the reduction of *lin-14* activity of hypomorphic alleles is due to further downregulation via the positive feedback circuit.

Importantly, the reason a *lin-46* mutation does not suppress a *lin-14* null allele is twofold: first, *lin-14* normally acts through the feedback circuit and *lin-28* to control the L2/L3 fate choice, as we have shown (Table 3); and second, the *lin-28*-repressive component of the feedback circuit is not engaged early in the first larval stage of a *lin-14* mutant, as has been shown previously (Seggerson et al., 2002). Therefore, the effects of *lin-28* and *lin-46* on the L2/L3 fate decision are independent of the feedback circuit because they occur in the early L1 when *lin-14* is absent. Supporting this conclusion is our observation that *lin-46(lf)* causes a severe retarded phenotype in a *lin-14(0)* background at 15°C (Table 4). Thus, the different genetic interactions *lin-46* has with *lin-14* and *lin-28* reflect the underlying change in the relationship between *lin-14* and *lin-28* that occurs at the end of the L1 stage with the engagement of the *lin-4*-independent feedback circuit (Fig. 5A) (Seggerson et al., 2002).

lin-46 affects L3 and adult fates independently

Our data also indicate that the role of *lin-46* in regulating the L/A switch appears to be independent of its role in the L2/L3 fate decision. Specifically, *lin-46(lf)* animals grown at 20°C display normal cell lineage patterns early, but at the same temperature there is significant failure of the L/A switch at the L4 stage (Tables 1 and 4; Fig. 1). Thus, the failure of the L/A switch in *lin-46(lf)* animals can occur without a prior lineage defect. Because *lin-46:GFP* reporters are expressed in the L4 stage (Fig. 4B), this independence of early and late retarded defects may reflect that *lin-46* acts twice, once to promote L3 fates and again later to promote adult-specific fates. However, we found that the cold-sensitive period of *lin-46(lf)* strain for the adult fates is prior to the L3 stage. This finding suggests that *lin-46* normally acts only once at a branch-point in the heterochronic pathway where some of its targets influence L3 fates immediately, and other targets, which are more sensitive to its absence, govern adult fates (Fig. 5A). This latter possibility is consistent with *lin-46* acting at a step immediately downstream of *lin-28*. In our model, therefore, *y* may represent multiple factors, some of which affect the L2/L3 fate decision and the regulation of *lin-14*, and others which affect the L/A switch through the other known heterochronic genes (Fig. 5A). One possibility is that *lin-28* and *lin-46* directly affect the microRNA let-7, which then regulates other genes that control the L/A switch (Fig. 5A). This is consistent with the failure of *lin-46(lf)* to suppress the precocious phenotypes of the later-acting genes *lin-41* and *lin-57* (Table 2). Further analysis will determine whether *lin-46* and *lin-28* indeed affect the expression or activity of *let-7*. Because let-7 or let-7-like microRNAs have been hypothesized to regulate *lin-14*, it is an intriguing possibility that some of factors that constitute *y* are microRNAs.

LIN-46 may form a scaffold for a multiprotein assembly

The homology of the LIN-46 protein to MoeA and gephyrin further provides a basis for explaining its function. The crystal structure of MoeA shows it to exist as a homodimer, and it is likely that proteins with sequence similarity to MoeA do so as well (Schrag et al., 2001; Xiang et al., 2001). We have observed that the LIN-46 protein strongly interacts with itself in a yeast two hybrid assay, further suggesting LIN-46 forms multimers (L. Tang and E.G.M., unpublished). Gephyrin is believed to dimerize with itself via its C-terminal MoeA-like region, and trimerize with its N-terminal MogA-like region (see Fig. 3). The ability of gephyrin to multimerize and form a scaffold is the basis for its ability to cluster and aid the activity of interacting proteins (Schwarz et al., 2001). Although gephyrin is known to act both in synaptic transmission and in

molybdenum co-factor biosynthesis, LIN-46 does not appear to have a role in either of these processes in *C. elegans*; possibly its paralog, MOC-1, has these roles. We speculate that a general feature of proteins related to gephyrin, therefore, may be their ability to form scaffolds that avail them to participating in diverse biological roles, including development.

Based on several lines of evidence, including the homology to gephyrin, we propose a molecular model for LIN-46 in which it acts as a scaffolding protein for a multiprotein complex that determines cell fates (Fig. 5B). Supporting this model is the striking cold sensitivity of *lin-46* null alleles (Table 2). Cold sensitivity can be a feature of mutations affecting the dynamics of multiprotein structures, such as microtubules and bacteriophage capsids (Huffaker et al., 1988; Bazinet et al., 1990). LIN-46 may potentiate the activity of a complex that includes cell fate regulators or other developmental timing factors. LIN-46 is not essential for the activity of this complex, because development of a *lin-46* null mutant is nearly normal at 20°C, but the absence of LIN-46 protein may substantially reduce the activity of the complex at low temperature, possibly by causing the retention of a negative regulator of the complex. Because the LIN-28 protein appears to be a specific mRNA-binding protein (Moss et al., 1997), the expression of some of the components of the complex may be repressed when *lin-28* is normally active (Fig. 5B). The suppression of the loss of *lin-28* activity would therefore result from balance between the overexpression of components of the complex, and reduced efficiency of their function.

In summary, we believe *lin-46* occupies a unique position in the heterochronic pathway and furthers our understanding of the complex genetic network that governs the temporal regulation of developmental events. The fact that the LIN-46 protein resembles gephyrin expands the biological function of this protein family to development. LIN-46 is likely to interact with other proteins in controlling cell fate choices, and the identities of these proteins, which may also be regulatory targets of LIN-28, will provide further insight into how the temporal components of cell fates are determined.

References

Abrahante, J. E., Miller, E. A. and Rougvie, A. E. (1998). Identification of heterochronic mutants in *Caenorhabditis elegans*. Temporal misexpression of a collagen::green fluorescent protein fusion gene. *Genetics* **149**, 1335-1351.

Abrahante, J. E., Daul, A. L., Li, M., Volk, M. L., Tennessen, J. M., Miller, E. A. and Rougvie, A. E. (2003). The *Caenorhabditis elegans* Hunchback-like gene *lin-57/hbl-1* controls developmental time and is regulated by microRNAs. *Dev. Cell* **4**, 625-637.

Ambros, V. and Horvitz, H. R. (1984). Heterochronic mutants of the nematode *Caenorhabditis elegans*. *Science* **226**, 409-416.

Ambros, V. and Horvitz, H. R. (1987). The *lin-14* locus of *Caenorhabditis elegans* controls the time of expression of specific postembryonic developmental events. *Genes Dev.* **1**, 398-414.

Ambros, V. (1989). A hierarchy of regulatory genes controls a larva-to-adult developmental switch in *C. elegans*. *Cell* **57**, 49-57.

Ambros, V. (2000). Control of developmental timing in *Caenorhabditis elegans*. *Curr. Opin. Genet. Dev.* **10**, 428-433.

Antebi, A., Culotti, J. G. and Hedgecock, E. M. (1998). *daf-12* regulates developmental age and the dauer alternative in *Caenorhabditis elegans*. *Development* **125**, 1191-1205.

Arasu, P., Wightman, B. and Ruvkun, G. (1991). Temporal regulation of *lin-14* by the antagonistic action of two other heterochronic genes, *lin-4* and *lin-28*. *Genes Dev.* **5**, 1825-1833.

Bazinet, C., Villafane, R. and King, J. (1990). Novel second-site suppression of a cold-sensitive defect in phage p22 procapsid assembly. *J. Mol. Biol.* **216**, 701-716.

Bloom, L. and Horvitz, H. R. (1997). The *Caenorhabditis elegans* gene *unc-76* and its human homologs define a new gene family involved in axonal outgrowth and fasciculation. *Proc. Natl. Acad. Sci. USA* **94**, 3414-3419.

Euling, S. and Ambros, V. (1996). Heterochronic genes control cell cycle progress and developmental competence of *C. elegans* vulva precursor cells. *Cell* **84**, 667-676.

Feng, G., Tintrup, H., Kirsch, J., Nichol, M. C., Kuhse, J., Betz, H. and Sanes, J. R. (1998). Dual requirement for gephyrin in glycine receptor clustering and molybdoenzyme activity. *Science* **282**, 1321-1324.

Hong Y., Lee R. C. and Ambros V. (2000). Structure and function analysis of LIN-14, a temporal regulator of postembryonic developmental events in *Caenorhabditis elegans*. *Mol. Cell Biol.* **20**, 2285-2295.

Horvitz, H. R., Brenner, S., Hodgkin, J. and Herman, R. K. (1979). A uniform genetic nomenclature for the nematode *Caenorhabditis elegans*. *Mol. Gen. Genet.* **175**, 129-133.

Huffaker, T. C., Thomas, J. H. and Botstein, D. (1988). Diverse effects of beta-tubulin mutations on microtubule formation and function. *J. Cell Biol.* **106**, 1997-2010.

Jeon, M., Gardner, H. F., Miller, E. A., Deshler, J. and Rougvie, A. E. (1999). Similarity of the *C. elegans* developmental timing protein *lin-42* to circadian rhythm proteins. *Science* **286**, 1141-1146.

Kamdar, K. P., Shelton, M. E. and Finnerty, V. (1994). The *Drosophila* molybdenum cofactor gene Cinnamon is homologous to three *Escherichia coli* cofactor proteins and to the rat protein gephyrin. *Genetics* **137**, 791-801.

Kneussel, M. and Betz, H. (2000). Clustering of inhibitory neurotransmitter receptors at developing postsynaptic sites: The membrane activation model. *Trends Neurosci.* **23**, 429-435.

Koh, K. and Rothman, J. H. (2001). *elt-5* and *elt-6* are required continuously to regulate epidermal seam cell differentiation and cell fusion in *C. elegans*. *Development* **128**, 2867-2880.

Lee, R. C., Feinbaum, R. L. and Ambros, V. (1993). The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell* **75**, 843-854.

Lin, S. Y., Johnson, S. M., Abraham, M., Vella, M. C., Pasquinelli, A., Gamberi, C., Gottlieb, E. and Slack, F. J. (2003). The *C. elegans* hunchback homolog, *hbl-1*, controls temporal patterning and is a probable microRNA target. *Dev. Cell* **4**, 639-650.

Moss, E. G., Lee, R. C. and Ambros, V. (1997). The cold shock domain protein *lin-28* controls developmental timing in *C. elegans* and is regulated by the *lin-4* RNA. *Cell* **88**, 637-646.

Reinhart, B. J. and Ruvkun, G. (2001). Isoform-specific mutations in the Caenorhabditis elegans heterochronic gene lin-14 affect stage-specific patterning. *Genetics* **157**, 199-209.

Reinhart, B. J., Slack, F. J., Basson, M., Pasquinelli, A. E., Bettinger, J. C., Rougvie, A. E., Horvitz, H. R. and Ruvkun, G. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* **403**, 901-906.

Rougvie, A. E. and Ambros, V. (1995). The heterochronic gene *lin-29* encodes a zinc finger protein that controls a terminal differentiation event in *Caenorhabditis elegans*. *Development* **121**, 2491-2500.

Ruvkun, G., Ambros, V., Coulson, A., Waterston, R., Sulston, J. and Horvitz, H. R. (1989). Molecular genetics of the *Caenorhabditis elegans* heterochronic gene *lin-14*. *Genetics* **121**, 501-516.

Ruvkun, G. and Giusto, J. (1989). The *Caenorhabditis elegans* heterochronic gene *lin-14* encodes a nuclear protein that forms a temporal developmental switch. *Nature* **338**, 313-319.

Schrag, J. D., Huang, W., Sivaraman, J., Smith, C., Plamondon, J., Larocque, R., Matte, A. and Cygler, M. (2001). The crystal structure of

Escherichia coli MoeA, a protein from the molybdopterin synthesis pathway. *J. Mol. Biol.* **310**, 419-431.

Schwarz, G., Schrader, N., Mendel, R. R., Hecht, H. J. and Schindelin, H. (2001). Crystal structures of human gephyrin and plant CNX1 G domains: Comparative analysis and functional implications. *J. Mol. Biol.* **312**, 405-418.

Seggerson, K., Tang, L. and Moss, E. G. (2002). Two genetic circuits repress the *C. elegans* heterochronic gene *lin-28* after translation initiation. *Dev. Biol.* **243**, 215-225.

Slack, F. J., Basson, M., Liu, Z., Ambros, V., Horvitz, H. R. and Ruvkun, G. (2000). The *lin-41* RBCC gene acts in the *C. elegans* heterochronic pathway between the *let-7* regulatory RNA and the *lin-29* transcription factor. *Mol. Cell* **5**, 659-669.

Stallmeyer, B., Nerlich, A., Schiemann, J., Brinkmann, H. and Mendel, R. R. (1995). Molybdenum co-factor biosynthesis: The *Arabidopsis thaliana* cDNA CNX1 encodes a multifunctional two-domain protein homologous to a mammalian neuroprotein, the insect protein Cinnamon and three *Escherichia coli* proteins. *Plant J.* **8**, 751-762.

Sulston, J. E. and Horvitz, H. R. (1977). Post-embryonic cell lineages of the nematode *Caenorhabditis elegans*. *Dev. Biol.* **56**, 110-156.

Timmons, L., Court, D. L. and Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* **263**, 103-112.

Wightman, B., Burglin, T. R., Gatto, J., Arasu, P. and Ruvkun, G. (1991). Negative regulatory sequences in the *lin-14* 3′ untranslated region are necessary to generate a temporal switch during *Caenorhabditis elegans* development. *Genes Dev.* **5**, 1813-1824.

Wood, W. B. (ed.) (1988). *The Nematode* Caenorhabditis elegans. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Xiang, S., Nichols, J., Rajagopalan, K. V. and Schindelin, H. (2001). The crystal structure of *Escherichia coli* MoeA and its relationship to the multifunctional protein gephyrin. *Struct. Fold. Des.* **9**, 299-310.