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

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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.


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 circuity 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, 1991; 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, unc-76(e911), which has no developmental timing effects (A.S.-R.P. and E.G.M., unpublished) (Bloom and Hirvitz, 1997). The presence of the lin-46 mutant allele in strains was confirmed upon outcrossing.

Strains

The lin-28(0f) 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 aexL (pHH1[lin-46:GFP] pRF4[rol-6(su1006)]) ME20 lin-46(ma164) V, ME21 lin-46(ma164) V, ME42 lin-46(ma164) unc-76(e911) V; lin-14(n35)] 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; wls78, ME90 lin-28(n719) I; wls78; lin-14(n536n540)/+ X, ME91 wls78; lin-46(ma164) unc-76(e911) V, ME92 lin-28(n719) I; wls78; lin-46(ma164) unc-76(e911) V, ME94 aex33 (pLT18 [lin-46:ΔGFP] pRF4[rol-6(su1006)]) ME97 lin-28(n719) I; wls78; lin-46(ma164) unc-76(e911) V; lin-14(n356n540)/+ X, ME98 wls78; lin-14(na135)/X, ME99 wls78; lin-46(ma164) unc-76(e911) V; lin-14(na135)/X, ME101 lin-41(na104) I, lin-46(ma164) V, ME105 wls78; lin-14(n179ts) X, ME107 wls78; lin-46(ma164) unc-76(e911) V; lin-14(n179ts) X, CT8 lin-41(na104) I, MT2257 lin-42(n1089) II, MT1848 lin-14(n360) X, PV5 lin-28(ga54) I, RG733 ced-1(e1735) I; wls78 and VT284 lin-14(ma135)/szT1 X, wls78; [pDP8/MM016B (unc-119) + pJS191 (ajm-1::gfp + pMF1(scn::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 wls78, which contains scm::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 (pJJ1) was made by introducing GFP in frame into the Eegl site, nine codons from the stop codon, in the Bgl/II-NgoMI subclone shown in Fig. 3A. A transcriptional fusion construct (pLT18) 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 EcoRV 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 dsDNA (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
mutations that suppress the precocious developmental phenotypes of lin-14(0) and lin-28(0) 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(0) are due in part to precocious vulva precursor cell (VPC) divisions and vulva development (Euling and Ambros, 1996). We observed that lin-28(0); lin-46(0) 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(0) animals have fewer seam cells than wild type, because they skip L2 lineage patterns that generate these additional seam cells; lin-28(0); lin-46(0) 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(0); lin-46(0) 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(0) by the lin-46 mutation (Table 1, line 3; Fig. 1). Furthermore, lin-46(0) completely suppressed the precocious L/A switch, as indicated by synthesis of adult alae, caused by lin-28(0) (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(0); 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, line 7). We found that a mutation in lin-46(0) suppressed lin-14B defects, but not the lin-14A defects, of the lin-14(n179ts) allele. Specifically, lin-46(0); 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(0); 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

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

*Strains used: N2, ME20, PV5, ME1, MT1848, ME12, VT284, ME42, CT8, ME101, MT2257, ME111, 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-14(A+B–) 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.

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

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Number of seam cells† (n)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 lin-14(0)</td>
<td>10 (2) 15 (16)</td>
</tr>
<tr>
<td>2 lin-46; lin-14(0)</td>
<td>10 (2) 15 (12)</td>
</tr>
<tr>
<td>3 lin-28; lin-14(0)</td>
<td>10 (2) 10 (10)</td>
</tr>
<tr>
<td>4 lin-28; lin-46; lin-14(0)</td>
<td>10 (3) 15 (20)</td>
</tr>
</tbody>
</table>

*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 acts independently of lin-14 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.

Important, 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,
lin-46(lf) appears to be a property of a strain lacking lin-46, which, as described below, are likely to be null by molecular criteria, it seems unlikely that this phenotype is due to molecular lesions alone, rather than a property of the molecular lesions themselves. Specifically, 32% of mutant alleles of lin-46 showed cold-sensitivity for this defect (Table 1, line 7). Because 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(lf) showed 32% penetrance at 20°C, 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 differentially 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 7). 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 differentially sensitive to its level of activity.

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-41 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(lf) 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 differentially sensitive to its level of activity.

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

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

*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.
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
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.

**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). Furthermore, 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
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.

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(n797s) 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(l0) background at 20°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 aid 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.

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