

The *lin-41* RBCC Gene Acts in the *C. elegans* Heterochronic Pathway between the *let-7* Regulatory RNA and the LIN-29 Transcription Factor

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Summary

Null mutations in the *C. elegans* heterochronic gene *lin-41* cause precocious expression of adult fates at larval stages. Increased *lin-41* activity causes the opposite phenotype, reiteration of larval fates. *let-7* mutations cause similar reiterated heterochronic phenotypes that are suppressed by *lin-41* mutations, showing that *lin-41* is negatively regulated by *let-7*. *lin-41* negatively regulates the timing of LIN-29 adult specification transcription factor expression. *lin-41* encodes an RBCC protein, and two elements in the *lin-41* 3' UTR are complementary to the 21 nucleotide *let-7* regulatory RNA. A *lin-41::GFP* fusion gene is downregulated in the tissues affected by *lin-41* at the time that the *let-7* regulatory RNA is upregulated. We suggest that late larval activation of *let-7* RNA expression downregulates LIN-41 to relieve inhibition of *lin-29*.

Introduction

Heterochronic mutations have revealed a dedicated pathway that coordinates the temporal sequence of cell division and differentiation in many *C. elegans* cell types and tissues. These mutations cause cells to adopt fates normally expressed at earlier or later stages of development. The heterochronic genes *lin-4*, *lin-14*, *lin-28*, *lin-42*, and *lin-29* form a genetic pathway to control stage-specific patterns of cell division and differentiation (Ambros and Horvitz, 1984; Ambros and Moss, 1993; Slack and Ruvkun, 1997). The upstream genes in the pathway regulate the normally L4 stage expression of the LIN-29 transcription factor that activates adult-specific cell differentiation. Loss-of-function mutations in

the heterochronic genes *lin-14*, *lin-28*, and *lin-42* cause precocious expression of LIN-29 during the L3 stage, whereas loss-of-function mutations in the gene *lin-4* abolish upregulation of *lin-29* (Bettinger et al., 1996).

The *lin-14* gene regulates cell fate decisions during the early larval stages by generating a temporal gradient of LIN-14 protein during the L1 stage (Ruvkun and Giusto, 1989). High LIN-14 protein levels during the L1 stage specify L1 fates, and low LIN-14 levels specify L2 fates (Ambros and Horvitz, 1987). Because LIN-14 is a nuclear protein, it is likely to regulate the expression of genes that mediate L1-specific versus L2-specific patterns of cell lineage. The downregulation of LIN-14 is mediated during the late L1 stage (Feinbaum and Ambros, 1999) by the *lin-4* regulatory RNA (Lee et al., 1993), which is complementary to several sites in the *lin-14* 3' UTR (Wightman et al., 1991, 1993; Ha et al., 1996).

The *lin-14* gene functions in temporal patterning during the L1 stage, long before the subsequent L4 stage upregulation of LIN-29, which controls the expression of adult-specific characters (Ambros and Horvitz, 1984). We screened genetically for novel heterochronic genes that act to couple activity of *lin-14* at the L1 stage to *lin-29* at the L4 stage. We sought such genes by genetic suppression of the heterochronic mutant phenotype of a relatively late-acting heterochronic mutant, *let-7*. *let-7* encodes a 21 nucleotide regulatory RNA that is expressed during the L3 and later stages and regulates temporal events at these late stages (Reinhart et al., 2000). *let-7* mutations cause reiterated late larval cell lineage transformations and interfere with normal temporal activation of LIN-29 expression (Reinhart et al., 2000). We identified seven *lin-41* alleles as genetic suppressors of the *let-7* retarded heterochronic and lethal phenotypes. Here we describe the molecular basis for the temporal patterning activity of the *lin-41* heterochronic gene. We present molecular and genetic evidence that *lin-41* acts in a genetic pathway of temporal control between the *let-7* regulatory RNA and the *lin-29* transcription factor.

Results

Isolation of Alleles of a Novel Heterochronic Gene, *lin-41*

let-7 mutations cause reiteration of L4 stage cell lineages (Reinhart et al., 2000) and a highly penetrant bursting from the vulva lethal phenotype at the L4/adult molt, presumably because of temporal misspecification of hypodermal tissues (Table 1) (Meneely and Herman, 1979; Reinhart et al., 2000). All previously identified precocious heterochronic mutations tested (*lin-14*, *lin-28*, and *lin-42*) partially suppress the retarded heterochronic (Reinhart et al., 2000) and the larval lethal phenotypes of *let-7* mutants (Table 1). The suppression of a lethal phenotype provides a powerful selection for new heterochronic mutants.

We performed F1 and F2 screens for mutations that

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Table 1. *lin-41* Mutations Suppress the Sterility and Heterochronic Phenotypes of *let-7* Mutants

Strain ^a	Percentage with Progeny	Avg. Number of Progeny	Percentage of Animals with Alae at the L3 Molt (#)	Percentage of Animals with Alae at the L4 Molt (#)
Wild-type	100	280 ^a	0 (40)	100 (14)
<i>let-7(n2853)</i> ^b	5	0	0 (20)	0 (20)
<i>lin-41(n2914)/+; let-7(n2853)</i>	100	64	ND	45 (22)
<i>lin-41(ma104)/+; let-7(n2853)</i>	100	64	ND	52 (21)
<i>let-7(mn112) unc-3(e151)</i>	7	0	0 (20)	0 (20)
<i>lin-41(n2914); let-7(mn112) unc-3(e151)</i>	0	0	ND	ND
<i>lin-41(ma104); let-7(mn112) unc-3(e151)</i>	95	19	0 (18)	70 (20)
<i>lin-41(mg185); let-7(mn112) unc-3(e151)</i>	90	25	ND	ND
<i>lin-41(mg184); let-7(mn112) unc-3(e151)</i>	100	47	16 (24)	89 (19)
<i>lin-41(ma104)</i>	100	30	54 (48)	100 (45)
<i>lin-41(ma104)/nDf24</i>	0	0	ND	ND
<i>lin-41(n2914)</i>	0	0	52 (62)	97 (35)
<i>lin-41(n2914)/nDf24</i>	0	0	ND	ND
<i>lin-14(n540 n536) let-7(mn112) unc-3</i>	90	14	44 (36)	70 (46)
<i>lin-28(n719); let-7(mn112) unc-3</i>	100	34	7 (60)	17 (58)
<i>lin-42(n1089); let-7(mn112) unc-3</i>	90	45	13 (66)	31 (86)
<i>lin-41(n2914)/unc-29 lin-11</i>	ND	ND	0 (15)	100 (25)
<i>nDf24/unc-13 lin-11</i>	ND	ND	0 (30)	100 (30)
<i>lin-41(ma104); lin-29(n333)</i>	ND	ND	0 (25)	0 (62)

^a All experiments were performed at 20°C, except those involving the temperature-sensitive *let-7(n2853)* allele, which were performed at 25°C. Twenty L3 animals were placed on separate plates, except for *let-7(mn112) unc-3* for which 60 animals were picked. Wild-type animals have an average brood of 280 (Byerly et al., 1976).

^b *n2853* animals were moved from 15°C to 25°C.
ND, not determined.

suppress the lethality of *let-7(n2853)* and identified 50 such mutations as detailed in Experimental Procedures. The F1 screen was expected to produce only dominant suppressor mutations, while the F2 screen was expected to produce both dominant and recessive suppressor mutations. One class of mutants, represented by seven independent isolates, was distinguished because they showed semidominant suppression of the lethality (Figure 1, Table 1, and data not shown) and the heterochronic defects (Table 1 and data not shown) of *let-7* mutations. We also performed a screen for new precocious heterochronic mutants in a strain that has been shown to cause high frequencies of transposon insertion and point mutations (Collins et al., 1987) and isolated one mutant, *ma104*. Genetic mapping and complementation tests showed that these mutations are all alleles of the same gene, *lin-41* (see below).

***lin-41* Mutants Display a Precocious Heterochronic Defect**

The *lin-41* mutations caused recessive precocious heterochronic expression of an adult-specific hypodermal cell fate (Table 1). In wild-type animals, the lateral hypodermal seam cells divide with a stem cell-like pattern during the L1, L2, L3, and L4 stages before exiting the cell cycle and terminally differentiating after the L4 molt—a process termed the larval-to-adult (L/A) switch. These cells secrete a cuticular structure known as lateral alae at the adult stage (Figure 1). In mutants homozygous for the *lin-41(n2914)* null allele (see below) as well as in *lin-41(ma104)* mutants, 50% of animals exhibited precocious terminal differentiation of the seam cells at the L3 molt (Table 1). An average of five seam cells (out of a possible 15) displayed the precocious phenotype in *lin-41(n2914)* animals (*n* = 15 animals) at the L4 stage. We examined lateral hypodermal seam cells (V cells and

descendants) at different stages during development and found that seam cell development in hermaphrodites proceeded normally until the L3 molt, when some seam cells precociously underwent the L/A switch (Figure 2).

lin-41(n2914) animals carrying a *lin-41(+)* transgenic array (C12C8 in Figure 3A) and presumably containing a higher than normal dosage of *lin-41*, showed a retarded phenotype in the hypodermis (59%, *n* = 53 animals), while control animals did not (data not shown). In retarded animals, most hypodermal seam cells failed to execute the L/A switch (i.e., exit the cell cycle and terminally differentiate) at the L4 molt and instead reiterated the larval fate and divided again (Figures 2B–2E). In some cases, the animals died by bursting through the vulva in a manner that resembled the lethality caused by loss of *let-7* function (Reinhart et al., 2000). A similar retarded phenotype was observed in wild-type animals carrying a full-length *lin-41* gene fused to the green fluorescent protein (GFP) (see Figure 5D legend), while control animals carrying just the injection marker did not display a retarded phenotype (data not shown). Thus, overexpression of *lin-41* caused a heterochronic defect opposite to that of animals missing *lin-41* gene activity, showing that LIN-41 is necessary and sufficient for the repression of adult-specific fates during earlier larval stages. *lin-41* may act as a genetic switch to regulate developmental timing in the hypodermis. These data also argue that *lin-41* is the major *let-7* regulated output for heterochronic patterning.

lin-41(n2914) and *lin-41(mg187)* behaved genetically as null alleles; both caused a complete recessive sterility as homozygotes or in trans to a deficiency that removes *lin-41*, *nDf24* (Table 1, data not shown). *lin-41(ma104)* did not behave as a null allele; *lin-41(ma104)* animals were fertile while *lin-41(ma104)/nDf24* animals had a

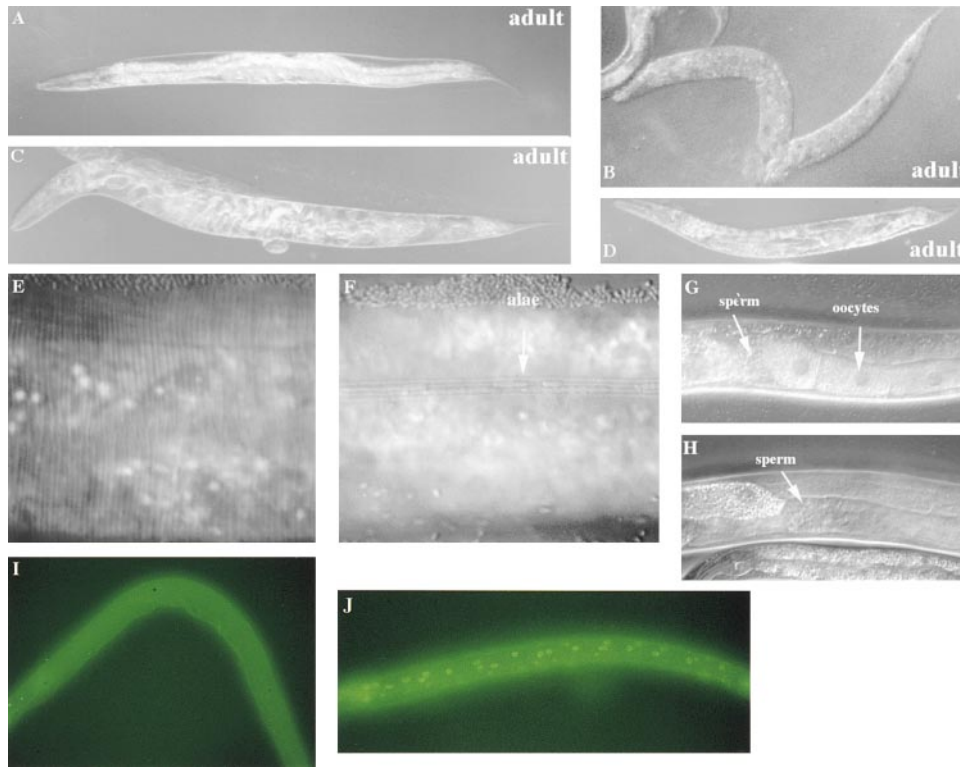


Figure 1. The *lin-41* Phenotype

(A–D) The *lin-41* phenotype (magnification 50 $\times$). (A) Wild-type adult. (B) *let-7(n2853)* adults die by bursting through the vulva before producing progeny. (C) A *lin-41(n2914)/+; let-7(n2853)* adult is viable, but shows eggs accumulating in the animal. (D) A *lin-41(n2914); let-7(n2853)* adult is viable, but sterile and slightly Dpy. (E) Lateral view of the adult cuticle from a *let-7(n2853)* mutant showing the lack of horizontal adult alae caused by the heterochronic defect (1000 $\times$). (F) Lateral view of the adult cuticle from a *lin-41(mg184); let-7(n2853)* animal showing restored adult alae (arrow). (G and H) *lin-41* mutants lack oocytes (magnification 400 $\times$). Wild-type adult (G) viewed by Nomarski optics showing one arm of the gonad with sperm and oocytes. *lin-41(n2914)* adult (H) gonad with sperm but no oocytes. (I and J) *lin-41* is required for proper timing of appearance of LIN-29 (magnification 630 $\times$). Wild-type L3 stage animals (I), which did not accumulate detectable levels of LIN-29 protein as viewed with antibodies to LIN-29. *lin-41(n2914)* L3 stage animal (J), which expressed high levels of LIN-29 protein, normally seen only at the L4 and adult stages. Anterior is to the left, and except for (J) ventral is down. In (J), ventral is toward the viewer.

more severe, sterile phenotype (Table 1). The classification of *lin-41(n2914)* as a null allele is supported by molecular analysis (see below). Observation of the gonads in *lin-41(n2914)* mutant animals using Nomarski optics revealed that sperm production appeared to be normal but that oocyte production was severely impaired (Figure 1). The defect in oocyte production is likely to be the cause of the sterility.

LIN-41 Is a Member of the RBCC Family

lin-41(n2914) maps to chromosome I just to the left of *mec-8*. (Figure 3, Experimental Procedures). Cosmids spanning the genetic region to the left of *mec-8* were tested for complementation of a *lin-41* mutation in transgenic lines. The sterility and precocious heterochronic phenotype of *lin-41(n2914)* animals were partially rescued by cosmid C12C8, but not by the neighboring overlapping cosmid F26H9 (Figure 3A). A 14.5 kb PCR product that includes only one identified gene, C12C8.3 (accession number EMBL Z81467) (Hodgkin et al., 1995), also rescued the *lin-41* phenotype (Figure 3A).

We identified mutations associated with *lin-41* alleles in C12C8.3. *lin-41(n2914)* is a deletion of 50 nucleotides (Figures 4B and 4C) that is predicted to cause a

frameshift that truncates the LIN-41 protein N-terminal to conserved regions and is predicted to produce a polypeptide of 138 amino acids instead of the normal products of 1143 and 1146 amino acids (see below). The genetic evidence that *lin-41(n2914)* is a null allele supports the prediction that this mutation truncates essential regions from LIN-41. The *lin-41(ma104)* allele has a Tc1 transposon inserted at amino acid 713. Five other *lin-41* mutations cause substitutions in the C-terminal conserved region of the protein (Figure 3 legend, Figure 4). *lin-41(mg187)* results in an alteration on a Southern blot probed with C12C8 (data not shown), suggesting that this mutation is a DNA rearrangement. We did not identify the endpoints of this lesion.

By cDNA analysis, *lin-41* is predicted to encode two nearly identical proteins, LIN-41A and LIN-41B, of 1143 and 1146 amino acids respectively, the result of an alternate splice acceptor site before the nonconserved region in exon 9 (Figure 3B) (accession numbers AF195610 and AF195611, respectively). LIN-41 is a member of a large family of RBCC (ring finger, B box, coiled coil) proteins (Figure 4A). Both LIN-41 products contain an N-terminal RING finger (Freemont, 1993) (a zinc-chelating domain thought to be involved in protein–protein

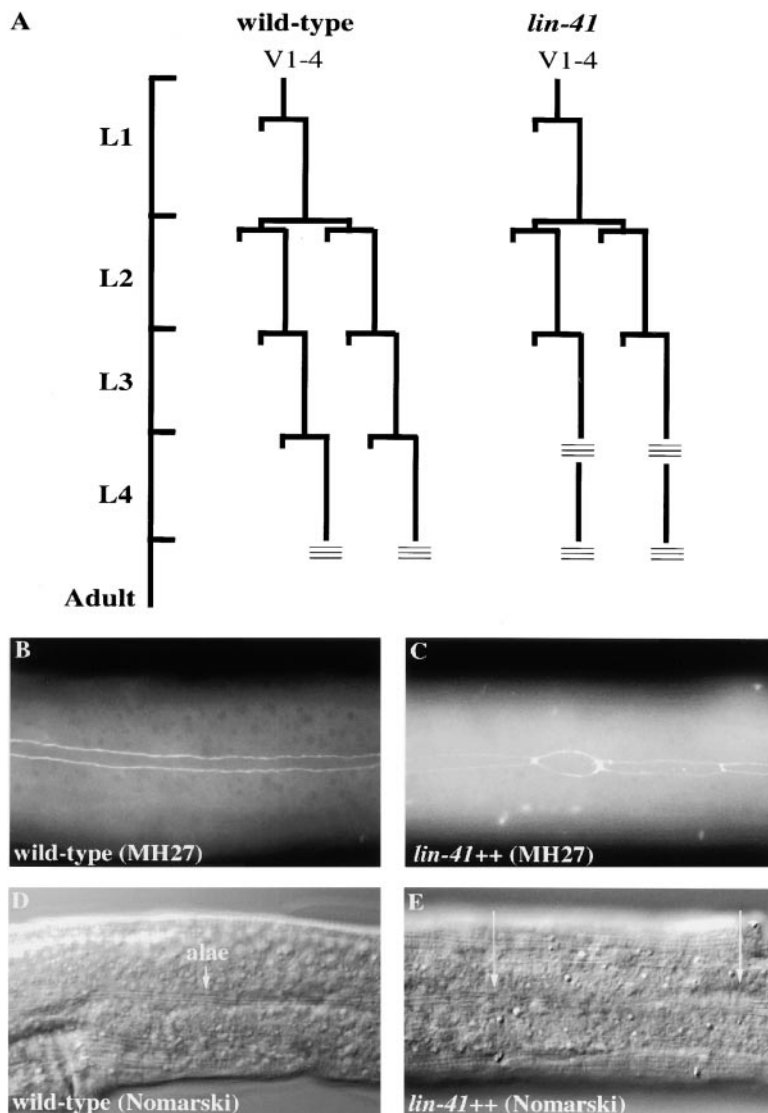


Figure 2. Cell Lineage Defects in *lin-41* Mutants

(A) Cell lineages of the lateral hypodermal V cells in wild-type and *lin-41(ma104)* animals. The vertical axis indicates the four postembryonic larval stages divided by molts. In wild-type animals, the V1–4 cells divide near the beginning of each larval stage until the final molt, when they terminally differentiate and secrete adult-specific cuticular alae (termed the L/A switch), indicated by the triple bars. In *lin-41(ma104)* animals, the V1–4 cells can precociously execute the L/A switch at the L3 molt. By anatomical observation, *lin-41(n2914)* null mutants displayed the same late cell lineage defect. Specifically, we synchronized *lin-41(n2914)/unc-29 lin-11* animals by hatching eggs in the absence of food, and the hypodermal seam cells of these animals (1/4 of which were predicted to be homozygous mutant for *lin-41*) were counted and found to be normal at early larval stages (# animal sides counted: mid-L1 [n = 99]; mid-L2 [n = 23]; mid-L3 [n = 19]). In addition, seam cells were also counted from homozygous *lin-41(n2914)* (mid-L4, n = 8 animal sides) and early adult (n = 6 animal sides) animals and were found to be normal. *lin-41* mutants had a molting defect and sometimes failed in ecdysis at the final molt (data not shown). (B–D) *lin-41* overexpression causes a retarded phenotype. In wild-type animals at the L4/adult molt, seam cells terminally differentiate, fuse (B) as observed with MH27 antibody, which recognizes a cell junction molecule (Labouesse, 1997), and secrete cuticular alae (D) indicated by a small arrow. In animals with increased *lin-41(+)* gene dosage (*lin-41++*), most seam cells reiterate the L4 stage larval fate at this time, divide again, and fail to fuse (B), or produce adult alae (E). A break in the alae is indicated between two large arrows. Magnification is 1000 $\times$.

and/or protein–nucleic acid interactions), a pair of B boxes (Freemont, 1993) (additional zinc chelating domains), and a coiled coil domain (Figure 3B). LIN-41 contains a C-terminal domain that consists of six copies of a 44 amino acid repeat (Figures 4A and 4B). This domain is often found associated with RBCC domains, and we have called this domain the NHL domain since we first recognized this domain in the NCL-1 (Frank and Roth, 1998), HT2A (Fridell et al., 1995), and LIN-41 proteins (Slack and Ruvkun, 1998). Five *lin-41* alleles are missense mutations in conserved residues in the repeats, suggesting that these repeats are important for LIN-41 function (Figure 4B). The NHL domains of RBCC proteins mediate protein–protein interactions (Fridell et al., 1995; Slack and Ruvkun, 1998; El-Husseini and Vincent, 1999). This domain as well as the other LIN-41 domains may mediate protein–protein interactions.

Possible LIN-41 orthologs were detected in vertebrate EST and *Drosophila* genomic DNA sequence databases. A mouse gene defined by two partial cDNA clones (accession numbers AA930787 and AA919390) is the closest relative to LIN-41 (44% identity; 61% similarity; p

value 3×10^{-87} over 495 amino acids, with most of the conservation in the NHL domain) (Figure 4B), and LIN-41 is the closest relative in any database to the protein encoded by these ESTs. Similarly, a zebrafish EST (accession number AI794385) encodes a protein with 47% identity and 64% similarity (p value 5×10^{-31}) to LIN-41 over 138 amino acids. A possible *Drosophila* LIN-41 ortholog with high conservation in the NHL domain (48% identity; 67% similarity over 183 amino acids; TBLASTN p value 5×10^{-62}) as well as less dramatic but significant conservation in the RING finger (27% identity; 50% similarity over 51 amino acids; TBLASTN p value 2.7×10^{-5}), B box 1 (33% identity; 36% similarity over 112 amino acids; TBLASTN p value 7×10^{-6}), and B box 2 and coiled coil (24% identity; 46% similarity over 174 amino acids; TBLASTN p value 7.1×10^{-6}) domains was detected in the translated sequence of a *Drosophila* genomic BAC clone (accession number AC004280). The major region conserved between the vertebrate partial cDNAs, the *Drosophila* gene, and LIN-41 is the NHL domain, consistent with many of the *C. elegans lin-41* mutations mapping to this domain. In addition, both

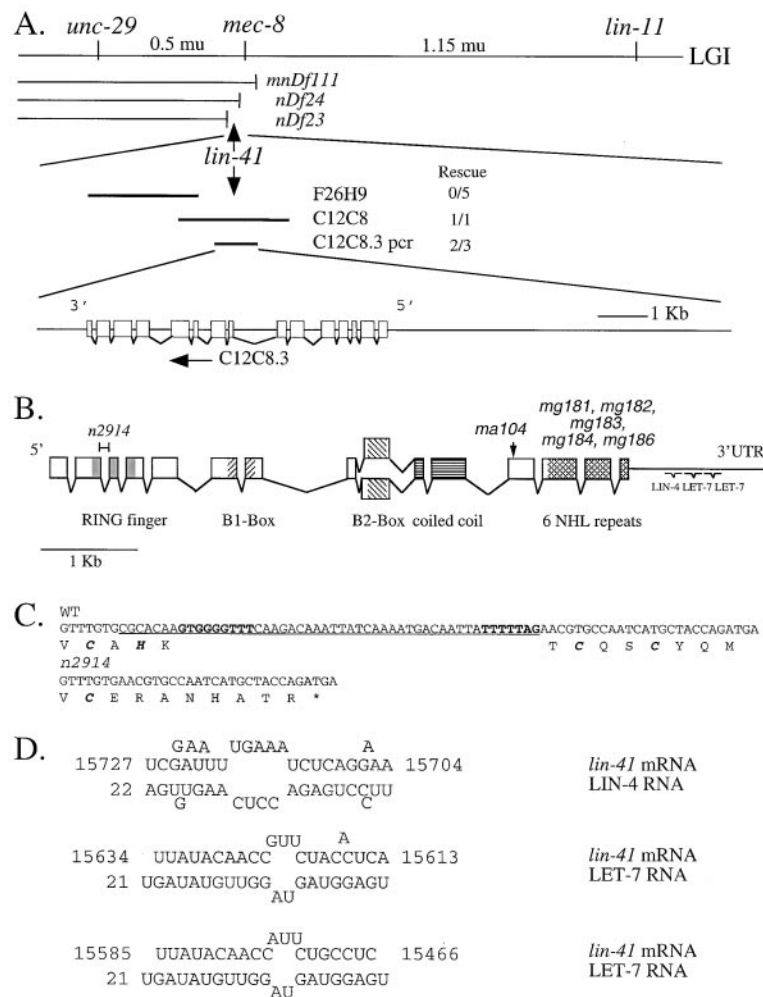


Figure 3. The *lin-41* Genetic Region

(A) *lin-41* was mapped to LG I between the right end points of deficiencies *nDf24* and *nDf23*. Cosmid C12C8 and a 14.5 kb PCR product containing only one identified gene, C12C8.3, partially rescued the sterile and heterochronic phenotype of *lin-41*—85% of C12C8 transgene-containing *lin-41(n2914)* animals from one line were rescued, and this line was fertile, although with a reduced brood size relative to wild type (data not shown). The neighboring cosmid F26H9 did not rescue. (B) The intron/exon structure of *lin-41* and notable domains. An alternative splice acceptor site results in two transcripts: *lin-41A* (above) is 12 nucleotides shorter than *lin-41B* (below). The relative positions of *lin-41* alleles are shown: *n2914* is a deletion from 21767 to 21817, *ma104* is a transposon insertion (Tc1) at position 17754, and the remaining mutations cause substitutions in the NHL domain (*mg181*, C-to-T at position 16985 [R-to-W]; *mg182*, *mg183*, G-to-A at position 16649 [R-to-Q]; *mg184*, C-to-T at position 17180 [R-to-C]; *mg186*, C-to-T at position 16849 [T-to-I]; numbering is based on cosmid C12C8—accession number EMBL Z81467). The RING finger, B box, coiled coil, and NHL domains are indicated. Potential binding sites for the *lin-4* and *let-7* RNAs are shown in the 3'UTR. (C) The molecular lesion in the *lin-41(n2914)* null allele. In the *lin-41(n2914)* mutant, the wild-type sequence underlined is deleted, leading to a frameshift predicted to result in a premature stop codon. The bold residues indicate splice donor and acceptor sites of an intron in the RING finger region of *lin-41*. The Cys and His residues that make up part of the RING finger are in italics. (D) Potential RNA:RNA duplexes between *lin-41* and *let-7* and between *lin-41* and *lin-4*, as identified by a combination of manual searching and computer analysis using the FOLDRNA program of the GCG software package (Devreux et al., 1984).

the possible *Drosophila* and vertebrate LIN-41 orthologs (but not more distantly related RBCC proteins such as KIAA0517) conserve a very similar pattern of amino acid residues on the NHL domain, also endorsing their assignment as orthologs.

While these possible LIN-41 orthologs have unknown functions and biochemical mechanisms, other RBCC superfamily members suggest possible mechanisms of *lin-41* gene function. The RBCC protein 52 kDa SS-A/Ro lupus autoantigen is cytoplasmic and associates with RNA (Chan et al., 1991). The RBCC protein HT2A binds to the HIV Tat transactivator, itself an RNA binding protein (Fridell et al., 1995). The *C. elegans* NCL-1 RBCC protein is a cytoplasmic protein that regulates ribosomal RNA and rRNA synthesis (Frank and Roth, 1998) through an unknown mechanism. Thus, LIN-41 is a potential RNA binding protein that could directly bind to, for example, the *lin-29* mRNA (see below). In contrast, the RBCC mammalian oncoproteins PML (de The et al., 1991; Kakiyama et al., 1991; Wang et al., 1998) and TIF1 (Le Dourarin et al., 1995) are nuclear factors that are thought to regulate transcription. Recently, RING domains have been found in subunits of the E3 ubiquitin ligase (Joazeiro et al., 1999; Seol et al., 1999; Tyers and Willems, 1999),

suggesting an alternative model in which LIN-41 may regulate LIN-29 stability.

lin-41 Is Required for Temporal Regulation of LIN-29 Expression

The expression of the LIN-29 zinc finger transcription factor is normally activated at the L4 and later stages in hypodermal cells and is necessary for the L4-to-adult stage differentiation of these cells (Ambros and Horvitz, 1984; Bettinger et al., 1996). Our genetic and molecular epistasis studies support a model in which *lin-41* negatively regulates *lin-29* to control the cessation of the molting cycle and the switch to adult hypodermal cell fates. The precocious heterochronic defect of *lin-41* mutants required a wild-type copy of the *lin-29* gene, since *lin-41*; *lin-29* double mutants did not display a precocious defect and rather displayed the retarded defect characteristic of *lin-29* mutants (Table 1). This finding suggests that *lin-41* negatively regulates *lin-29* in the hypodermis.

Molecular studies also favor the model in which *lin-41* negatively regulates *lin-29*. LIN-29 protein was precociously expressed in *lin-41(n2914)* mutants at the L3 stage and, in some animals, at the L2 stage (Figures

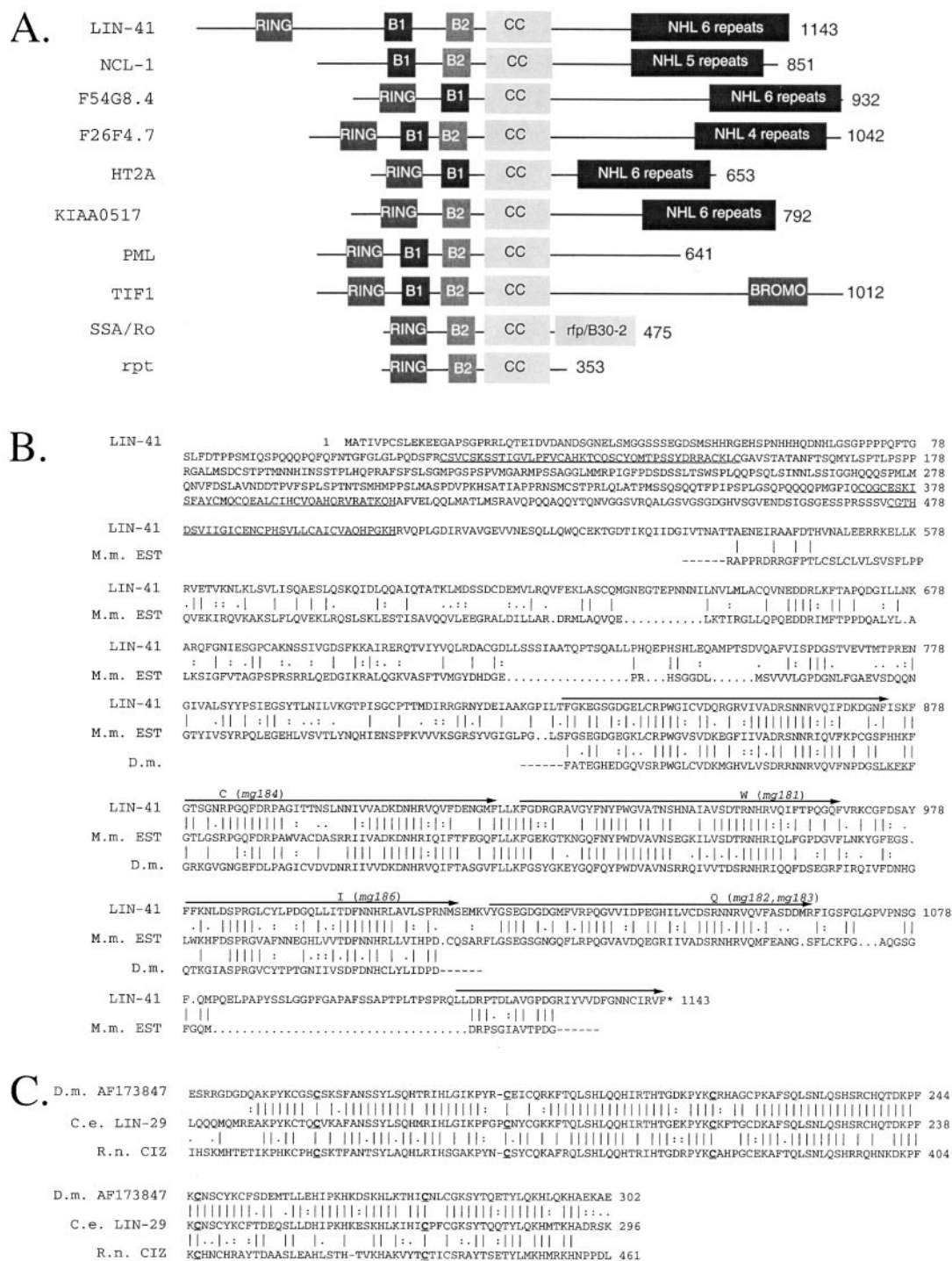


Figure 4. LIN-41 Relatives

(A) LIN-41 is a divergent member of the RBCC family. (B) The amino acid sequence of LIN-41A in an alignment with the partial sequence of both a mouse gene (corresponding to the ESTs AA930787 and AA919390) and a predicted *Drosophila* gene (AC004280). The RING finger, B1 box and B2 box regions are underlined in succession. The NHL repeats are indicated by arrows; "-" indicates that the sequence is likely to continue but is not known at this time; "." indicates gaps in the alignment; "*" indicates the end of an amino acid sequence. The amino acid changes caused by mutations in five *lin-41* mutants are indicated. *mg186* may disrupt a potential Ser/Thr phosphorylation site. TBLASTN against GenBank with the partial mouse gene shown above gave the following hits: LIN-41 p value 3×10^{-74} ; *Drosophila* AC004280 9×10^{-60} ; KIAA0517 1×10^{-41} . Accession numbers NCL-1 (GenBank AF047027), F54G8.4 (SWISS-PROT Q03601), F26F4.7 (GenBank U12964), HT2A (SWISS-PROT Q13049), KIAA0517 (DDBJ AB011089), PML (GenBank S50913), TIF1 (GenBank AF009353), SSA/Ro (GenBank M62800), and rpt (SWISS-PROT P15533). (C) Alignment of *C. elegans* LIN-29 zinc finger region with one of 2 possible *Drosophila* LIN-29 proteins (GenBank accession # AF173847 p value 3.8×10^{-67}) and Rat CIZ (DBJ AB019281 p value 3×10^{-49}). The other possible *Drosophila* LIN-29 homologue

1I and 1J). This finding suggests that the precocious phenotype of *lin-41(lf)* animals is the result of precocious *lin-29* activity, and hence the normal role of *lin-41* is to help restrict *lin-29* activity to the L4.

Expression Pattern of *lin-41*

To determine which cells express *lin-41*, we fused the green fluorescent protein (GFP) gene to the N terminus of the full-length *lin-41* gene (Figure 5C) and introduced this construct into wild-type *C. elegans*. This construct rescued a *lin-41* mutant and caused a gain-of-function phenotype in wild-type animals (Figure 5 legend), suggesting that the construct encoded a functional product. LIN-41/GFP expression was observed in most *C. elegans* neurons (Figure 5A), in body wall and pharyngeal muscles, and in the lateral hypodermal seam cells (Figure 5B). LIN-41/GFP was predominantly cytoplasmic. In neurons, muscle, and pharyngeal cells, LIN-41/GFP was expressed at all stages, from late embryogenesis until adulthood. In hypodermal seam cells, LIN-41/GFP expression faded during the L4 stage (Figure 5D) at a time when genetic analysis suggests that activation of the *let-7* regulatory RNA inhibits *lin-41* gene activity. Molecular evidence favors a direct role for *let-7* RNA in regulation of *lin-41* (see below).

LIN-41/GFP was also expressed in the somatic gonad throughout postembryonic development, and was expressed in the spermatheca, the distal tip cells, and certain uterine cells in late larval and adult animals (data not shown). The somatic gonad might be the site of action of LIN-41 in promoting oocyte development (Figure 1).

The 3'UTR of *lin-41* Is Sufficient for Downregulation of *lin-41*

let-7 encodes a small regulatory RNA that is necessary for upregulation of *lin-29* expression and for specification of adult stage cell fates (Reinhart et al., 2000). *let-7* expression begins just prior to the L4 stage, when *lin-41* is downregulated. Given that *lin-41* alleles were identified as suppressors of *let-7* alleles and that two zygotic doses of *lin-41* are necessary for the *let-7* lethality and retarded defects, we postulated that the *let-7* RNA may directly regulate *lin-41*. We identified two sequences in the *lin-41* 3'UTR complementary to the *let-7* RNA (Figure 3D). The *lin-41* 3'UTR was placed 3' to the *E. coli lac-Z* gene driven by the hypodermally expressed *col-10* promoter. This reporter gene was temporally regulated in a similar manner to the *lin-41* gene itself. β -galactosidase activity was observed in larval animals but was mostly absent in adult animals (Figure 5C). The adult stage downregulation of the *col-10/lacZ* fusion gene was dependent on the *lin-41* 3'UTR, because a control fusion gene bearing the nonheterochronic gene *unc-54* 3'UTR was expressed at all stages including the adult stage (Wightman et al., 1993). *let-7* and the *let-7* complementary sites in the *lin-41* 3'UTR were required for the downregulation (Reinhart et al.,

2000). These experiments show that the *let-7* complementary sites in the *lin-41* 3'UTR mediate the downregulation of LIN-41 during the L4 and later stages in the hypodermis.

The heterochronic gene *lin-4* also encodes a small RNA that binds to complementary sequences in the 3'UTR of the mRNAs of *lin-14* and *lin-28* to downregulate their expression. The *lin-41* 3'UTR also contains a site with complementarity to the *lin-4* RNA (Figure 3D). However, *lin-4* may not play a significant role in regulating *lin-41*, as the *lin-4(lf)* retarded phenotype was not affected by removal of *lin-41*. Specifically, the *lin-41(lf); lin-4(lf)* double mutant animals displayed the *lin-4* heterochronic phenotype in the hypodermis and vulva (data not shown).

Discussion

Based upon the *lin-41* null phenotype and the epistasis relationships among *lin-41*, *let-7*, and *lin-29* mutations, we conclude that *lin-41* functions in temporal patterning during late larval stages, and that *lin-41* acts downstream of the *let-7* regulatory RNA and upstream of the LIN-29 transcription factor. Loss-of-function mutations in *lin-41* cause precocious terminal differentiation of hypodermal seam cells one larval stage earlier than normal, while overexpression of *lin-41* causes terminal differentiation to be delayed. Thus, *lin-41* is necessary and sufficient to prevent terminal differentiation of seam cells and to promote their cell division.

Consistent with this proposed regulatory role, *lin-41* encodes a member of the RBCC family of regulatory proteins, some of which have been implicated in RNA binding or control of RNA function. For example, the RBCC family member 52 kDa SS-A/Ro, an autoantigen in Sjogren's syndrome, is cytoplasmically localized (Pourmand et al., 1998) and binds to a particular small RNA (Chan et al., 1991), while the *C. elegans* NCL-1 RBCC protein regulates nucleolus size and rRNA abundance (Frank and Roth, 1998). Additionally, the MDM2 oncoprotein (with a RING finger but no BCC domain) binds RNA directly through the RING finger domain (Elenbaas et al., 1996). The biochemical assignment of RBCC protein function to RNA regulation, plus our finding that a *lin-41::GFP* fusion gene is cytoplasmically localized, suggest that LIN-41 may regulate the translation of other heterochronic genes. LIN-41 may have additional targets in the gonad that control fertility (see below). RING finger proteins are also important subunits of the E3 ubiquitin ligase (Joazeiro et al., 1999; Seol et al., 1999; Tyers and Willems, 1999).

The wild-type activity of LIN-29 is required for the precocious defect of *lin-41* mutations (Table 1) and the time of LIN-29 protein expression is regulated by *lin-41* (Figure 1), suggesting that the *lin-29* mRNA may be a direct target of *lin-41*. During wild-type development, LIN-29 protein is first detected during the L4 stage, when it specifies the adult-specific expression of particular

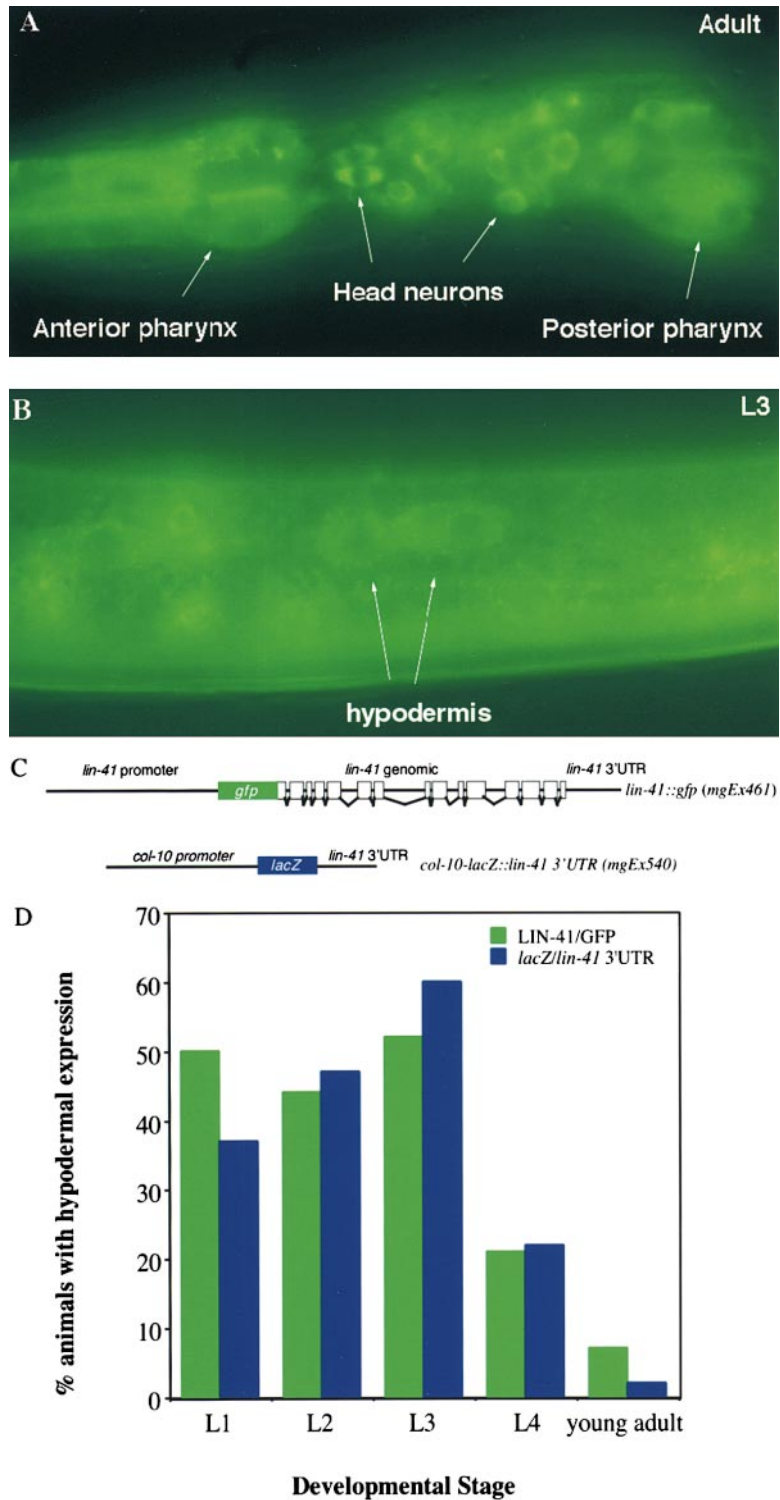


Figure 5. Expression Pattern of LIN-41/GFP (*mgEx461*) in Wild-Type Animals

(A) Expression in the cytoplasm of pharyngeal muscles and head neurons of an adult animal. (B) Expression in lateral hypodermal seam cells in an L3 animal (magnification 1000 $\times$). (C) Schematic of *lin-41::gfp* and *col-10::lacZlin-41* 3'UTR constructs. Not shown to scale. (D) LIN-41/GFP is temporally regulated in the seam cells, and *lin-41* expression is controlled by the 3'UTR. Green bars show the percentage of wild-type animals with lateral hypodermal LIN-41/GFP expression (# of animals scored: L1 [n = 12], L2 [n = 25], L3 [n = 31], L4 [n = 28], early adult [n = 28]). Not every seam cell expressed LIN-41/GFP. All of the adults expressing LIN-41/GFP in the lateral hypodermis displayed a retarded heterochronic phenotype. Blue bars show the percentage of wild-type animals displaying β -galactosidase expression from a *col-10/lacZ/lin-41* 3'UTR (*mgEx540*) construct in the hypodermis (# of animals scored: L1 [n = 19], L2 [n = 42], L3 [n = 25], L4 [n = 18], early adult [n = 73]).

collagen genes, the cessation of the molting cycle, and fusion rather than division of hypodermal seam cells (Ambros and Horvitz, 1984). However, the *lin-29* mRNA accumulates much earlier, starting at the L2 stage (Rougvie and Ambros, 1995; Bettinger et al., 1996). Thus, during normal development, the translation of *lin-29* mRNA appears to be negatively regulated until the L4

stage, restricting the time of the L4-to-adult transition to the time of LIN-29 protein expression. An attractive model is that LIN-41 directly binds to the *lin-29* mRNA and inhibits its translation during early larval stages (Figure 6). LIN-41 contains domains that are implicated in RNA binding, and is expressed in the correct cellular compartment and at the correct time (L2 and L3 stage)

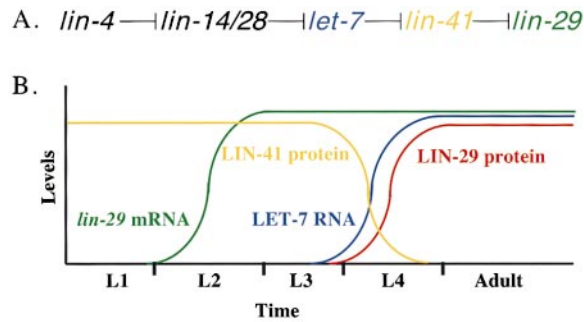


Figure 6. Model of Heterochronic Gene Activities in the Hypodermis (A) Genetic data support a model whereby the heterochronic gene *let-7* negatively regulates *lin-41*, which in turn negatively regulates *lin-29*. *let-7* is likely to respond to the earlier action of *lin-14/lin-28*. The simplest interpretation of the data is consistent with a linear pathway as shown. (B) *lin-29* mRNA appears during the L2 stage, but LIN-29 protein is first observed during the L4 stage. Molecular data suggest that *lin-41* activity is required for the post-transcriptional repression of *lin-29* during the L2 and L3 stages. *lin-41* expression may itself be translationally regulated by the appearance of the *let-7* RNA. *lin-41* and *lin-29* are represented in the other animals, raising the possibility that the mechanism of temporal patterning may be conserved.

to function in this role. The precocious heterochronic *lin-41* mutant phenotype may be caused by a loss of the normal *lin-41* mediated repression of *lin-29* mRNA translation during the L2 and L3 stages. The RING finger of LIN-41 suggests an alternative model that it may regulate LIN-29 stability. Although *lin-29* is expressed in the L2 stage in *lin-41(lf)* animals, there is no adult alae formation at the L2 molt. This suggests that either *lin-29* is not sufficient for adult fates in the L2, and/or that *lin-29* is regulated posttranslationally.

lin-41 is not only necessary for the earlier repression of LIN-29 expression, but we speculate that it is also sufficient to induce that repression at later stages, since high *lin-41* gene dosage causes a delay in the larval-to-adult switch. Thus, the downregulation of *lin-41* gene activity during the L4-to-adult transition may be critical to the upregulation of *lin-29* gene activity. A likely trigger for *lin-41* downregulation during the L4 stage, which we observed in the hypodermis with a *lin-41::GFP* fusion gene (Figures 5 and 6), is the upregulation of the *let-7* regulatory RNA during the L3 and L4 stages. *lin-41* is a major target of *let-7* regulation; *lin-41* mutations strongly suppressed the retarded heterochronic and lethal phenotypes of *let-7* mutants, and high *lin-41* gene dosage caused the same heterochronic and lethal phenotypes as did loss of *let-7*. *let-7* encodes a small RNA that is expressed beginning during the L3 stage and functions during the L4 stage to control indirectly the translation of the LIN-29 protein, which in turn regulates the switch to adult hypodermal fates (Reinhart et al., 2000). Consistent with a direct regulation of *lin-41* by *let-7* is our finding of *let-7* complementary sequences in the *lin-41* 3'UTR, suggesting that *let-7* directly negatively regulates *lin-41* by binding to the 3'UTR of *lin-41*. The time of *let-7* expression and function appears to be just before the time of LIN-41 protein downregulation in the hypodermis and function during the L4-to-adult molt.

The *lin-41* 3'UTR was sufficient to downregulate a heterologous reporter gene (Figure 5), and we found that *let-7* and the *let-7* complementary sequences in the *lin-41* 3'UTR were required for this downregulation (Reinhart et al., 2000). These observations strongly suggest that *let-7* functions to bind directly to the 3'UTR of *lin-41* mRNA and inhibit *lin-41* expression. Thus, *lin-41* transduces temporal information from a regulatory RNA to a transcriptional output, LIN-29.

We propose that in a *let-7* mutant, LIN-41 protein is misexpressed in time, and that this misexpression causes the *let-7* heterochronic defects and lethality. Since animals heterozygous for the *lin-41* null mutation in trans to a wild-type allele can potentially suppress the heterochronic and lethal phenotypes of a *let-7* null mutant, *lin-41* is dosage-sensitive for the retardation of *lin-29* gene action in the *let-7* mutant. This is consistent with the hypothesis that LIN-41 has a structural or stoichiometric function as opposed to a catalytic function (Snustad, 1968). Perhaps the concentration of LIN-41 relative to a threshold is critical, and the role of *let-7* is to repress LIN-41 to below-threshold levels.

lin-41 is unlikely to be the sole input to *lin-29* temporal regulation. The precocious phenotype of *lin-41* null mutant animals is neither 100% penetrant nor 100% expressive; not all animals exhibit a precocious phenotype and in those animals that do, not every seam cell expresses the precocious L/A switch defect. This observation suggests that additional genes can partially substitute for LIN-41 function. For example, there may be other *let-7* regulatory outputs. In addition, there are three RBCC-NHL proteins predicted from the *C. elegans* genome sequence (Figure 5A) that may function in a partially redundant manner to provide additional temporal inputs to *lin-29*.

The heterochronic pathway involves at least three cases of posttranscriptional regulation. *lin-14* and *lin-28* messenger RNAs are expressed at all stages of post-embryonic development, yet the proteins are expressed only at specific times (Wightman et al., 1993; Moss et al., 1997). The *lin-4* small RNA with sequence complementarity to the 3'UTRs of *lin-14*, *lin-28*, and the *let-7* small RNA with complementarity to *lin-41* are thought to regulate translation of these mRNAs. Similarly, *lin-29* mRNA is present from the L2 stage, but translated in the hypodermis only during the L4 stage and later stages (Rougvie and Ambros, 1995), after the levels of LIN-41 RBCC protein decline.

The inhibition of translation also plays a role in the control of the *tra-2* and *fem-3* genes, which regulate the switch between sperm and oocyte cell fates in *C. elegans* (Goodwin et al., 1993; Zhang et al., 1997). Although we have not examined directly whether *lin-41* hermaphrodites are defective in the sperm/oocyte switch, the lack of oocytes is consistent with such a defect, and implies that LIN-41 could play a role in the translational regulation involved in this germline cell fate choice. LIN-41/GFP is expressed in multiple cell types in the somatic gonad, where it could act to affect this switch.

Of the known *C. elegans* heterochronic genes, *lin-41* (Figure 4B) and *lin-29* (Figure 4C) (Rougvie and Ambros, 1995) show the most extensive sequence similarity to genes from other phyla. The mammalian and *Drosophila*

genes with a high degree of sequence identity to *lin-41* and *lin-29* define possible orthologs that may have similar functions in temporal patterning. Time will tell whether any of these orthologs also play roles in the control of developmental timing.

Experimental Procedures

Phenotype Analysis

To observe the extent of alae formation in larval and adult stages, living animals were observed using Nomarski optics. L4 stage animals and young adults were identified by their gonadal development (Hirsh and Vanderslice, 1976), which is not affected by the heterochronic mutations used in this study (Ambros and Horvitz, 1984). The extent of alae formation was judged on a single side of each animal. Anti-LIN-29 antibody staining was performed as described (Bettinger et al., 1996). Animals were grown at 15°C prior to staining. Cell lineage analysis was performed as described (Sulston and Horvitz, 1977).

Isolation and Characterization of Novel Precocious Mutants

let-7(n2853ts) mutants were grown at the permissive temperature (15°C) and subjected to ethylmethane sulfonate (EMS) mutagenesis. In two separate screens, the F1 progeny (F1 screen for dominant suppressors) or F2 progeny (F2 screen for recessive suppressors), respectively, of mutagenized animals were raised at the nonpermissive temperature (25°C) and reproducing animals were picked from a background of *Let-7* lethal mutant animals. F1 screen: from 12,000 mutagenized haploid genomes, 10 independently isolated nonlethal animals were obtained. F2 screen: from approximately 10⁵ mutagenized haploid genomes, 40 independently isolated nonlethal animals were obtained. Additional spontaneously arising suppressor mutants were identified in the course of propagating unmutagenized *let-7(n2853)* animals. These mutations fall into a number of different classes: one class (*mg181*, *mg182*, *mg183*, *mg184*, *mg186*, *mg187*, and *n2914*), which shows dominant suppression of the *let-7* lethality, is the subject of this paper; the others will be described elsewhere (F. J. S., and G. R., unpublished data). *let-7(n2853)* animals heterozygous for the *lin-41* alleles, *mg187* and *n2914*, were slightly egg-laying defective (*Egl*) and were observed to segregate approximately 1/4 dumpy (*Dpy*) sterile (*Ste*) animals (*lin-41*; *let-7*), as well as 1/4 dead *Let-7* animals (*lin-41*(+); *let-7*), suggesting that the *mg187* and *n2914* mutations semidominantly suppressed the lethality of *let-7*, and that they displayed a recessive *Dpy Ste* phenotype. *mg181*, *mg182*, *mg183*, *mg184*, and *mg186* are not recessive sterile, but can also dominantly suppress *let-7* (data not shown). *lin-41(ma104)* was isolated from the mutator strain TR679 as a precocious heterochronic mutant.

Mapping of *lin-41* and Complementation Tests

lin-41(n2914) was mapped to the *unc-29 lin-11* interval on LGI by standard genetic tests: from *lin-41(n2914)/unc-29(e1072) lin-11(n1281)* animals, 8/10 *Lin-11* non-*Unc* recombinants segregated *Lin*(n2914) animals. *lin-41(ma104)* was shown to be linked to *unc-29* on LGI by standard genetic tests. *lin-41(ma104)* was shown to map to the right of *lin-28*: from *lin-41(ma104)/dpy-5 lin-28 1/45 Lin*(ma104) animals through *Dpy Non-Lin-28* recombinants, and 1/45 *Lin*(ma104) animals through *Dpy Lin-28* recombinants. This result suggests that *lin-41(ma104)* maps very close either to the left or to the right of *lin-28*.

The heterochronic and fertility defects of *lin-41* are recessive. Deficiencies *nDf30*, *mnDf112*, and *nDf24* failed to complement *lin-41* mutations for the *Dpy* sterile phenotype, but *nDf23* complements *lin-41(n2914)*. These data position *lin-41* to a very small region of the genome, between *aph-1* and *mec-8*. *lin-41(n2914)* failed to complement *lin-41(ma104)*, but complemented mutations in other genes in this region of the genetic map.

Cloning of *lin-41*

Cosmids were injected as pools into the gonads of *lin-41(n2914)/unc-29 lin-11* animals (at 5 ng/μl). As a transformation marker, 50 ng/μl of pKP13 (*goa-1::gfp* [Segalat et al., 1995]) was injected with

each pool. Pools were said to have rescued if transformed lines segregated non-*Dpy* non-*Ste* (i.e., non-*Lin-41*) animals that never segregated *Lin-11 Unc-29* animals. When *Dpy Ste* (*Lin-41*) animals were observed, these animals had lost the transformation array, indicated by loss of the GFP staining. A 14.5 kb PCR product that includes 7.4 kb of DNA upstream of the *lin-41* initiator methionine and the whole *lin-41* genomic region to the polyadenylation site was prepared with primers C12C8(26) (ACTGAATTCTTAAAAAAC AATTATTCC) and C12C8(27) (GAGTAAGTTGAGTGTCGAGTT AACG) and tested for rescue at 10 ng/μl.

Analysis of *lin-41* cDNA Sequence and Construction of *lin-41::gfp*

We obtained two cDNAs (yk20b11 [accession numbers D32693 D35405] and yk307c10 [accession number C67656]) corresponding to the 3' regions of *lin-41* from a *C. elegans* EST collection and completed the sequences of these clones. To obtain the *lin-41* 5' region, we performed PCR on the cDNA libraries RB1 and RB2 with a primer designed to the predicted ATG start codon of *lin-41*. We determined DNA sequences and found that four of five subcloned cDNAs contained the splice variation found in LIN-41A, while one had the splice variation found in LIN-41B. We obtained two mouse cDNAs corresponding to AA930787 and AA919390 from the IMAGE consortium and completed the sequencing of the cloned inserts. A full-length *lin-41::gfp* transgene that fuses GFP in frame to the very N terminus of LIN-41, containing 7.3 kb of upstream DNA, the *gfp* coding sequence, the *lin-41* genomic coding sequence, and the complete *lin-41* 3'UTR was generated by overlap extension PCR. DNA from the start codon of *lin-41* to position -7.3 kb was fused to GFP, and this was subsequently fused to the 7.1 kb *lin-41* genomic coding region and 3'UTR. This fusion gene rescued a *lin-41(ma104)* mutant. DNA was injected into the gonad of wild-type animals at 10 ng/μl with 100 ng/μl pRF4 as an injection marker. Oligonucleotide sequences are available upon request.

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Accession Codes

The accession numbers for the novel sequences reported in this paper, LIN-41A and LIN-41B, are AF195610 and AF195611, respectively.