Temporal regulation of *lin-14* by the antagonistic action of two other heterochronic genes, *lin-4* and *lin-28*

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Heterochronic genes form a regulatory pathway that controls the temporal sequence of the *Caenorhabditis elegans* postembryonic cell lineage. One of these genes, *lin-14*, encodes a nuclear protein that constitutes a temporal developmental switch. During wild-type development, *lin-14* protein is abundant during early larval stage 1 (L1) to specify L1-specific cell lineages but is nearly undetectable at L2 and later stages to specify L2-specific and later cell lineages. To determine the roles played by other genes in executing this temporal switch, we have analyzed how *lin-14* expression is regulated by other heterochronic genes. *lin-4* is required to down-regulate *lin-14* protein levels during the L1 stage, whereas *lin-28* positively regulates *lin-14* protein levels. The *lin-4* gene product is a candidate for interacting with the negative regulatory element in the 3'-untranslated region of *lin-14*. *lin-29* mutations do not affect *lin-14* protein levels, consistent with *lin-29* acting downstream of *lin-14*. Switching off *lin-14* expression during the L1 stage is not triggered by the passage of time per se but, rather, is normally dependent on feeding or the feeding-dependent initiation of postembryonic cell division.

[Key Words: *lin-14*; *lin-4*; *lin-28*; *lin-29*; heterochronic genes; *C. elegans*; developmental switch]

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Development in multicellular organisms is controlled by a network of genes that specify the temporal and spatial pattern of cell division and differentiation (Horvitz 1988; Ingham 1988). In *Caenorhabditis elegans*, heterochronic genes control the temporal identities of specific postembryonic cells: Mutations in these genes cause particular cells to adopt identities normally associated with cells at earlier or later stages of development (Chalfie et al. 1981; Ambros and Horvitz 1984). The heterochronic gene *lin-14* plays a major role in this control of temporal development: Gain-of-function (gf) mutations that inappropriately increase *lin-14* activity at late stages impose reiterated larval stage 1 (L1)-specific cell lineages at post-L1 stages, and mutations that decrease *lin-14* activity cause blast cells during the L1 stage to express precociously L2-specific cell lineages (Ambros and Horvitz 1984).

Genetic and molecular studies have shown that *lin-14* specifies cell identities through a temporal gradient of the *lin-14* nuclear protein (Ambros and Horvitz 1987; Ruvkun and Giusto 1989). *lin-14* gene activity and the level of *lin-14* protein is high in late embryos and L1 stage animals and decreases rapidly during mid-L1 stage to specify the switch from early to late identities of blast cells. Gain-of-function *lin-14* mutations cause inappropriately high levels of *lin-14* protein at all developmental stages (Ruvkun and Giusto 1989) which, in turn, cause reiterated execution of early cell lineages at late devel-

opmental stages. These mutations delete or rearrange negative regulatory sequences in the 3'-untranslated region (3' UTR) of the *lin-14* gene, resulting in the abnormal accumulation of *lin-14* protein late in development (Wightman et al., this issue). Loss-of-function (lf) *lin-14* mutations that produce no *lin-14* protein affect the same cell lineages but cause the opposite transformations in cell fate—late cell lineages are precociously executed at early developmental stages.

From genetic analyses several other heterochronic genes have been found to affect the temporal pattern of *C. elegans* development. The *lin-4(e912)* mutation, as *lin-14(gf)* mutations, causes reiteration of L1-specific lineages in many postembryonic cell lineages. *lin-28* mutations affect L2 and later lineages (Chalfie et al. 1981; Ambros and Horvitz 1984). *lin-29* controls the switch from L4 to adult stage hypodermal cell identities (Ambros 1989). *lin-4* and *lin-14* also regulate the stage at which the initiation of dauer larvae occurs, whereas *lin-28* and *lin-29* do not (Liu and Ambros 1989). This observation suggests that *lin-4* and *lin-14* could regulate more cell lineages or cell types than *lin-28* and *lin-29*. Genetic epistasis studies have ordered these genes into a pathway: *lin-4* negatively regulates *lin-14* and perhaps *lin-28*, either or both of which, in turn, negatively regulate *lin-29*.

On the basis of genetic analysis, *lin-4*, *lin-28*, and *lin-*

29 could participate either in the generation or the interpretation of the *lin-14* temporal switch. Here, we explore how the *lin-14* temporal switch is regulated by these other heterochronic genes. On the basis of comparison of the cellular and temporal expression pattern of *lin-14* in mutants and wild type, we find that *lin-28* and *lin-4* act antagonistically to regulate *lin-14*. Conversely, *lin-29* mutations do not affect *lin-14* levels; thus, *lin-29* is a candidate downstream gene that may be regulated by *lin-14*. We also explored how the down-regulation of *lin-14* protein is normally triggered. We find that the temporal down-regulation of *lin-14* protein levels is not initiated by the passage of time per se but, instead, depends on feeding or the initiation of the postembryonic lineage during the L1 stage.

Results

In wild-type animals, the *lin-14* protein is expressed at a high level in the nuclei of specific somatic cells of late stage embryos and early L1 larvae, but not at subsequent developmental stages of wild-type animals (Fig. 1a; Ruvkun and Giusto 1989). All late embryos and early- to mid-L1 stage animals show intense *lin-14* protein staining in hypodermal, intestinal, body wall muscle, nerve ring, and ventral nerve cord nuclei (Fig. 1a). During the late L1 stage, hypodermal and intestinal nuclei show little or no immunofluorescence, whereas neuronal and muscle cells show faint *lin-14* immunofluorescence (Ruvkun and Giusto 1989). By the L2 stage, *lin-14* protein is undetectable in most cell types and only weakly detectable in some neurons (Fig. 1a). Quantitation of immunoblots shows that the level of the *lin-14* protein relative to a pharyngeal myosin control decreases ≥ 25 -fold from L1 to L2 (B. Wightman and G. Ruvkun, in prep.).

The heterochronic genes *lin-4* and *lin-28* affect the temporal regulation of *lin-14*

To study the regulatory effects of the genetically interacting genes *lin-4*, *lin-28*, and *lin-29* on *lin-14*, we analyzed the temporal and cellular expression patterns of *lin-14* protein in these heterochronic mutants.

The *lin-4(e912)* mutant, as with *lin-14(gf)* mutants, displays a retarded phenotype with reiteration of L1-specific lineages at L2 and later stages (Chalfie et al. 1981). The *Lin-4* retarded phenotype is dependent on *lin-14* gene activity (Ambros 1989). These genetic results suggest that *lin-4* negatively regulates *lin-14*, although not necessarily at the level of gene expression. We examined the *lin-14* immunostaining pattern in *lin-4(e912)* to ascertain how *lin-4* regulates *lin-14*. The *lin-4(e912)* allele appears to be a null mutant based on genetic criteria (R. Feinbaum and V. Ambros, pers. comm.). Anti-*lin-14* antibodies detect *lin-14* protein in *lin-4* L1 animals in all nuclei that are detected in wild type. As judged by immunofluorescence intensity, the level of *lin-14* protein was similar to wild type at this stage. Unlike wild type, however, *lin-14* protein is detected in nuclei at L2, L3, L4, and adult stages (Fig. 1c). This expression of high *lin-14* protein levels after the L1 stage was very similar to that observed in the *lin-14(gf)* mutants, consistent with the similar lineage defects observed (Ambros and Horvitz 1984). As judged by immunofluorescence intensity, the level of *lin-14* protein at post-L1 stages in *lin-4(e912)* animals is about half that in the *lin-14(n355gf)* mutants (Fig. 1b) and similar to less severe *lin-14(n536gf)* mutants. As with the *lin-14(n536n540)* null mutant, no *lin-14* protein staining at any stage was observed in a *lin-4(e912)*; *lin-14(n536n540)* mutant, showing that the late stage staining in a *lin-4* mutant is caused by misexpression of the *lin-14* gene and not expression of an im-

Figure 1. In situ immunofluorescent detection of *lin-14* nuclear protein in wild-type and heterochronic mutants. Specimens were fixed and incubated with affinity-purified anti-*lin-14* antibodies, followed by a secondary fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit antibody and 4,6-diamido-2-phenylindole (DAPI) stained to visualize all nuclei. Immunofluorescent photographs were taken with FITC (F) filters to show *lin-14* protein staining or DAPI (D) filters to show all nuclei. Representative nuclei of a cell type are identified by arrows; (G) gonad; (H) hypoderm; (I) intestine; (M) body wall muscle; (N) nerve ring; (V) ventral cord neurons. Photomicrographs are generally lateral views, in one of two planes. If a hypodermal cell is pointed out, the plane of focus is lateral and large nuclei are all hypodermal (H) and small nuclei are neuronal (N). If intestinal (I) and ventral cord neurons (V) are labeled, the plane of focus is medial and all large nuclei are intestinal and the line of small nuclei ventrally are the ventral nerve cord and small dorsal nuclei are muscle. The head of the animal is identified in most photomicrographs by the intensely staining nerve ring nuclei (N). (a) (Top) FITC; wild-type early L1 stage (L1), late L1 stage (unlabeled), and L3 stage (L3) animals. Lateral plane showing bright *lin-14* protein staining in most nuclei at early L1, somewhat dimmer staining at late L1, and absence of staining at L3. (Bottom) DAPI; same animals with same plane of focus. We have observed very dim *lin-14* protein staining in some neuronal nuclei of L2 and later animals in some preparations (M. Finney, unpubl.). (b) (Top left) FITC; lateral view of *lin-14(n355gf)* mutant early L1 stage, showing intense staining of hypodermal, ventral nerve cord, and nerve ring nuclei. (Top right) FITC; ventral view of *lin-14(n355gf)* L2 stage, showing *lin-14* protein in the ventral cord neurons and in muscle nuclei. Both gain-of-function mutants show inappropriate accumulation of *lin-14* protein in L2 and later stages, although the *n536* allele is weaker than the *n355* allele and *lin-14* protein staining is consistently less intense in *lin-14(n536)* mutant animals. (c) (Left) FITC; *lin-4(e912)* L1 stage focused at the midline, showing *lin-14* protein staining in the nuclei of ventral cord neurons, intestine, hypoderm, and tail ganglion neurons. (Right) FITC; lateral view of *lin-4(e912)* L2 stage, showing *lin-14* protein in hypodermal and postdeirid neuronal nuclei. (d) (Top left) FITC; *lin-28(n719)* with three animals, two late stage embryos, and an early L1 stage. All animals show accumulation of *lin-14* protein in nerve ring, ventral cord, hypoderm, intestine, and muscle nuclei, like wild type at these early developmental stages. (Top middle) FITC; *lin-28(n719)* early L1 stage, showing a decrease in *lin-14* protein staining in the hypodermal and intestinal nuclei compared with neuronal and body wall muscle nuclei. (Top right) FITC; *lin-28(n719)* L2 stage, showing no *lin-14* protein staining. (e) (Top left) FITC; *lin-29(n333)* L1 stage that displays *lin-14* protein in the same nuclei as wild type. (Top right) FITC; *lin-29(n333)* L2 stage, showing no *lin-14* staining.

Figure 1. (See facing page for legend.)

munologically related protein (data not shown). These data show that *lin-4* regulates *lin-14* gene activity by controlling the level of the *lin-14* protein.

In contrast to *lin-4*, *lin-28* null mutations result in precocious expression of L3 or L4 stage-specific cell lineages in hypodermal blast cells during the L2 stage after a normal L1 stage-specific cell lineage (Ambros and Horvitz 1984). Genetic epistasis analysis showed that *lin-28* acts downstream of *lin-4* but could not determine whether *lin-28* acts upstream or downstream of *lin-14* (Ambros 1989).

To examine the regulatory effect of *lin-28* on *lin-14*, we tested the effect of a *lin-28(n719)* mutation on *lin-14* protein accumulation. By genetic criteria, the *lin-28(n719)* allele is a null mutant (V. Ambros, pers. comm.). By immunostaining, *lin-28(n719)* embryos show a pattern of *lin-14* protein accumulation very similar to wild type (Fig. 1d). In early L1 *lin-28(n719)* animals, *lin-14* nuclear staining is normal in the ventral cord neurons, body wall muscle, and nerve ring cells, but hypodermal and intestinal nuclei show much weaker staining than wild type (Fig. 1d). By mid-L1 stage, *lin-14* protein is very faint in all cell types of *lin-28(n719)* animals (data not shown). As in wild type, by the L2 stage *lin-14* protein is undetectable in this mutant in all cell types and lineages (Fig. 1d).

These data suggest that *lin-28* is not required for the initial expression of the *lin-14* gene but is necessary for the continued expression or persistence during the L1 stage of *lin-14* protein, particularly in hypodermal and intestinal cells.

lin-29 mutations do not affect the regulation of *lin-14*

lin-29 gene activity is necessary for a switch from L4 to adult hypodermal cell identities and, thus, only affects one of the temporal events regulated by *lin-14* (Ambros

and Horvitz 1984). However, the time at which the *lin-29*-mediated switch occurs is regulated by *lin-14*: The L4-to-adult switch never occurs in *lin-14(gf)* mutants, and this switch occurs one stage precociously in *lin-14(lf)* mutants (Ambros 1989). These genetic data predict that *lin-29* is regulated by *lin-14* so that mutations in *lin-29* should have no effect on *lin-14* regulation. Observation of the regulation of *lin-14* expression in a *lin-29* mutant confirmed this prediction. The *lin-29(n333)* mutant, which by genetic criteria appears to be null (V. Ambros, pers. comm.), displayed a *lin-14* staining pattern similar to wild type in L1 stage animals and, as in wild type, displayed no *lin-14* protein after L1 (Fig. 1e).

lin-28 regulates *lin-14* independently of *lin-4* and the *lin-14* negative regulatory element

Because *lin-4* and *lin-14(gf)* mutations result in higher levels of the *lin-14* protein at post-L1 stages while *lin-28* mutations have the opposite effect on regulation of *lin-14* protein level, we examined the temporal regulation of *lin-14* in *lin-28(n719)*; *lin-4(e912)* and *lin-28(n719)*; *lin-14(n536gf)* double mutants, both of which display a precocious phenotype as in *lin-28* single mutants. Immunofluorescent analyses of embryos and early L1 stage larvae of both double mutants showed normal *lin-14* protein staining (Fig. 2a,b). At L2 and later stages, the *lin-14* protein is undetectable, as observed in *lin-28(n719)* but unlike *lin-4* or *lin-14(gf)* mutants (Fig. 2a,b).

Thus, a functional *lin-28* gene product is necessary for the high level of *lin-14* protein normally observed at the L1 stage in wild-type animals and at all postembryonic stages in *lin-14(gf)* and *lin-4* mutants. These data show that the *lin-28*-mediated positive regulation of *lin-14* protein levels does not act via *lin-4* or via the regulatory element in the *lin-14* 3' UTR that is deleted in the *lin-14(n536gf)* mutant.

Figure 2. In situ immunofluorescent detection of *lin-14* nuclear protein in double mutants. (a) (Top left) FITC; *lin-4(e912)*; *lin-28(n719)*, showing an early L1 stage tenderly wrapped around an early embryo. *lin-14*-protein staining is not detectable in the early embryo, whereas the early L1 animal shows normal *lin-14* protein staining. (Top right) FITC; *lin-4*; *lin-28* L2 stage, showing no *lin-14* protein at this stage, as with *lin-28* mutants but unlike *lin-4* mutant animals. (b) (Top left) FITC; *lin-14(n536gf)*; *lin-28(n719)*, showing newly hatched L1 and L2 late stage embryos, all of which accumulate *lin-14* protein at these early stages. (Top right) FITC; L2 stage *lin-14*; *lin-28* animal, showing no *lin-14* protein at this stage. (See legend to Fig. 1 for abbreviations.)

Down-regulation of lin-14 protein levels is triggered by developmental cues and not real time

lin-14 protein levels normally begin to decrease within a few hours of hatching during mid-L1 as postembryonic cell division is initiated. We investigated whether the down-regulation of *lin-14* is dependent on the passage of time per se or on postembryonic developmental events by observing the regulation of *lin-14* protein in L1 animals in which development was suspended. When newly hatched L1 larvae are starved, they do not undergo postembryonic development but, instead, can remain suspended at this stage for up to 5 days (data not shown). Upon feeding, they resume development, executing normal L1-specific and subsequent cell lineages.

Immunofluorescent analysis of developmentally suspended L1 larvae at various time intervals of up to 125 hr after hatching showed that there was no decline in the *lin-14* protein levels as compared with early L1 stage animals (Fig. 3a). In comparison, during normal development, animals mature to adulthood in ~60 hr and have an undetectable level of *lin-14* protein in nearly all cells within 15 hr of hatching.

L1 larvae starved for 20, 30, or 50 hr were then fed for 12 hr. About 20% of the animals continued development into the L2 stage, and these animals showed a rapid decline in *lin-14* protein levels similar to that occurring during normal development (Fig. 3b). Therefore, upon feeding, animals can resume postembryonic development and trigger the down-regulation of *lin-14* protein levels.

Figure 3. In situ immunofluorescent detection of *lin-14* nuclear protein in starved wild-type animals. (Top) FITC; (bottom) DAPI. (a) Lateral view of wild-type L1 stage starved for 48 hr, showing *lin-14* protein in hypodermal, nerve ring, and muscle nuclei. Animals starved up to 125 hr were similarly arrested at the L1 stage and showed no variation in the pattern of *lin-14* protein staining (data not shown). (b) Medial view of wild-type L1 stage starved in the presence of 1 mg/ml of cycloheximide for 48 hr, showing no detectable *lin-14* protein. Animals treated in this manner were alive and still fairly mobile. (c) Lateral view of wild-type late L1 stage, 12 hr after feeding 48-hr-starved L1 larvae. *lin-14* protein is undetectable in hypodermal nuclei and barely detectable in nerve ring nuclei, similar to wild type (photo is overexposed to show weak *lin-14* protein in neuronal nuclei). (See legend to Fig. 1 for abbreviations.)

We determined whether the presence of *lin-14* protein in L1 animals at 48 hr of starvation was due to stability of *lin-14* protein or to continued translation of *lin-14* mRNAs. After 24–48 hr of starvation in 1 mg/ml of cycloheximide, animals were still alive; and after fixation, animals showed weak or no *lin-14* protein staining compared with control animals (Fig. 3c). These animals showed no significant decrease in *lin-14* transcript levels compared with control animals (data not shown). These data suggest that *lin-14* protein or a protein that stabilizes *lin-14* protein is continually translated in these developmentally arrested L1 animals.

lin-28 is necessary for maintenance of lin-14 protein levels in developmentally arrested L1 larvae

Because *lin-14* protein levels remain elevated in developmentally arrested wild-type L1 animals, we examined whether a functional *lin-28*, *lin-29*, or *lin-4* gene is necessary to maintain high levels of *lin-14* protein in these animals. *lin-28(n719)* arrested L1 animals displayed little or no *lin-14* protein staining after 48 hr of starvation (Fig. 4a). These data suggest that *lin-28* is necessary for the persistence or continued expression of *lin-14* protein in suspended L1 larvae.

In contrast to *lin-28* animals, *lin-4(e912)* mutant animals (Fig. 4b), *lin-14(n355gf)* mutants (data not shown), and *lin-29* mutants (data not shown) displayed normal *lin-14* protein levels and a normal cellular pattern of expression in developmentally arrested L1 larvae. Therefore, the *lin-14* 3'UTR, and *lin-4* and *lin-29* gene activities are not necessary to maintain high levels of *lin-14* protein in arrested L1 animals. As in wild type, in the presence of cycloheximide, the *lin-14* protein level in 48-hr-arrested *lin-4* L1 larvae decreased to nearly undetectable (Fig. 4c). This suggests that the inappropriate accumulation of *lin-14* protein in a *lin-4* mutant is not caused by increased stability of *lin-14* protein in this mutant. Thus, the *lin-4* gene activity is probably not functioning as (or regulating) a protease during the L1 stage.

lin-28; *lin-4* and *lin-28*; *lin-14(gf)* double mutants, which normally express *lin-14* protein only in the early L1 stage, showed no *lin-14* protein staining after 48 hr of starvation (data not shown), as with *lin-28* mutant animals but unlike *lin-4*, *lin-14(gf)*, or wild-type animals. These data show that the *lin-28* gene activity is necessary for continued *lin-14* protein accumulation in starved L1 animals even if the negative regulatory element in *lin-14* is deleted or the *lin-4* gene activity is missing.

lin-28 and lin-14 do not control initiation of postembryonic development

Normally, *lin-14* protein levels fall just as postembryonic cell division begins during the L1 stage. Because postembryonic development is initiated in *lin-14(gf)* mutants, although the level of *lin-14* protein does not decrease normally, down-regulation of *lin-14* protein is not necessary to initiate postembryonic development. To

Figure 4. In situ immunofluorescent detection of *lin-14* nuclear protein in starved heterochronic mutant animals. (Top) FITC; (bottom) DAPI. (a) Lateral view of *lin-28(n719)* L1 stage starved for 48 hr, showing no detectable *lin-14* protein. In the presence of cycloheximide, *lin-28* mutant animals similarly do not contain *lin-14* protein (data not shown). (b) Medial view of *lin-4(e912)* L1 stage starved for 48 hr, showing *lin-14* protein in nerve ring, muscle, intestinal, and ventral cord nuclei, as in wild-type L1 animals. (c) Lateral view of *lin-4(e912)* L1 stage starved for 48 hr in the presence of 1 mg/ml of cycloheximide, showing no detectable *lin-14* protein. (See legend to Fig. 1 for abbreviations.)

test whether decreased *lin-14* protein levels are sufficient to initiate the postembryonic lineage, we observed whether postembryonic cell division occurs in starved *lin-28* or *lin-14(lf)* mutant L1 larvae. We monitored whether postembryonic cell divisions occurred in the suspended *lin-28(n719)* (Fig. 4a) and *lin-14(n179)* (data not shown) mutants. DAPI staining of fixed specimens was used to survey for production of the postembryonic Pn.a ventral cord neurons and Vn.a and Vn.p seam cells from the V1 to V6 lineages (Fig. 4a). The starved L1 larvae from these mutants displayed no postembryonic cell divisions after 48 hr, showing that development was suspended, although there was no *lin-14* protein in these animals. These data show that *lin-28* and *lin-14* do not regulate the initiation of postembryonic cell division. Furthermore, the data show that lack of *lin-28* gene activity does not down-regulate the *lin-14* protein levels as a consequence of inappropriately initiating postembryonic cell division.

Discussion

The *lin-14* protein gradient controls a major switch in the temporal pattern of the *C. elegans* cell lineage. The transition during the L1 stage from a high to a low level of *lin-14* protein causes a number of blast cells to switch from L1-specific patterns of cell lineage during the early L1 stage to distinct L2-specific cell lineages that they execute at the next larval stage. Failure to down-regulate the *lin-14* protein level after the L1 stage causes reiterations of L1-specific cell lineages (and lack of later cell lineages) at later stages, whereas failure to express high levels of *lin-14* protein causes precocious expression of L2-specific cell lineages by blast cells at the L1 stage (Ambros and Horvitz 1987). We have shown here that the heterochronic genes *lin-4* and *lin-28* play a major role in the generation of the *lin-14* temporal gradient, whereas *lin-29* is not involved in establishing this gradient.

lin-4 negatively regulates *lin-14*

The *lin-4(e912)* mutation, which causes reiterations of L1-specific cell lineages at later larval stages, as with *lin-14(gf)* mutations, results in inappropriately high lev-

els of *lin-14* protein at L2, L3, and later developmental stages (Figs. 1 and 5). The *lin-4* gene activity must therefore be necessary for the proper down-regulation of *lin-14* protein levels.

Our results suggest that *lin-4* may regulate *lin-14* via the negative regulatory element in the 3' UTR of *lin-14*. Two *lin-14(gf)* mutants contain a rearrangement (*n355*) or a 607-bp deletion (*n536*) in the 3' UTR that abrogates the proper down-regulation of *lin-14* protein at late larval stages (Wightman et al., this issue). The similarity of the *lin-4* and *lin-14(gf)* phenotypes, both at the level of cell lineage changes and at the level of inappropriate *lin-14* regulation, suggests that the *lin-4* product may bind or activate a factor that binds to the *lin-14* 3' UTR to down-regulate production of *lin-14* protein (Fig. 6). The simplest model for such a regulatory event is that the *lin-4* gene product is expressed or activated during the L1 stage to bind directly or indirectly to the *lin-14* mRNA which, in turn, down-regulates production of *lin-14* protein during the late L1 stage (Fig. 6). The time at which *lin-4* acts has not been determined.

lin-28 positively regulates *lin-14*

Loss-of-function mutations in *lin-28* are also heterochronic but affect only post-L1 stage hypodermal cell lineages. L1-specific lineages occur normally during the L1 stage in a *lin-28(n719)* null mutant; but at the L2 stage, hypodermal blast cells can express L3- or even L4-specific cell lineages (Fig. 5; Ambros and Horvitz 1984). In hypodermal blast cells, *lin-28* gene activity is necessary for the reiterated L1 lineage at L2 and later stages caused by excess *lin-14* gene activity in *lin-4* and *lin-14(gf)* mutants (Ambros 1989). This genetic result suggests that *lin-28* may be necessary for the production of *lin-14* gene activity or may act downstream of *lin-14* in hypodermal blast cells. However, *lin-28* affects fewer stage-specific events than *lin-4* or *lin-14*. For example, the time of appearance of the alternative L3 dauer larval stage is controlled by *lin-14* and *lin-4* but not by *lin-28* (Liu and Ambros 1989). These data suggested to Liu and Ambros that the heterochronic genes form a branched regulatory pathway. Such a branched pathway suggests that *lin-4* and *lin-14* could affect a broader number of cell types or act at a distinct time from *lin-28*.

Figure 5. Summary of the temporal and cellular expression profile of *lin-14* protein in wild-type, *lin-14(gf)*, *lin-14(lf)*, *lin-4*, *lin-28*, and *lin-29* heterochronic mutants. Shown are the lineage changes induced by these mutations, and superimposed in dark line are those cells that also express the *lin-14* protein. Wild type expresses *lin-14* protein only during the L1 stage. *lin-14(n355gf)* and *lin-4(e912)* express the *lin-14* protein at all developmental stages and, therefore, reiterate L1-specific cell lineages. *lin-14(n536n540)* shows no detectable *lin-14* protein so that precocious L2-specific lineages occur during the L1 stage. *lin-28(n719)* shows *lin-14* protein only during the early L1 stage, albeit weakly, but not after it, so that a normal L1-specific lineage occurs, but L3 and later specific lineages occur precociously during the L2 stage. *lin-29(n333)* expresses *lin-14* normally during the L1 stage so that L1-specific lineages and subsequent L2-, L3-, and L4-specific lineages are normally expressed. This mutant fails to express adult-specific traits and reiterates L4-specific cell identities regardless of whether *lin-14* is mutant or not.

Analysis of the temporal regulation of *lin-14* protein levels in a *lin-28* null mutant showed that *lin-28* either regulates *lin-14* gene expression positively or is required for the stability of the *lin-14* protein. The temporal and cellular profile of *lin-14* protein accumulation begins normally in the *lin-28(n719)* mutant, but by the early L1 stage the level of *lin-14* protein is decreased relative to wild type in the hypodermal and intestinal cell lineages but not in neuronal and muscle cells (Fig. 1d). Although the difference in cell type expression of *lin-14* observed in *lin-28* mutants could account for the difference in *lin-28* and *lin-14* phenotypes with respect to dauer formation, other observations show that *lin-28* affects *lin-14* protein levels in all cell types. For example, unlike wild type, the *lin-14* protein level declines rapidly in all cell types of *lin-28* L1 larvae that are developmentally arrested by starvation (Fig. 4). In addition, *lin-28* gene activity is necessary for the inappropriate high level of *lin-14* protein in all cells of post-L1 stage *lin-4* and *lin-14(gf)* mutants. These data rule out a simple model where *lin-28* positively regulates *lin-14* only in a specific subset of cells.

The temperature-sensitive period for the *lin-14*-mediated specification of L1-specific cell lineages in the hypodermis is just before those lineages occur during the early L1 stage (Ambros and Horvitz 1987). In *lin-28* mutants, *lin-14* protein levels begin to fall in hypodermal cells during the time that the gene must function to specify L1-specific cell lineages. Because *lin-28* mutants execute normal L1-specific cell lineages, either the falling level of *lin-14* protein in these blast cells is sufficient to specify L1-specific cell lineages in those cells or *lin-14* specifies L1-specific cell lineages to the hypodermal blast cells non-cell-autonomously.

Although these data suggest that *lin-28* plays a role in the regulation of *lin-14*, other observations argue that *lin-28* also functions independently of *lin-14*. At the L2 stage, some hypodermal blast cells in *lin-28* mutants execute L4-specific fates, whereas *lin-14(lf)* null mutants execute L3-specific fates (Ambros and Horvitz 1984). That is, at L2 and later stages, *lin-28* mutants are more precocious than *lin-14(lf)* mutants. Furthermore, *lin-28; lin-14(lf)* double mutants execute L4-specific fates during the L2 stage more often than either single mutant does (Ambros 1989). Therefore, *lin-28* probably has functions in addition to regulation of the *lin-14* protein.

lin-4 and *lin-28* antagonistically regulate *lin-14*

lin-4 and *lin-28* act antagonistically to set the level of the *lin-14* protein during the L1 stage and later (Fig. 6). Immunofluorescence analyses of *lin-28; lin-4* and *lin-28; lin-14(gf)* double mutants showed that in both cases, accumulation of *lin-14* protein at post-L1 stages depends on *lin-28* activity (Fig. 2). These results are consistent with the genetic epistasis of *lin-28* to *lin-4* and *lin-14(gf)* mutations (Ambros 1989). These data distinguished between two models for down-regulation of *lin-14* during the L1 stage in wild type: (1) During the L1 stage, *lin-4* down-regulates *lin-28* which, in turn, fails to up-regulate *lin-14*; or (2) *lin-28* positively regulates *lin-14* expression or stability during all postembryonic stages but *lin-4*-mediated negative regulation normally overrides *lin-28* after the L1 stage. Because *lin-28* is required for post-L1 stage accumulation of *lin-14* protein even in *lin-14(gf)* mutants, which presumably have normal *lin-4* gene activity, *lin-28* must not be down-regulated by *lin-4* gene activity in this strain. We hypothesize that the logic is

Figure 6. A model for the generation of the *lin-14* protein gradient. In early L1, or starved L1, wild-type animals, *lin-14* protein is actively translated and specifies L1-specific cell lineages, perhaps by activating L1-specific genes and/or repressing L2-specific and later gene activities including *lin-29*. The normal temporal pattern of *lin-14* protein level is regulated by the heterochronic genes *lin-4* and *lin-28* that act antagonistically to define the *lin-14* protein gradient. *lin-28* is required either to activate *lin-14* expression positively or to stabilize the *lin-14* protein during the L1 stage of wild-type development and during L2 and later stages of development in *lin-4* and *lin-14(gf)* mutants. A decrease in *lin-14* protein levels during the mid-L1 stage is triggered by feeding and/or by postembryonic developmental events. This triggering event may activate *lin-4* expression or activity to regulate *lin-14* negatively. The *lin-4* product could bind to the *lin-14* negative regulatory element (NRE) or regulate another factor that binds to the *lin-14* NRE. The normal disappearance of the *lin-14* protein at the L2 stage prevents the expression of L1-specific cell lineage genes and allows the expression of L2-specific and subsequent cell lineage genes, including *lin-29*, which specifies the switch from larval to adult stage cuticle formation (Ambros 1989). Arrows denote positive regulation; bars denote negative regulation.

the same in wild type: *lin-4* does not act via *lin-28* to down-regulate *lin-14* during wild-type development either. The data also argue that *lin-28* positive regulation cannot be mediated by sequences in the *lin-14* 3' UTR deleted or disrupted by *lin-14(gf)* mutations because these mutants are still affected by *lin-28*. Rather, these data favor the second model that *lin-28* positively regulates *lin-14* protein levels independently from *lin-4/lin-14* 3' UTR negative regulation.

Down-regulation of lin-14 is triggered by postembryonic developmental cues

We showed that the normal down-regulation of *lin-14* protein level during the L1 stage is not determined by the

passage of a particular period of time from hatching. Rather *lin-14* down-regulation is triggered by an unknown postembryonic event that can be blocked by starvation; wild-type L1 animals that are developmentally suspended at the L1 stage show no decline in *lin-14* protein levels (Fig. 3). Treatment with cycloheximide showed that translation is necessary for maintenance of *lin-14* protein and suggests that it is continually synthesized in these suspended L1 larvae (Fig. 3). *lin-28* gene activity, but not *lin-4* and *lin-29*, is necessary to maintain high levels of *lin-14* protein before its down-regulation is triggered by feeding or postembryonic development. Upon feeding, animals that resume cell division and differentiation show the normal late L1 down-regulation of *lin-14* proteins (Fig. 3).

Down-regulation of *lin-14* protein levels does not cause initiation of postembryonic development, and the *lin-28* mutation does not cause a decrease in the *lin-14* protein level by initiating postembryonic development without the feeding cue. Starved L1 larvae from *lin-28(n719)* and *lin-14(n179)* mutants did not initiate postembryonic cell lineages without feeding, although these animals had undetectable *lin-14* protein levels (Fig. 3). Rather, these data suggest that feeding or the subsequent postembryonic development, or both, normally triggers the decrease in *lin-14* protein levels. The feeding/postembryonic development switch could cause an increase in *lin-4* activity to down-regulate the *lin-14* protein level.

The postembryonic event that triggers *lin-14* down-regulation could be the same one that releases the animal for further cell divisions, or it is possible that down-regulation of *lin-14* depends on a signal from a particular cell generated from lineages early during the L1 stage. It is known that sensory detection of bacteria or the dauer pheromone antagonistically controls the dauer/non-dauer switch during *C. elegans* development (Riddle 1988) and that specific neurons mediate this sensory/developmental pathway (Bargmann and Horvitz 1991). Similarly, the same sensory neurons could control the initiation of postembryonic development and *lin-14* down-regulation by feeding. On the other hand, simple increase in cell mass upon feeding could cause these changes. We are currently attempting to determine whether sensory detection of bacteria, ingestion of bacteria, or postembryonic development is necessary to trigger the *lin-14* temporal switch and the postembryonic cell lineage.

A model for the lin-14 temporal switch

The cellular and temporal expression patterns of *lin-14* proteins in wild-type and various heterochronic mutant strains have revealed aspects of how the *lin-14* protein gradient is generated (Fig. 6). In wild-type late embryos and early L1 stage animals, *lin-14* protein is actively translated. During the L1 stage, *lin-28* either regulates *lin-14* expression positively or stabilizes the *lin-14* protein. The *lin-14* protein specifies L1-specific cell lineages

by activating L1-specific genes and/or repressing L2-specific genes and later genes such as *lin-29*.

The normal down-regulation of *lin-14* protein is triggered by feeding and/or consequent postembryonic development early in the L1 stage. We suggest that *lin-4* or some other factor is activated during this time to down-regulate *lin-14* by the negative regulatory element in the 3' UTR. This factor could be activated by the sensation of food, or a specific cell generated or induced postembryonically may produce a signal that activates the factor.

Once the *lin-4* and *lin-28* genes are cloned, in situ expression studies will establish whether they are expressed in the same cells as *lin-14* and at the same time as the *lin-14* temporal switch. These studies will reveal whether these genes or their products are likely to interact directly with the *lin-14* gene, mRNA, or protein. In vitro experiments will be necessary to show whether the protein products of these genes interact directly.

Materials and methods

Strains

C. elegans strains were propagated as defined by Brenner (1974). All heterochronic mutant strains were isolated as described by Ambros and Horvitz (1984, 1987).

Immunofluorescence

C. elegans wild type (N2) and mutant animals were fixed and immunostained with anti-*lin-14* antibodies as described by Ruvkun and Giusto (1989), and animals were staged as described by Ambros and Horvitz (1987). Fluorescent nuclei were visualized with a Zeiss universal microscope as reported previously (Ruvkun and Giusto 1989). The *lin-14* gene encodes multiple proteins with distinct amino-terminal regions (Wightman et al., this issue). The antibodies used in this study were raised against the *lin-14A* protein. Because the fusion used to raise antibodies includes a large portion of the domain common to all protein products and recognizes nothing in a *lin-14* null mutant, it is likely that the antiserum recognizes all *lin-14* protein products.

Starvation

Embryos were prepared by bleach treatment, washed thoroughly, and resuspended in S-basal medium (Wood et al. 1988). Developmentally arrested L1 larvae were fixed for immunostaining after 24, 48, 96, or 125 hr of incubation without a bacterial food source. Embryos were also incubated in medium containing 0.5, 1, or 5 mg/ml of cycloheximide (Sigma) under similar conditions of starvation. To monitor total protein synthesis, embryo preparations were hatched in the presence of 1 mCi/ml of [³⁵S]methionine medium containing 0, 0.5, or 5 mg/ml of cycloheximide; hatched L1 larvae were solubilized for protein gel analysis as described above. Total protein was displayed on Laemmli gels by Coomassie blue staining, and total counts incorporated were measured by excising the entire lane from the gel and counting ³⁵S. Translation was inhibited by 0%, 60%, or 90%, respectively, under these conditions.

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