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Multiple mechanisms are involved in regulating the expression of the developmental timing regulator *lin-28* in *Caenorhabditis elegans*

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The timing of postembryonic developmental programs in *Caenorhabditis elegans* is regulated by a set of so-called heterochronic genes, including *lin-28* that specifies second larval programs. *lin-66* mutations described herein cause delays in vulval and seam cell differentiation, indicating a role for *lin-66* in timing regulation. A mutation in *daf-12*/nuclear receptor or *alg-1*/argonaute dramatically enhances the retarded phenotypes of the *lin-66* mutants, and these phenotypes are suppressed by a *lin-28* null allele. We further show that the LIN-28 protein level is upregulated in the *lin-66* mutants and that this regulation is mediated by the 3′UTR of *lin-28*. We have also identified a potential *daf-12*-response element within *lin-28* 3′UTR and show that two microRNA (miRNA) (*lin-4* and *let-7*)-binding sites mediate redundant inhibitory activities that are likely *lin-66*-independent. Quantitative PCR data suggest that the *lin-28* mRNA level is affected by *lin-14* and miRNA regulation, but not by *daf-12* and *lin-66* regulation. These results suggest that *lin-28* expression is regulated by multiple independent mechanisms including LIN-14-mediated upregulation of mRNA level, miRNAs-mediated RNA degradation, LIN-66-mediated translational inhibition and DAF-12-involved translation promotion.

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Introduction

Although spatial regulation of developmental pattern formation has attracted extensive research in the past two decades, study of temporal regulation has been limited to a few systems and remains a relatively open research field. A number of heterochronic genes have been shown to regulate developmental timing in *Caenorhabditis elegans*. Some of the key players, including the two first identified microRNAs (miRNA) *lin-4* and *let-7*, are evolutionarily conserved (Rougvie, 2001; Pasquinelli and Ruvkun, 2002; Ambros, 2004). *lin-14* and *lin-28* are two critical timing regulators of stage-specific developmental programs, as opposite heterochronic phenotypes are associated with their loss-of-function (*lf*) and gain-of-function (*gf*) mutations. *lin-14* acts to specify the first larval (L1) developmental program: the L1 program is skipped in *lin-14 (lf)* mutants but reiterated in *lin-14 (gf)* mutants (Ambros and Horvitz, 1984). Similarly, *lin-28* acts to specify the second larval (L2) developmental program: the L2 program is skipped in *lin-28* (*lf*) mutants but reiterated in animals expressing a *lin-28 (gf)* transgene (Moss *et al*, 1997). The LIN-14 and LIN-28 proteins are abundant from the late embryo to L1 stage, but their expression decreases after L2 and is further reduced to undetectable levels in the L4 and adult stages (Wightman *et al*, 1993; Moss *et al*, 1997; Seggerson *et al*, 2002).

The heterochronic gene *lin-4* encodes an miRNA that acts in later larval and adult stages to repress the expression of *lin-14* and *lin-28* (Lee *et al*, 1993; Wightman *et al*, 1993; Moss *et al*, 1997). In addition, *lin-14* and *lin-28* are also known to positively regulate each other (Arasu *et al*, 1991; Moss *et al*, 1997). *lin-28* encodes an approximately 25-kDa protein with two types of RNA-binding motifs: a so-called cold shock domain and a pair of retroviral-type CCHC zinc-finger domains (Moss *et al*, 1997). The mammalian homologues of *lin-28* are expressed at early developmental stages and they have a long 3′UTR with sequences that are complementary to the *lin-4* and *let-7* miRNA homologues (Moss and Tang, 2003). *lin-14* encodes a transcription factor (Ruvkun and Giusto, 1989).

Previous work indicated that *lin-4* activity alone is not sufficient to suppress the expression of *lin-28* in later larval stages (Seggerson *et al*, 2002). One other gene known to be involved in regulating *lin-28* is *daf-12* that encodes a nuclear hormone receptor (Antebi *et al*, 2000). A recessive *gf* mutation, *daf-12 (rh61)*, causes a prominent developmental delay phenotype and accumulation of LIN-28 protein in late stages, although the delay phenotypes were not associated with a null allele (Antebi *et al*, 1998, 2000; Seggerson *et al*, 2002).

Key timing regulators such as *lin-4*, *lin-14* and *lin-28* have been shown to regulate the timing of vulval cell division (Ambros and Horvitz, 1984; Euling and Ambros, 1996). *lin-14 (lf)* and *lin-28 (lf)* mutations cause precocious vulval cell divisions: vulval cells divide one stage earlier than in wild type (WT), presumably owing to skipping the L1 and L2 programs in the *lin-14 (lf)* and *lin-28 (lf)* mutants, respectively. On the other hand, *lin-4 (lf)* and *lin-14 (gf)* mutations cause retarded or eliminated vulval cell divisions. *C. elegans* vulval differentiation is regulated by several well-known signalling pathways including the RTK/RAS/MAPK pathway that induces three of six vulval precursor cells to become vulval cells (Sternberg, 2005). LIN-31 and LIN-1 are two transcription factors that act at the end of the signalling pathway to specify vulval cell fate.

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In an effort to identify genes acting downstream or with *lin-31* to specify vulval cell fate, we have carried out a genetic screen for suppressors of the multivulva (Muv) phenotype of *lin-31 (lf)*. Interestingly, we found a number of suppressors that displayed heterochronic mutant phenotypes. These mutations are alleles of five genes including *ain-1* (Ding *et al*, 2005), *alg-1* and *lin-66* (this study). In this paper, we describe the genetic and molecular analysis of *lin-66* and provide evidence that *lin-66* likely acts to inhibit *lin-28* translation. We also analyzed the roles of *daf-12*, miRNA and *lin-14* in regulating *lin-28* expression, and show they mediate multiple independent mechanisms.

Results

lin-66 (lf) mutations suppress the multivulva phenotype of *lin-31 (lf)*

In our screens for suppressors of the Muv phenotype of *lf* alleles of *lin-31* (Ding *et al*, 2005; Morita *et al*, 2005), two of the 12 mutations, *ku423* and *ku424*, displayed similar phenotypes and were mapped to the same chromosome region. In the *lin-31 (n301)* mutant background, *ku423* or *ku424* causes a fully penetrant egg-laying defect in hermaphrodites ($n = 244$ and 160, respectively). When these two alleles were isolated away from the *lin-31 (n301)* mutation, they displayed a striking larval lethality; 95% of the mutants die at the late L4 stage ($n = 256$), accompanied with a burst of the gonad through the vulva (Figure 1A). This lethal phenotype is very similar to that of *let-7(lf)* mutants. A small percentage of the homozygous animals escaped from lethality but all of them failed to lay eggs.

lin-66 encodes a novel protein that is ubiquitously expressed

Both *ku423* and *ku424* are recessive alleles. Using single-nucleotide polymorphism (SNP) mapping, DNA-mediated rescue and DNA sequencing (Materials and methods), we determined that B0513.1, a transcription unit annotated by the genome project (WormBase.com), is the *lin-66* gene (Supplementary Figure S1). *ku423* and *ku424* were determined to contain a nonsense mutation in exon 5 (Q364 to stop) and a first exon splicing mutation (GT to AT), respectively (Supplementary Figure S1). The molecular lesions of the two mutations and the fact that each displayed indistinguishable phenotypes strongly suggest that both *ku423* and *ku424* are null or strong *lf* mutations. We named the gene defined by these two mutations as *lin-66* (*lin*eage defective 66). *ku423* is used for most of the genetic analysis.

lin-66 encodes a 627-amino-acid novel protein. LIN-66 is highly homologous (80% identity in amino acids) to a protein in the closely related nematode species *C. briggsae*. To assess the expression pattern of *lin-66*, we constructed a transgenic strain that expresses a *lin-66*::*GFP* translational fusion protein (Materials and methods). This transgene fully rescued the *lin-66 (lf)* phenotypes (data not shown). The GFP expression was detected in many tissues throughout the development (Supplementary Figure S1). The fusion protein was cytoplasm-localized (Supplementary Figure S1), which is consistent with its activity on the *lin-28* 3′UTR and a role in translational inhibition (see below).

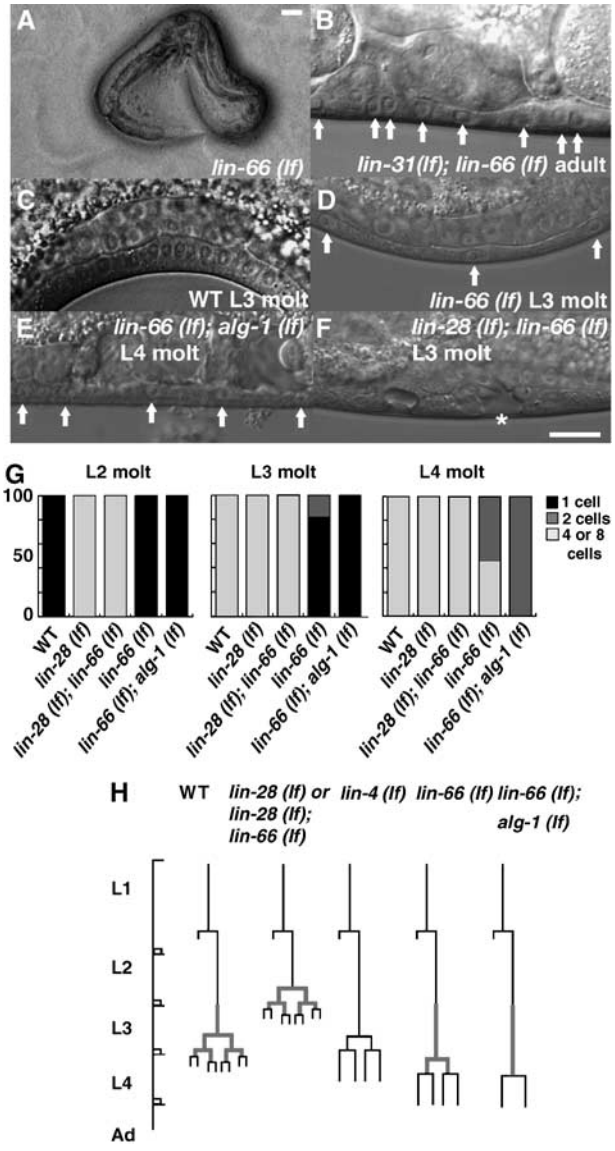

Figure 1 *lin-66 (lf)* causes defective vulval development. (**A**) A *lin-66 (ku423)* L4 dying larva with the gonad bursting through the vulva. Ninety-five percent of the homozygous mutants from heterozygous mother die at this stage. Bar, ~50 μm. (**B**) A *lin-31 (n301); lin-66 (ku423)* adult animal showing that Pn.p cells failed to differentiate into vulval cells and form vulval invaginations. Arrows indicate the Pn.p derivatives. (**C, D**) L3 larva of WT and *lin-66 (ku423)* mutants showing that the first round of vulval cell divisions was delayed in the mutant animals. Arrows in (D) indicate one-cell stage Pn.p cells. (**E**) An L4 molting larva showing that the vulval cell division is severely delayed in the *lin-66 (ku423); alg-1 (gk214)* double mutants, as the Pn.p cells (arrows) are still at the two-cell stage. Bar, ~10 μm. (**F**) A *lin-28 (n719); lin-66 (ku423)* double mutant L3 larva displaying a precocious vulval division phenotype. The vulval morphology in this worm is normally seen only in L4 larva. (**G**) Graphical representations of the percent of vulval cells at each division stage (derived from P5–7.p) at three larval stages. Twenty or more animals were examined for each strain at each developmental stage. (**H**) Schematic summary of the timing of the division of Pn.p cells destined to become vulval cells in indicated strains. Thick line indicates the *egl-17*::*GFP* expression in P6.p.

lin-66 (lf) mutations cause a delay or elimination of vulval cell divisions

To assess the cause of the suppression of the *lin-31 (lf)* Muv phenotype that was scored under a dissecting microscope, we

followed the cell lineage of P5.p, P6.p and P7.p (P(5–7).p) in *lin-31 (n301); lin-66 (ku423)* double mutants using Nomarski optics. In WT worms, P(5–7).p cell lines start dividing during the L3 stage (Sulston and Horvitz, 1977) (Figure 1C and H). In contrast, in *lin-31 (n301); lin-66 (ku423)* double mutants, cell divisions of these cells often started during the late L4 stage or did not occur at all (Figure 1B). This phenotype is similar to the developmental delay phenotype associated with certain heterochronic mutants such as the *lin-4 (lf)* mutants (Ambros and Horvitz, 1984). Therefore, *lin-66 (lf)* likely suppresses the Muv phenotype of *lin-31 (lf)* by delaying or eliminating the cell division of vulval cells.

We then analyzed the phenotypes of *lin-66 (ku423)* single mutant animals. *lin-66 (ku423)/lin-66 (ku423)* homozygous progeny from *lin-66 (ku423)/+* heterozygous mothers displayed a weaker cell division delay in P5.p, P7.p and their derivatives when compared with the *ku423/ku423* progeny from *ku423/ku423* mothers (data not shown). This weaker phenotype is consistent with the existence of maternal *lin-66* gene product in the homozygous animals from the heterozygous mothers. Interestingly, *ku423/ku423* progeny from *ku423/+* mothers displayed a stronger lethality at the late L4 stage (95%, $n=256$) when compared with the *ku423/ku423* progeny from *ku423/ku423* mothers (46%, $n=317$). As *ku423/ku423* animals are egg-laying defective, resulting in the progeny hatched internally experienced starvation after the hatching, the weaker lethality of the *ku423/ku423* animals from *ku423/ku423* animals may be due to starvation-induced phenotypic suppression (Rougvie, 2005).

We further analyzed vulval cell divisions of *lin-66 (ku423)* homozygotes from the homozygous mothers under Nomarski optics. In WT hermaphrodites, the first round of cell divisions of P(5–7).p occur during the mid-L3 stage and the next round of cell divisions occur at the L3 molting stage. At the L4 molting stage, P(5–7).p derivatives terminally differentiate into vulval cells. (Sulston and Horvitz, 1977) (Figure 1C and H). We observed that P(5–7).p cell divisions were severely delayed or eliminated in *lin-66 (ku423)* animals (Figure 1D and H). For example, 82% of P(5–7).p cells failed to complete the first cell division (stayed at the one-cell stage) in the L3 molt (Figure 1D and H). Moreover, in the *lin-66 (ku423)* mutants, most of the P(5–7).p cells failed to complete cell divisions and no vulval structure was observed in adults (Figure 1G; data not shown).

lin-66 (lf) mutants display a retarded heterochronic phenotype during seam cell differentiation

In WT worms, the lateral hypodermal seam cells divide with a stem-cell-like pattern during larval stages. They then exit the cell cycle and terminally differentiate after L4 molting—a process termed as the 'larval to adult (L/A) switch' (Ambros, 1989) (Figure 2G). These cells secrete a cuticular structure known as the lateral alae during the adult stage. We observed that *lin-66 (lf)* animals failed to generate alae after the L4 molt (Table I), suggesting that *lin-66* has a role in regulating seam cell division and differentiation.

In WT larvae, the majority of the 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 (Figure 2G). Therefore, these divisions do not result in changes in seam cell numbers. However, during the L2 stage, certain seam cells (V1–4, V6, H1) undergo one round of symmetric cell divisions before the asymmetric cell division, which results in an increase of the number of seam cells on each side of the animal by six (Rougvie, 2001) (Figure 2G). At the L/A switch, WT animals usually have 16 unfused seam cells (Figure 2A). Mutations in heterochronic genes often alter the seam cell division pattern. For example, *lin-4 (lf)* displays a retarded mutant phenotype in which the animals repeat the L1 seam cell division program (never entering the L2 stage). Consequently, there are only 10 seam cells even at the adult stage. In contrast, *lin-28 (lf)* displays a precocious phenotype in which the animals skip the L2 seam cell division program and instead enter the L3 program prematurely. By eliminating the cell number duplication for the six seam cells at the L2 stage, the seam cell number for L3, L4 and adult animals is also reduced to 10 (Ambros and Horvitz, 1984; Pepper *et al*, 2004) (Table II). Consistent with the retarded phenotype in the vulval lineage, *lin-66 (lf)* mutants displayed a retarded seam cell differentiation phenotype, based on the observations using *SCM::GFP(QwIS79)* as a seam cell marker (Koh and Rothman, 2001). *lin-66 (ku423)* animals had a normal seam cell number at L1 and L2, but at the L3 stage seam cell numbers continued to increase to 29 on average, indicating that some of the cells divide symmetrically as in the L2 stage (Figure 2B and C and Table II). This result is consistent with the idea that the L2 seam cell program is reiterated in the L3 stage in *lin-66 (lf)* mutants.

The defect in seam cell division, failure in alae formation, lethality and the vulval division defects were all suppressed when the *lin-66 (lf)* animals developed through the

Table I Genetic interaction of *lin-66* with known heterochronic genes on alae formation

Genotype[a]	Alae synthesis (%) (*n*)		
	L3 molt	L4 molt	Ad/L5 molt[b]
WT (N2)	0 (20)	100 (20)	ND
lin-66 (ku423)	ND	0 (14)	100[c] (41)
lin-31 (n301); lin-66 (ku423)	ND	0 (31)	100[c] (15)
lin-31 (n301)	ND	100% (31)	ND
lin-14 (ma135)	100 (23)	ND	ND
lin-14 (ma135); lin-66 (ku423)	0 (29)	100[c] (25)	ND
lin-28 (n719)	100 (18)	ND	ND
lin-28 (n719); lin-66 (ku423)	100 (52)	ND	ND
hbl-1 (RNAi)	83 (35)	100[c] (15)	ND
lin-66 (ku423); hbl-1 (RNAi)	0 (24)	100[c] (35)	ND
lin-41 (ma104)	60[c] (25)	100[c] (25)	ND
lin-41 (ma104); lin-66 (ku423)	33[c] (33)	100[c] (36)	ND
lin-42 (n1089)	80[c] (50)	100[c] (38)	ND
lin-42 (n1089); lin-66 (ku423)	3[c] (32)	100[c] (21)	ND
lin-29 (n333)	ND	0 (25)	0 (28)
lin-29 (n333); lin-66 (ku423)	ND	0 (28)	0 (30)
lin-4 (e912)	ND	0	0
daf-12 (rh61)	ND	100[c] (41)	ND
lin-66 (ku423); daf-12 (rh61)	ND	0 (32)	0 (42)
alg-1 (ok214)	ND	59% (49)	100% (16)
lin-66 (ku423); alg-1 (ok214)	ND	ND	0% (33)
lin-46 (ma164)	ND	100	100%
lin-66 (ku423); lin-46 (ma164)	ND	ND	0% (26)

[a]The newly synthesized cuticle was examined for the presence of alae by Nomarski microscope.
[b]Adult stage or fifth molting stage for retarded mutants that undergo an extra molting stage.
[c]Some animals had gaps in their alae.
ND, not determined.

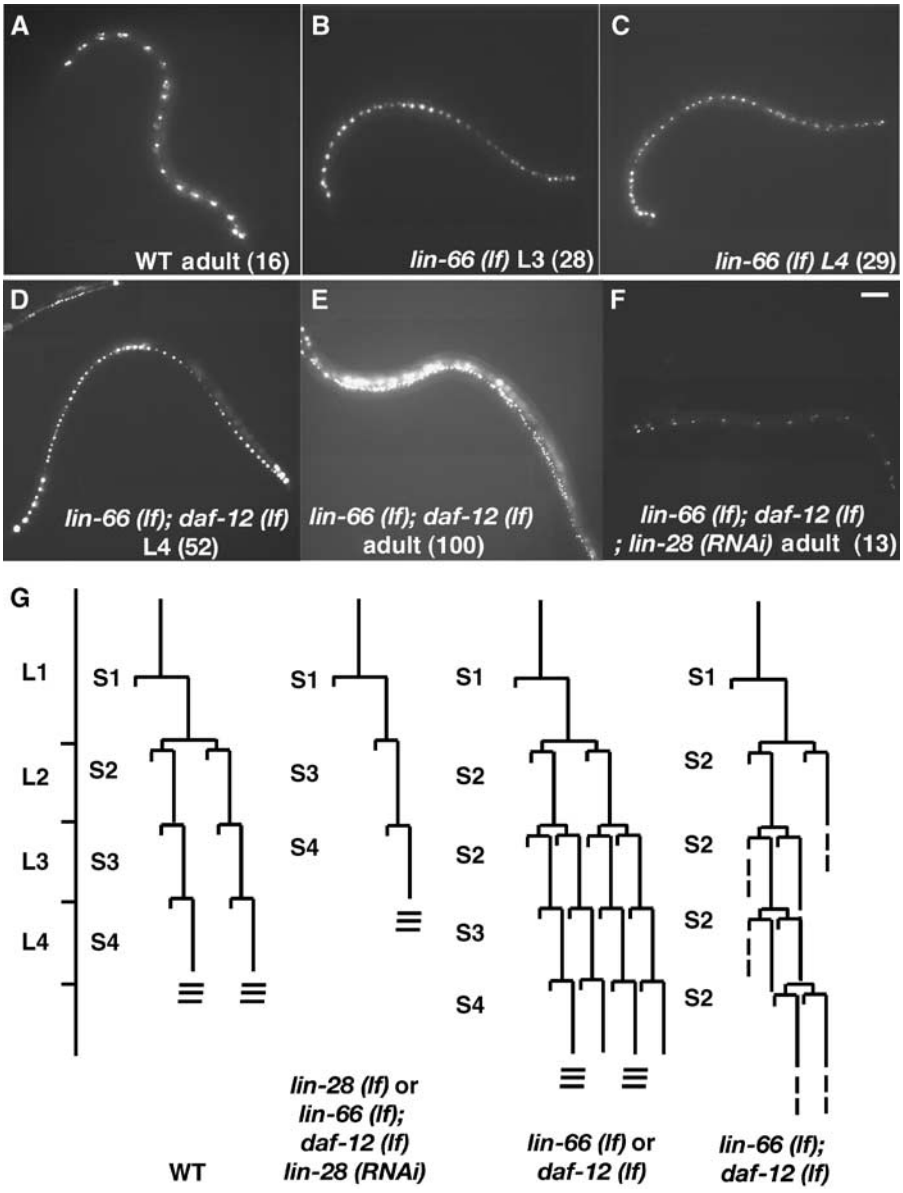

Figure 2 Effect of *lin-66 (lf)* and its interaction with *daf-12* and *lin-28* mutations on seam cell differentiation. (**A–F**) Fluorescent images of lateral sides of animals of genotypes and stages as indicated. The fluorescence indicates expression of SCM-1::GFP that marks the seam cells. (*n*), number of GFP-positive cells counted in each image. *ku423*, *rh61* and *n719* are alleles of *lin-66*, *daf-12* and *lin-28*, respectively. Bar, ~50 μm. (**G**) Schematic summary of the differentiation pattern of certain seam cells (V1–V4, V6 and (H) during post-embryonic development. The *lin-28 (lf)* and *daf-12 (rh61)* pattern was reported previously (Ambros and Horvitz, 1984; Antebi *et al*, 1998). Sn refers to Ln-specific cell lineage/program in hypodermal cell differentiation (Ambros and Horvitz, 1984). Three horizontal lines at the end of lineage stand for adult alae formation.

alternative dauer larval stage (data not shown). Phenotypic suppression by progression through the dauer stage is a distinctive feature of many heterochronic mutants (Liu and Ambros, 1989).

lin-66 and *daf-12* double mutants display a strong retarded seam cell phenotype

daf-12 encodes a nuclear receptor that has been shown to be involved in the heterochronic genetic pathway (Antebi *et al*, 1998, 2000; Grosshans *et al*, 2005). *daf-12 (rh61)* was thought to be a recessive *gf* mutation that causes a retarded seam cell defect similar to that of *lin-66 (lf)* (repeating the L2 program once; Figure 2G; Table II). To examine the functional relationship between *daf-12* and *lin-66*, we constructed and examined *lin-66 (lf); daf-12 (rh61)* double mutants. If *lin-66* and *daf-12* function in a linear pathway, the double mutant animals would be expected to display the same phenotype as that of a single mutant because *lin-66 (ku423)* is most likely a null allele. Strikingly, in *lin-66 (ku423); daf-12 (rh61)* double mutants, the seam cell number continued to increase during the L3, L4 and adult stages to about 100 cells, indicating that the L2 program was repeated multiple times in the double mutants (Figure 2D and E; Table II). Consequently, *lin-66 (ku423); daf-12 (rh61)* double mutants never generate the adult alae even in the L5 molt (Table I). We have also constructed double mutants containing *lin-66 (lf)* and two likely *daf-12* null alleles, *rh61rh411* and *m20* (Antebi *et al*, 2000). Each of the two *daf-12(null)* alleles alone

Table II *lin-66 (lf);daf-12 (rh61)* and *lin-66 (lf); alg-1 (lf)* double mutants display a strong L2 reiteration phenotype

	No. of *SCM*::*GFP*-positive cells/lateral side at each stage (*n*)				
	L1	L2	L3	L4	Ad/L5
WT	10 (7)	16 (12)	16 (11)	16 (17)	16 (19)
lin-66 (ku423)	10 (22)	16.5 (59)	28.6 (39)	29.1 (50)	29.1 (25)
daf-12 (rh61)	10 (10)	16.6 (22)	25.7 (21)	26.6 (25)	27.9 (14)
lin-66 (ku423); daf-12 (rh61)	10 (12)	17.0 (20)	28.8 (25)	50.6 (28)	78 (6)
daf-12 (rh61rh411)	10 (8)	16.7 (8)	19 (7)	20.7 (14)	21.5 (22)
lin-66 (ku423); daf-12 (rh61rh411)	10 (6)	16.2 (6)	28.7 (6)	49.4 (13)	93 (7)
daf-12 (m20)	10 (5)	16.3 (8)	18.3 (7)	21 (7)	19.6 (19)
lin-66 (ku423); daf-12 (m20)	10 (8)	16.5 (6)	28.4 (7)	48.7 (6)	83.7 (10)
lin-4 (e912)	10 (10)	10 (12)	10 (14)	10 (6)	10 (10)
lin-66 (ku423); alg-1 (ok214)	9.8 (13)	16.7 (12)	27.7 (10)	45.2 (6)	83.2 (12)
lin-28 (RNAi)	ND	ND	ND	ND	11.0 (25)
lin-66 (ku423); alg-1 (ok214); lin-28 (RNAi)	ND	ND	ND	ND	11.7 (20)
lin-66 (ku423); daf-12 (rh61); lin-28 (RNAi)	ND	ND	ND	ND	12.3 (13)

Seam cell nuclei were counted on one side of the animal of the indicated genotype and stage.
RNAi was performed by injecting dsRNA into the adult gonad. The seam cell number of the next generation was counted.
ND, not determined.

caused a small increase in seam cell number in late larval and adult stages, whereas both alleles drastically enhanced the seam cell phenotype of the *lin-66* mutation (Table II), consistent with a role of *daf-12(+)* in inhibiting the L2 program (see Discussion). These results suggest that these two genes act through parallel pathways to regulate the timing of seam cell differentiation.

lin-66 acts in parallel to alg-1/argonaute in regulating developmental timing

In *C. elegans, alg-1* and *alg-2* encode members of the argonaute protein family that are part of the RISC complexes involved in miRNA or siRNA maturation and function (Grishok *et al*, 2001; Bartel, 2004). ALG-1 and ALG-2 are highly homologous to each other and were shown to be specifically involved in miRNA functions including miRNA-mediated timing regulation (Grishok *et al*, 2001). Injection of the full-length *alg-1* dsRNA, which is likely to partially inactivate *alg-2*, has been shown to cause the reiteration of the L2 seam cell division program at L3 and an increase in the average number of seam cells from 16 to 21 (Grishok *et al*, 2001; Bartel, 2004). We have also isolated an *alg-1* allele in the same screen that isolated *lin-66* alleles. This mutation, *ku421*, was determined to have a nonsense mutation in the first exon and is thus likely to be another null or severe *lf* allele. However, neither an *alg-1* nor an *alg-2* null allele causes a dramatic increase in seam cell numbers in later larval stages (Grishok *et al*, 2001) (data not shown).

We investigated the relationship of *lin-66* with *alg-1* and *alg-2*. Strikingly, a *lin-66 (ku423); alg-1 (gk214)* double mutant displayed a strong L2 reiteration of the seam cell phenotype similar to that of the *lin-66 (ku423); daf-12 (rh61)* double mutant (Table II). In addition, the *alg-1* allele also significantly enhanced the vulval cell division delay phenotype of *lin-66 (ku423)*; 100% of P(5–7).p cells at the L3 molting stage were at the one-cell stage and 100% of the cells at the L4 molting stage were at the two-cell stage (Figure 1E, G and H; Table II).

These results suggest that *lin-66* may also function in parallel to *alg-1* to regulate developmental timing (see Discussion). In comparison, double mutants containing *lin-66 (lf)* and a null allele of *alg-2 (ok304)* displayed a seam cell phenotype that is similar to that of the *lin-66 (lf)* single mutant. This result may suggest that the *alg-2* function, which overlaps with part of the *alg-1* function (Grishok *et al*, 2001), plays a less prominent role in specification of the L2 program. This, however, does not exclude a possibility that *alg-2* is involved in the *lin-66*-mediated function.

RTK/RAS-regulated egl-17::GFP *expression in vulval precursor cells appears to be normal in lin-66 mutants*

We have shown that in *lin-66 (lf)* single and *lin-66 (lf); alg-1 (lf)* double mutants, the first round of cell divisions of P(5–7)p cells was delayed. This delay could be due to a delay or impairment of the RTK/RAS signalling activity that induced vulval division and differentiation. To investigate this possibility, we analyzed the expression of *egl-17*::*GFP* in *lin-66 (lf)* and *lin-66 (lf); alg-1 (lf)* mutants. In WT animals, *egl-17*::*GFP* expression can be detected at the early L3 stage in the P6.p cell and this expression is in response to RTK/RAS signalling (Burdine *et al*, 1998; Ambros, 1999). We observed normal *egl-17*::*GFP* expression in P6.p at the early L3 stage in *lin-66 (lf)* and *lin-66 (lf); alg-1 (lf)* mutants (Supplementary Figure S2), even though the vulval cell divisions were delayed in these strains. This result suggests that in the *lin-66 (lf)* and in *lin-66 (lf); alg-1 (lf)* mutants, the timing of the RTK/RAS signalling event was not obviously altered.

lin-66 acts upstream of lin-28 to regulate developmental timing

Genetic properties of *lin-28* and *lin-66* suggest that these two genes may interact to regulate L2 programs. Not only is the *lin-66 (lf)* mutant seam cell phenotype (L2 repeating once) opposite that of *lin-28 (lf)* (L2 bypassing), the *lin-66 (lf); daf-12 (rh61)* double mutant seam cell phenotype (L2 reiteration multiple times) is similar to the *gf* mutant phenotype of overexpressing *lin-28* (Moss *et al*, 1997). We thus examined the epistatic relationship between the likely null mutations in the two genes. We found that a *lin-28 (n719); lin-66 (ku423)* mutant displayed the same seam cell phenotype as that of *lin-28 (n719)*, suggesting that *lin-28* acts either downstream of or in parallel to *lin-66* to specify the L2 seam cell program.

We also examined the genetic interaction between the two genes in vulval cells. *lin-28 (lf)* mutations were previously shown to cause a precocious mutant phenotype (Euling and Ambros, 1996). We found that the delay of vulval cell divisions *lin-66 (lf)* was also suppressed by the *lin-28 (n719)* allele, a result consistent with the idea that *lin-66* acts upstream of *lin-28* (Figure 1F).

It has previously been shown that the *lin-28 (lf)* mutant phenotype in seam cells was also epistatic to that of *daf-12 (rh61)* (Antebi *et al*, 1998) and that LIN-28 protein expression was increased in *daf-12* mutants (Seggerson *et al*, 2002). Strikingly, the multiple L2 repeat phenotype of the *lin-66 (ku423); daf-12 (rh61)* double mutant was completely suppressed by RNAi of *lin-28* (Figure 2F, Table II). These results, when combined with the genetic interaction data between *lin-66* and *daf-12*, are consistent with a model in which *daf-12* and *lin-66* act in parallel to regulate the activity of *lin-28* (Figure 3).

As mentioned above, *lin-28 (lf)* mutations cause a precocious phenotype in which seam cells bypass the L2 program. In comparison, *lin-14 (lf)* causes a different precocious phenotype: the seam cells bypass the L1 program, which is opposite to that of *lin-4 (lf)* (reiterating the L1 program) (Lee *et al*, 1993; Wightman *et al*, 1993). Furthermore, both *lin-14* and *lin-28* have been shown to be regulated by *lin-4* miRNA (Lee *et al*, 1993; Wightman *et al*, 1993; Moss *et al*, 1997). We then examined the interaction between *lin-66* and *lin-14*. The *lin-66 (ku423); lin-14 (lf)* double mutants displayed a seam cell phenotype that reflected a mutual suppression of mutating the two genes (Table I). This suggests that *lin-66* does not act upstream of *lin-14*, which is consistent with the hypothesis that *lin-66* acts to regulate *lin-28*.

Next, we analyzed the genetic interaction between *lin-66* and *hbl-1*, knowing that *hbl-1* also regulates the L2/L3 transition and is regulated by the *let-7*-like miRNAs, *mir-48, mir-84* and *mir-241* (Abrahante *et al*, 2003; Lin *et al*, 2003; Abbott *et al*, 2005). Knocking down of *hbl-1* by RNAi in *lin-66 (ku423)* animals resulted in an alae formation phenotype that reflected a mutual suppression of mutating the two genes, suggesting that *lin-66* is unlikely to act upstream of *hbl-1* (Table I).

lin-46 has also been indicated to have a role antagonistic to *lin-28* in the L2 seam cell program (Pepper *et al*, 2004). We thus constructed a *lin-66 (ku433); lin-46 (ma164 lf)* mutant and found that the *lin-46* allele also significantly enhanced the alae formation phenotype (Table I), suggesting that the two genes may act in parallel to regulate the L2 program.

These genetic analysis led to a model in which *lin-66* negatively regulates *lin-28* activity to specify the L2 seam cell program, whereas *lin-14* and *hbl-1* act in parallel or upstream of *lin-66* and *lin-28* in regulating the L1/L2/L3 programs (Figure 3).

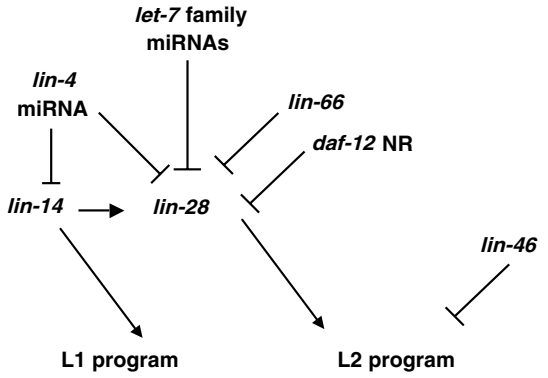

Figure 3 *lin-28* activity is regulated by multiple factors. The functional relationships between the genes shown in the figure are based on genetic data from previous analyses (see Seggerson *et al*, 2002) and this study. An arrow indicates positive regulation, whereas a T-bar indicates negative regulation. NR, nuclear receptor.

lin-66 (lf) causes an increase of LIN-28 protein level

To determine if *lin-66* regulates *lin-28* activity by regulating the expression of LIN-28, we examined the expression of a functional *lin-28*::*GFP* reporter transgene (Moss *et al*, 1997) in the *lin-66 (lf)* background. This transgene, with the GFP sequence inserted at the C-terminal end of the ORF, contains both the 5′ promoter sequence and the 3′UTR of *lin-28* and could rescue the phenotype of *lin-28 (lf)* (Moss *et al*, 1997). In a WT background, the expression of this *lin-28*::*GFP* reporter was never observed at the adult stage ($n=30$; Figure 4A). However, the expression is prominent in adult *lin-66 (ku423)* mutants (100%, $n=30$) (Figure 4B). Similar result is obtained from the analysis of the expression in vulval cells at the L3 stage (WT, 14%, $n=14$; *lin-66 (ku423* mutant, 92%, $n=24$) (Figure 4D and F). This result indicates that *lin-66* represses the expression of LIN-28 in late larval and adult stages. To confirm this and quantify the result, we examined the levels of the endogenous LIN-28 protein in *lin-66 (lf)* and *lin-66 (lf); alg-1 (lf)* double mutants by immunoblot analysis using an anti-LIN-28 antibody (gift from E Moss) (Seggerson *et al*, 2002). In WT, LIN-28 was abundant at L1, but its level at L3 was about 20-fold lower than that at L1 (data not shown). Such a difference is consistent with the previous report (Seggerson *et al*, 2002). In comparison, in *lin-66 (ku423)* L3 animals, the LIN-28 protein was detected at a level that is five-fold higher than that in WT L3 animals (Figure 4G and Supplementary Figure S3). In addition, the level of LIN-28 at L1 was also detected to be two-fold higher than that in WT. The increase is more prominent than that observed in *daf-12 (rh61)* mutants (three-fold), consistent with a stronger seam cell differentiation mutant phenotype of *lin-66 (lf)*. However, in the *alg-1 (gk214)* single mutant, the LIN-28 protein level at L3 was not significantly higher than that of WT (about 1.5-fold change) (Supplementary Figure S3). This finding is consistent with the hypothesis that *alg-1* plays a role in regulating another factor, likely *hbl-1*, for its role in L2/L3 fate specification (see Discussion).

lin-66 and daf-12 may regulate lin-28 expression through its 3′ UTR

To learn at what level *lin-66* regulates *lin-28* expression, we made transgenic worms that contain a *col-10 promoter (*p*col-10)*::*lacZ*::*lin-28* 3′UTR reporter construct (Figure 5A). The *col-10* promoter drives expression in hypodermal cell lineages (Wightman *et al*, 1993). In a WT background, prominent expression of *lacZ* from this reporter was observed in nearly 100% of the L1 larvae; however, no expression was detected in any adults ($n>200$) (Figure 5B and C), and weak expression was detected in 26% of L4 larvae ($n=116$). When a *unc-54* 3′UTR was used instead of the *lin-28* 3′UTR in a control construct, *lacZ* expression was detected in 93% of the adult animals ($n=123$). These results are consistent with the

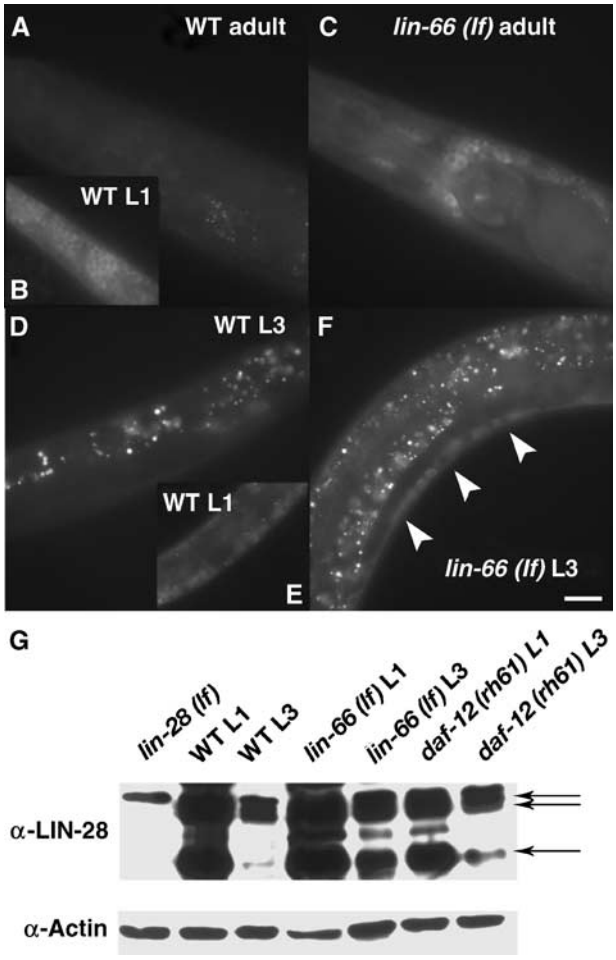

Figure 4 *lin-66* represses LIN-28 protein levels in late larval and adult stages. (**A–F**) Fluorescence images of animals of the genotype and stage as indicated. The fluorescence indicates the expression of an integrated *lin-28*::*GFP* transgene (Moss *et al*, 1997). In L3 animals, strong expression is seen in Pn.p cell derivatives (cells above the white line) only in *lin-66 (lf)* mutants. In adults, the GFP expression is essentially undetectable in WT but still seen in many neurons in the mutant. Bar, ~50 μm. (**G**) Western blot analysis of endogenous LIN-28 protein using an anti-LIN-28 antibody. Arrows indicate three LIN-28 protein bands determined in a previous study (Seggerson *et al*, 2002). A lighter exposure of the gel is shown in Supplementary Figure S3.

previous proposal that *lin-28* expression is regulated post-transcriptionally through its 3′UTR (Moss *et al*, 1997). After crossing the same p*col-10*::*lac-Z*::*lin-28* 3′UTR transgene into *lin-66 (ku423)* mutants, prominent expression of *lacZ* could clearly be detected in 20% of adult animals and 73% of the L4 animals (Figure 5D and F). Significant increase of the expression of the transgene was also observed in *daf-12 (rh61)* mutants (Figure 5F). These results suggest that *lin-66* and *daf-12* may regulate *lin-28* expression through its 3′UTR.

lin-66-mediated repression is likely independent of *miRNA- and daf-12*-response elements in the *lin-28* 3′UTR

Previous work has determined that the *lin-28* 3′UTR mediates regulation of LIN-28 expression by miRNA and *daf-12* (Moss *et al*, 1997; Seggerson *et al*, 2002). Although the *daf-12*-response element had not been identified, a single conserved binding site for *lin-4* miRNA and a single conserved binding site for *let-7* family miRNAs were identified (Figure 5E) (Moss *et al*, 1997; Reinhart *et al*, 2000). Regions conserved between *C. elegans* and another nematode species (*C. briggsae)* were also recognized (Figure 5E). To identify the *lin-66*-response element(s) and analyze its relationship with miRNA and *daf-12* regulation, we made a number of mutations in the *lin-28* 3′UTR of the p*col-10*::*lacZ*::*lin-28* 3′UTR reporter gene and analyzed the expression of these constructs at L1, L4 and adult stages (Figure 5F). First, when we introduced a mutation in the *lin-4* or *let-7*-like miRNA binding sites, a moderate increase of *lacZ* expression in adult animals was detected. Mutating both the *lin-4* and *let-7*-like miRNA-binding sites causes significantly enhanced *lacZ* expression, suggesting that these two sites may be responsive to a synergistic negative regulation (Bartel, 2004). However, the expression from the same transgene carrying mutations that disrupted either miRNA-binding site is further increased in the *lin-66 (ku423)* or *daf-12 (rh61)* mutant adults (Figure 5F). This result is consistent with the hypothesis that the regulation through these two miRNA sites may be independent of the regulation by *lin-66* and *daf-12*. Additionally, the expression of the transgene with both miRNA-binding sites disrupted might be near saturation in WT L4 larvae, as a significant increase in expression in the *lin-66 (lf)* background was not observed.

When another conserved region upstream of the miRNA-binding sites was mutated (four nucleotide substitutions; pKM63), the *lacZ* expression was also significantly increased in WT L4 larvae and adults (Figure 5F). The *lin-66 (lf)* mutation, but not the *daf-12 (rh61)* mutation, significantly enhanced the expression of *lacZ* (Figure 5F), suggesting that the region mutated in pKM63 may be involved in *daf-12*-mediated regulation that is likely independent of the *lin-66* regulation.

We have carried out a series of deletion analyses on the *lin-28* 3′UTR in the reporter construct, but failed to identify a specific DNA region that clearly displays the property of a potential *lin-66* response element (data not shown). Gel-shift assays were also unable to detect the binding of LIN-66 on the *lin-28* 3′UTR, suggesting the possibility that other factors may mediate the interaction between LIN-66 and the 3′UTR of *lin-28*. Therefore, the proposed *lin-66* regulation on the *lin-28* 3′UTR remains to be supported by further biochemical analysis.

lin-28 mRNA level is significantly changed by altering *lin-14* or miRNA function, but not *lin-66* or *daf-12* function

As previous work and the above data indicate that *lin-66, daf-12 (rh61)* and miRNAs repress *lin-28* expression through its 3′UTR, the repression may be caused by either RNA degradation or translational inhibition. To distinguish these two possibilities, we performed quantitative RT–PCR to compare the mRNA level in WT and mutant larvae. In WT, the level of *lin-28* mRNA was observed to decrease approximately seven-fold from the early L1 stage to the L3 stage (Figure 6A). This change is consistent with the result in a recent report (Bagga *et al*, 2005). In *lin-66 (ku423)* and *daf-12 (rh61)* animals, the levels of *lin-28* mRNA at early L1 and L3 stage were similar to that in WT worms (Figure 6A). These data indicate that *lin-66* and *daf-12* do not regulate *lin-28* expression through RNA

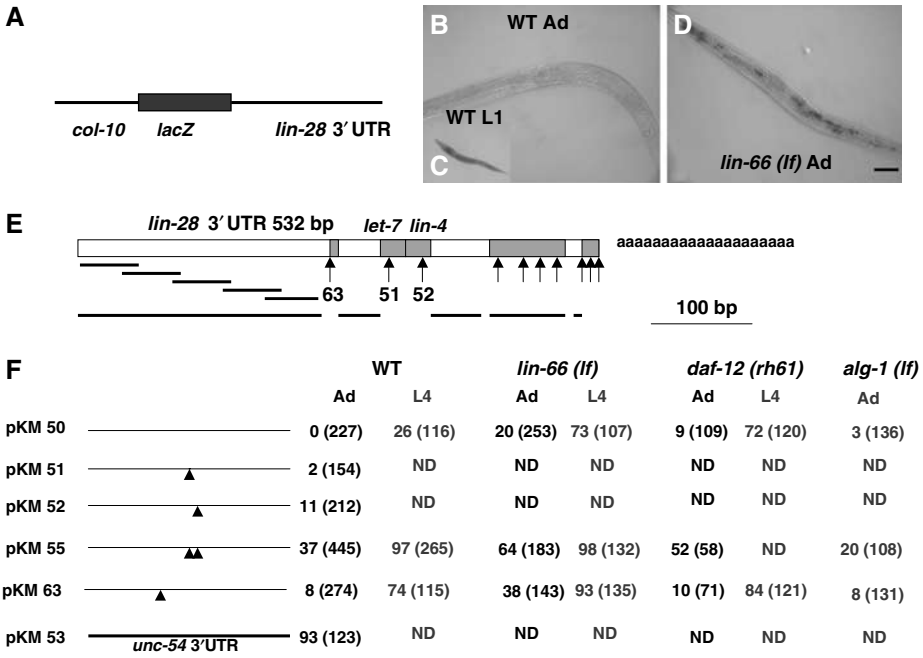

Figure 5 *lin-66* regulates LIN-28 expression through the 3′UTR region of *lin-28*. (**A**) Schematic illustration of the p*col-10*::*lacZ*::*lin-28* 3′UTR construct. (**B–D**) Photos showing LacZ staining of animals expressing the reporter construct (pKM50). The expression of the reporter construct is observable in the *lin-66 (ku423)* adult animal (D) but not in WT (B). The reporter construct is robustly expressed in WT L1 larvae (C). Bar, ~50 μm. (**E**) Schematic illustration of the 3′UTR region of *lin-28*. Gray filled boxes indicate areas conserved between *C. elegans* and *C. brigssae*. The *let-7*- and *lin-4*-binding sites are indicated. Arrows indicate substitution or small deletion mutations made in the area, whereas bars indicate deletion mutations. The numbers indicate the plasmid shown in (F). The data for those arrows and bars without numbers are not shown, as these mutations either did not change the expression of the reporter or did not significantly change the response of the expression to *lin-66*, *daf-12* or miRNA regulations. pKM50 is the intact 3′UTR of *lin-28*. pKM51 and pKM52 have a three nucleotide deletion in the *let-7* (ctc) and *lin-4*-binding site (ggg), respectively. pKM55 has deletions of both the *let-7*- and *lin-4*-binding sites. pKM63 has a four nucleotide substitution (caaa to accc) in the indicated conserved region. (**F**) Percent of animals that displayed the *lacZ* expression of indicated construct in at least some of the hypodermal cells in various mutant backgrounds in the L4 and adult stage. Number of animals counted is indicated. Nearly 100% lacZ expression was observed in L1 animals carrying each construct and in each mutant background (data not shown).

degradation, implying possible roles for these genes in protein translation, which is consistent with previously proposed translational inhibition of *lin-28* at late larval stages in WT animals (Moss *et al*, 1997).

As was observed in a previous study (Bagga *et al*, 2005), the *lin-28* mRNA level was drastically increased in *lin-4 (lf)* mutants (Figure 6A), consistent with the idea that *lin-4* miRNA represses *lin-28* expression by promoting RNA degradation. However, this increase could be due to an indirect regulation mediated by *lin-14*/transcription factor, as *lin-4* has a prominent role in repressing the expression of *lin-14,* which has a positive effect on *lin-28* expression (Seggerson *et al*, 2002). We addressed this question by quantitative PCR (qPCR) analysis. We show that the *lin-28* mRNA level is at a very low level in L3 larvae of *lin-4 (lf)*; *lin-14 (lf)* double mutants but at a high level in L3 larvae of *lin-14 (gf)* similar to that of the *lin-4 (lf)* mutants (Figure 6B). These results suggest that the majority of the increase in *lin-28* mRNA level in *lin-4 (lf)* was likely due to an increase in the level of the LIN-14 transcription factor, which is consistent with several observations in previous studies and this study (see Discussion).

To investigate whether RNA degradation is still a mechanism involved in regulating *lin-28* expression by miRNA, we performed qPCR analysis of mRNA transcribed from the *pcol-10*::*lacZ*::*lin-28 3′UTR* transgene that had deleted of both *lin-4* miRNA and *let-7* family miRNA-binding sites (Figure 5). We observed that in L3 animals, the level of mRNA transcribed from the reporter gene containing the deletion drastically increased, compared to that transcribed from the reporter gene without the deletion. This result supports the notion that RNA degradation is an important mechanism by which *lin-4* and *let-7*-family miRNAs repress gene expression (Bagga *et al*, 2005).

These qPCR results, along with the results from genetic and reporter-gene analyses, indicate that *lin-66*, *daf-12*, miRNAs and *lin-14* mediate four independent mechanisms to regulate the expression of *lin-28*.

Discussion

lin-66 likely acts in parallel to daf-12 and miRNAs to inhibit lin-28 expression

lin-28 functions to specify the L2 developmental programs and the downregulation of its expression is required for animals to exit the L2 programs in late larval stages (Moss *et al*, 1997). The similar retarded mutant phenotypes associated with *lin-66* and *daf-12*, especially the robust L2 seam cell reiteration phenotype of *lin-66 (lf); daf-12 (lf)* double mutants (Table II), clearly indicate that the mechanisms mediated by these two proteins play critical roles in repressing *lin-28* expression in late larval stages. LIN-66 and DAF-12 proteins appear to act independently on the *lin-28* 3′UTR to repress and promote translation, respectively.

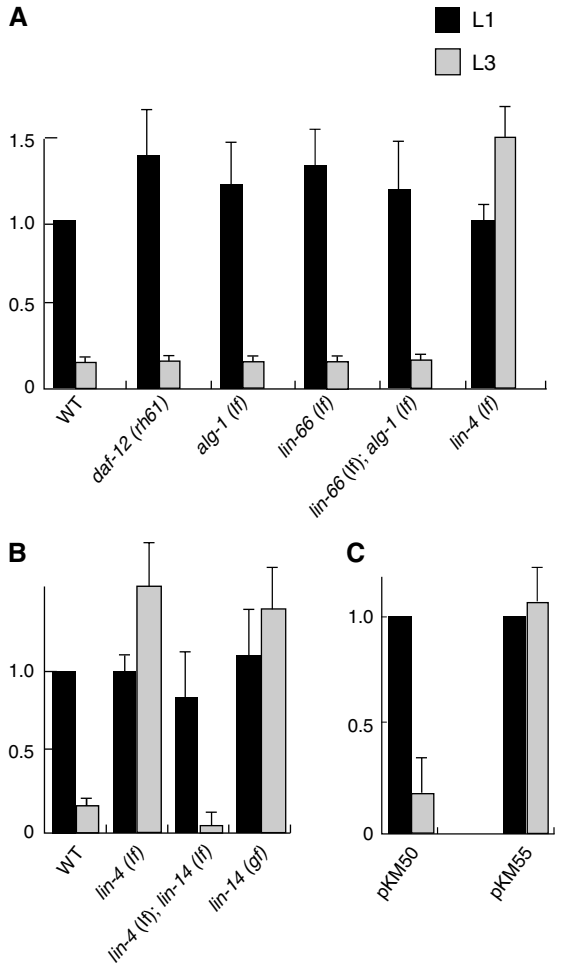

Figure 6 mRNA levels of *lin-28*. (**A–C**) *lin-28* mRNA levels in various strains indicated were determined by real-time RT–PCR. The alleles used are listed in Materials and methods. pKM50 and pKM55 are reporter constructs depicted in Figure 5, and they were integrated into the genome before the PCR analysis. Error bars indicate the s.d..

Previous work has indicated that *lin-28* is one of the targets of *lin-4* miRNA, and a potential *lin-4*-binding site on the *lin-28* 3′UTR was also identified (Moss *et al*, 1997). A deletion of the *lin-4*-binding site in the reporter construct caused only a small increase in expression, but this deletion significantly enhanced the phenotype of the deletion of the *let-7*-like-binding site (Figure 5), suggesting that multiple miRNAs may have partially redundant activities in repressing *lin-28* expression (Figure 3). The repression mediated by miRNAs appears also to be independent of *lin-66* and *daf-12* as the expression of a p*col-10*::*lacZ*::*lin-28* 3′UTR reporter with both the miRNA-binding sites mutated is still subject to regulation by *lin-66* and *daf-12*. Furthermore, miRNAs, but not *lin-66* and *daf-12*, appear to be involved in regulating the *lin-28* mRNA level. Consistent with the suggestion that *lin-66* may not be involved in general miRNA maturation or function, the levels of *lin-4* and *let-7* family miRNAs were shown to be normal in a *lin-66* mutant by Northern analysis (Supplementary Figure S4), and miRNA was not found to be associated with LIN-66::GFP protein in a co-immunoprecipitation (co-IP) experiment using an anti-GFP antibody (data not shown).

Using biochemical assays, we failed to see a direct interaction between LIN-66 and the *lin-28* 3′UTR (data not shown), leaving a good possibility that the LIN-66 interaction with the *lin-28* 3′UTR is indirect. Our co-IP test has so far failed to identify proteins that are associated with LIN-66 (data not shown).

It is interesting to learn that multiple mechanisms are critically involved in regulating the expression of *lin-28* in late larval stages (Figure 3). Disrupting or disturbing any of these mechanisms would cause a significant increase in the LIN-28 protein level and retarded phenotypes. This is in contrast to spatial regulation of expression of other developmental regulators where multiple pathways redundantly repress their transcription in certain tissues (Cui *et al*, 2006).

Roles of lin-4 miRNA and LIN-14 transcription factor in lin-28 expression

lin-28 mRNA level was shown in a recent report as well as in this study to be significantly increased in later larval stage in *lin-4 (lf)* mutants (Bagga *et al*, 2005) (Figure 6A). However, we only observed a minor effect resulting from deleting the *lin-4*-binding site in the *lin-28* 3′UTR. A likely scenario is that the major effect on *lin-28* expression seen in *lin-4 (lf)* is due to an indirect effect mediated by *lin-14* on *lin-28* transcription. *lin-14* is a major target of *lin-4* miRNA and *lin-4 (lf)* causes severe derepression of *lin-14* expression (Wightman *et al*, 1993). It has been shown that in a *lin-4 (lf)* mutant, *lin-28* protein level decreases if *lin-14* activity is reduced or eliminated, suggesting that *lin-14* promotes *lin-28* expression (Moss *et al*, 1997; Seggerson *et al*, 2002). Finally, we have shown in this study that *lin-28* mRNA levels are low in *lin-4 (lf); lin-14 (lf)* double mutants but high in a *lin-14 (gf)* mutant (Figure 6B), suggesting that the increase of *lin-28* mRNA levels in the *lin-4 (lf)* single mutant depends on *lin-14(+)*. However, we have not demonstrated that *lin-28* is a direct target of the LIN-14 transcription factor. Taken together, these data suggest that *lin-4* miRNA acts redundantly with *let-7* family miRNAs to promote *lin-28* mRNA degradation, whereas *lin-14* may upregulate *lin-28* mRNA either by transcription activation or through another indirect mechanism.

Function of daf-12 on lin-28 expression

The fact that both *daf-12 (rh61)* and a *daf-12 (null)* allele significantly enhanced the retarded phenotype of *lin-66 (lf)* suggests that the *daf-12 (rh61)* allele expresses a protein with a role that is opposite to that of DAF-12(+), which likely represses *lin-28* expression. *daf-12* encodes a nuclear receptor. It has been proposed that DAF-12 changes from a transcriptional repressor to an activator upon binding to a ligand (Antebi, 2006). The *rh61* mutant allele produces a truncated protein that is expected to lose the ability to bind to a ligand (Antebi *et al*, 2000), and thus may constitutively repress transcription, which likely accounts for the mutant effect. As *daf-12 (rh61)* causes the upregulation of *lin-28* expression and acts on the *lin-28* 3′UTR (Seggerson *et al*, 2002) (this study), this regulation could be indirect; *daf-12 (rh61)* may repress the transcription of a factor involved in interacting with the *lin-28* 3′UTR to repress its expression. Mutational analysis of a reporter construct and qPCR analysis suggest that *daf-12 (rh61)* acts on a site that is distinct from miRNA-binding sites and it may not act to degrade *lin-28* mRNA.

Functional relationships of lin-66 with alg-1 and hbl-1

We show in this paper that *lin-66 (lf)* and *alg-1 (lf)* double mutants display an L2 reiteration phenotype that is much stronger than that of either single mutation (Figures 1 and 2), suggesting that these two genes may have parallel activities in regulating the L2-to-L3 transition. *alg-1* and *alg-2* encode homologues of argonaute proteins and have been shown to be involved specifically in the maturation and function of miRNA (Grishok *et al*, 2001). It is thus possible that the *alg-1 (lf)* mutant effect reflects miRNA regulation on *lin-28*. However, Western blot analysis did not reveal a significant increase in the LIN-28 protein level in *lin-66 (lf); alg-1 (lf)* double mutants (Supplementary Figure S3). Furthermore, the expression of the p*col-10*::*lacZ*::*lin-28* 3′UTR reporter construct did not display a prominent increase in *alg-1 (lf)* mutants (Figure 5F).

An alternative explanation is that *alg-1* plays a more prominent role in the L2/L3 transition by regulating another factor, likely the *hbl-1* gene, which encodes a hunchback-like transcription factor and was shown to regulate the L2/L3 and L4/adult transitions (Fay *et al*, 1999; Abrahante *et al*, 2003; Lin *et al*, 2003). Recently, it has been shown that three *let-7* family miRNAs (*mir-48, mir-84* and *mir-241*) redundantly regulate the L2-to-L3 transition mainly by repressing *hbl-1* expression (Abbott *et al*, 2005). Therefore, the L2 reiteration phenotype of *alg-1 (lf)* is likely due, to a large extent, to the disruption of the regulation of *hbl-1* by these miRNAs. However, as our deletion analysis suggests that *let-7* family miRNAs also work together with *lin-4* miRNA to regulate *lin-28* expression, *alg-1* may also play a role in *lin-28* regulation.

lin-28 regulates the competence of VPC cell division timing

Euling and Ambros (1996) have previously shown that in WT, the VPC cell cycle contains a long G1 phase from mid-L1 to the end of the L2 stage. The first round of cell divisions of VPCs is completed at the mid-L3 stage (Figure 3G). In a *lin-28* mutant, the G1 phase of the VPCs is shortened and the first round of VPC divisions occurs at the mid-L2 stage (Euling and Ambros, 1996). We show in this paper that in *lin-66 (lf)* and *lin-66 (lf); alg-1 (lf)* double mutants, the first round of VPC divisions is delayed, suggesting a longer than normal G1 phase for the VPCs in these mutants.

It is worthy to mention that the delay in the first round of VPC division observed in *lin-66 (lf)* and *lin-66 (lf); alg-1 (lf)* was not previously observed in known retarded heterochronic mutants. In particular, the first round of VPC division occurred at a similar time in *lin-4 (lf)* and *lin-14 (gf)* mutants as that in WT (Chalfie *et al*, 1981; Euling and Ambros, 1996) (Figure 1H). In these animals, the second and third rounds of vulval cell divisions are randomized and these cells cannot respond properly to inductive signalling (Chalfie *et al*, 1981; Euling and Ambros, 1996). In comparison, vulval cell divisions in *lin-66 (lf)* mutants are delayed but still coordinated, often capable of generating a vulva in the adult stage. We speculate that the difference between *lin-66 (lf)/alg-1 (lf)* and *lin-4 (lf)/lin-14 (gf)* mutants reflects the difference between VPC programs in L1 and L2. In a *lin-66 (lf)* mutant, the high level of LIN-28 causes the L2 stage, at which VPCs are in G1 phase, to repeat once and VPC divisions to be delayed by one stage. As EGFR-mediated inductive signalling occurs at the L2 and L3 stages, vulval cells in *lin-66 (lf)* may still be able to respond to the signalling. On the other hand, both *lin-4 (lf)* and *lin-14 (gf)* cause hyperactive LIN-14 and reiteration of the L1 program in hypodermal cells. VPCs in the L1 stage may not be competent to properly respond to the inductive signal that only affects the second and third rounds of vulval divisions (Sternberg and Horvitz, 1986).

Materials and methods

General method and strain

Mutagenesis and genetic crosses were performed as described by Wood (1988). The following strains were used: WT *C. elegans* variety Bristol strain (N2), *lin-31 (n301); eff-1 (hy21), let-7 (mn112); unc-3 (e151), lin-4 (e912), lin-14 (n179ts), lin-14 (ma135), lin-14 (n355 gf), lin-28 (n719), llin-29 (n333), lin-41 (ma104), lin-42 (n1089), lin-46 (ma164), daf-12 (rh61), daf-12 (rh61rh411), daf-12 (m20), hbl-1 (ve12), alg-1 (ok214), alg-2 (ok304), egl-17*::*GFP (ayIs4), lin-28*::*GFP (VT808), SCM*::*GFP (QwIS79)*.

Mutagenesis, mapping and positional cloning

The screen for suppressors of *lin-31 (n301); eff-1 (hy21)* animals has been previously described (Morita *et al*, 2005). SNP mapping (Wicks *et al*, 2001) was performed to determine chromosomal locations of *ku423* and *ku424*. The gene was placed between cosmid F52H3 and F37H8. Microinjection transformation was performed to identify DNA sequences that were able to rescue the mutant phenotype. The DNA lesions were determined by directly sequencing genomic DNA.

Phenotypic analysis

Alae formation and VPC division timing were analyzed under Nomarski optics as described previously (Euling and Ambros, 1996). Cells positive for the seam cell marker (*scm-1*::*GFP*) were counted under Nomarski and fluorescence microscopy. The stages of development were determined by examining the body size, molting state, size and shape of the gonad arms, and the stage of germline development.

Western analysis

Lysates from synchronized worms (hatched L1 and L3) were prepared as described previously (Seggerson *et al*, 2002).

Construction and expression analysis of the pcol-10::*lacZ*::*lin-28 3′UTR transgene*

A p*col-10-lac-Z-lin-28* 3′UTR construct (pKM50) was generated as follows. The *lin-28* 3′UTR from a *lin-28* cDNA clone, yk117g6, was amplified by PCR and subcloned into the *Spe*I and *Apa*I sites of the pPD95.11 vector. The *col-10* promoter was PCR amplified from genomic DNA and then subcloned into the *Pst*I and *Bam*HI sites of the resulting plasmid to create pKM50. The deletion or substitution mutations within the *lin-28* 3′UTR region were introduced to the reporter construct using the Stratagene QuikChange site-directed mutagenesis kit and confirmed by direct sequencing. These constructs were injected at 2 ng/μl with the marker Myo-3::GFP into WT hermaphrodites. At least three independent lines were generated for each construct. These constructs were crossed into mutants to examine expression in various genetic backgrounds. Animals with green fluorescence were selected for *lacZ* staining following a standard protocol.

Real-time RT–PCR

Synchronized L1 and L3 worms were prepared using a standard alkaline hypochlorite method and by selecting the L3 animals under a dissecting scope (~500 worms for each sample). mRNA was prepared using Trizol (Invitrogen) and the reverse transcription reaction was performed using the SuperScript III Kit (Invitrogen). PCR reactions were performed using the SYBR Green JumpStart Taq ReadyMix (SIGMA) and the Rotor-Gene RG-3000 system (CORBETT RESEARCH). *eft-2* was used as an internal control (Bagga *et al*, 2005).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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References

Abbott AL, Alvarez-Saavedra E, Miska EA, Lau NC, Bartel DP, Horvitz HR, Ambros V (2005) The *let-7* microRNA family members *mir-48*, *mir-84*, and *mir-241* function together to regulate developmental timing in *Caenorhabditis elegans*. *Dev Cell* **9:** 403–414

Abrahante JE, Daul AL, Li M, Volk ML, Tennessen JM, Miller EA, Rougvie AE (2003) The *Caenorhabditis elegans hunchback*-like gene *lin-57/hbl-1* controls developmental time and is regulated by microRNAs. *Dev Cell* **4:** 625–637

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

Ambros V (1999) Cell cycle-dependent sequencing of cell fate decisions in *Caenorhabditis elegans* vulva precursor cells. *Development* **126:** 1947–1956

Ambros V (2004) The functions of animal microRNAs. *Nature* **431:** 350–355

Ambros V, Horvitz HR (1984) Heterochronic mutants of the nematode *Caenorhabditis elegans*. *Science* **226:** 409–416

Antebi A (2006) Nuclear hormone receptors in *C. elegans* (January 03, 2006). *WormBook*, ed, http://www.wormbook.org

Antebi A, Culotti JG, Hedgecock EM (1998) *daf-12* regulates developmental age and the dauer alternative in *Caenorhabditis elegans*. *Development* **125:** 1191–1205

Antebi A, Yeh WH, Tait D, Hedgecock EM, Riddle DL (2000) *daf-12* encodes a nuclear receptor that regulates the dauer diapause and developmental age in *C. elegans*. *Genes Dev* **14:** 1512–1527

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

Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, Eachus R, Pasquinelli AE (2005) Regulation by *let-7* and *lin-4* miRNAs results in target mRNA degradation. *Cell* **122:** 553–563

Bartel DP (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116:** 281–297

Burdine RD, Branda CS, Stern MJ (1998) EGL-17(FGF) expression coordinates the attraction of the migrating sex myoblasts with vulval induction in *C. elegans*. *Development* **125:** 1083–1093

Chalfie M, Horvitz HR, Sulston JE (1981) Mutations that lead to reiterations in the cell lineages of *C. elegans*. *Cell* **24:** 59–69

Cui M, Chen J, Myers TR, Hwang BJ, Sternberg PW, Greenwald I, Han M (2006) SynMuv genes redundantly inhibit *lin-3*/EGF expression to prevent inappropriate vulval induction in *C. elegans*. *Dev Cell* **10:** 667–672

Ding L, Spencer A, Morita K, Han M (2005) The developmental timing regulator AIN-1 interacts with miRISCs and may target the argonaute protein ALG-1 to cytoplasmic P bodies in *C. elegans*. *Mol Cell* **19:** 437–447

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

Fay DS, Stanley HM, Han M, Wood WB (1999) A *Caenorhabditis elegans* homologue of *hunchback* is required for late stages of development but not early embryonic patterning. *Dev Biol* **205:** 240–253

Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, Baillie DL, Fire A, Ruvkun G, Mello CC (2001) Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control *C. elegans* developmental timing. *Cell* **106:** 23–34

Grosshans H, Johnson T, Reinert KL, Gerstein M, Slack FJ (2005) The temporal patterning microRNA *let-7* regulates several transcription factors at the larval to adult transition in *C. elegans*. *Dev Cell* **8:** 321–330

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

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

Lin SY, Johnson SM, Abraham M, Vella MC, Pasquinelli A, Gamberi C, Gottlieb E, Slack FJ (2003) The *C elegans hunchback* homolog, *hbl-1*, controls temporal patterning and is a probable microRNA target. *Dev Cell* **4:** 639–650

Liu ZC, Ambros V (1989) Heterochronic genes control the stage-specific initiation and expression of the dauer larva developmental program in *Caenorhabditis elegans*. *Genes Dev* **3:** 2039–2049

Morita K, Hirono K, Han M (2005) The *Caenorhabditis elegans ect-2* RhoGEF gene regulates cytokinesis and migration of epidermal P cells. *EMBO Rep* **6:** 1163–1168

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

Moss EG, Tang L (2003) Conservation of the heterochronic regulator Lin-28, its developmental expression and microRNA complementary sites. *Dev Biol* **258:** 432–442

Pasquinelli AE, Ruvkun G (2002) Control of developmental timing by microRNAs and their targets. *Annu Rev Cell Dev Biol* **18:** 495–513

Pepper AS, McCane JE, Kemper K, Yeung DA, Lee RC, Ambros V, Moss EG (2004) The *C. elegans* heterochronic gene *lin-46* affects developmental timing at two larval stages and encodes a relative of the scaffolding protein gephyrin. *Development* **131:** 2049–2059

Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, Ruvkun G (2000) The 21-nucleotide *let-7* RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* **403:** 901–906

Rougvie AE (2001) Control of developmental timing in animals. *Nat Rev Genet* **2:** 690–701

Rougvie AE (2005) Intrinsic and extrinsic regulators of developmental timing: from miRNAs to nutritional cues. *Development* **132:** 37837–37898

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

Seggerson K, Tang L, Moss EG (2002) Two genetic circuits repress the *Caenorhabditis elegans* heterochronic gene *lin-28* after translation initiation. *Dev Biol* **243:** 215–225

Sternberg P (2005) Vulval development. URL:: http://www.wormbook.org

Sternberg PW, Horvitz HR (1986) Pattern formation during vulval development in *C. elegans*. *Cell* **44:** 761–772

Sulston JE, Horvitz HR (1977) Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev Biol* **56:** 110–156

Wicks SR, Yeh RT, Gish WR, Waterston RH, Plasterk RH (2001) Rapid gene mapping in *Caenorhabditis elegans* using a high density polymorphism map. *Nat Genet* **28:** 160–164

Wightman B, Ha I, Ruvkun G (1993) Posttranscriptional regulation of the heterochronic gene *lin-14* by *lin-4* mediates temporal pattern formation in *C. elegans*. *Cell* **75:** 855–862

Wood WB (1988) *The Nematode Caenorhabditis elegans*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press

